Exploring mechanisms of and therapeutic interventions for microvascular endothelial activation in shock
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Chapter 1

Introduction and Aim of this thesis
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Introduction

Circulatory shock, named “shock” in this thesis, refers to an acute, life-threatening medical condition associated with patients treated in intensive care units (ICU). Shock is the clinical form of circulatory failure of adequate oxygen delivery for aerobic cellular respiration that results in cellular dysfunction and tissue injury, and organ failure (1). According to the underlying causes, shock can be characterized in four classical subtypes, i.e., hypovolemic shock, cardiogenic shock, obstructive shock, and distributive shock. The first three shock subtypes are associated with a low fluid flow state and inadequate tissue oxygen and nutrient supply. Distributive shock is characterized by abnormal blood vessel responses to vasoconstriction and vasodilation (2). Patients can have a combination of more than one subtype of shock at any given time or occur consecutively (2). Hemorrhage and sepsis are two major causes of circulatory shock, hemorrhage accounting for 16% of cases and sepsis for 62% of cases (3).

Shock is a frequently diagnosed condition and present in more than 30% of patients in ICUs (2). Advances in clinical care have led to a decrease in early deaths of shock patients. However the survivors of shock often develop the failure of multiple organs at the same time, the so called multiple organ dysfunction syndrome (MODS) (4). Shock associated MODS is nowadays still the most common cause of death in critically ill patients in ICUs (4). The precise pathogenesis of MODS is not completely understood: it is commonly accepted that systemic inflammatory responses contribute to the development of organ dysfunction (5), and that endothelial cells actively engage in this pathophysiological response by coordinating the host response and the progression of multiple organ failure (6). Investigating the mechanisms underlying endothelial activation in shock is important as the endothelium might be a valuable potential target for the treatment of shock and shock associated organ dysfunction (7).

In the following parts of this thesis introduction, I will focus on hemorrhagic and septic shock-associated endothelial pro-inflammatory activation, and the presently known molecular mechanisms leading to these endothelial responses, as well as potential therapeutic interventions in hemorrhagic shock and sepsis.
Hemorrhagic shock

Hemorrhagic shock (HS) is defined as decreased tissue blood perfusion due to a significant reduction of effective circulating blood volume. Roughly half of trauma deaths are attributed to hemorrhage (8). Hemorrhage results in the inability of the heart to supply enough blood to the tissues and organs. Initial compensatory mechanisms start to give priority to provide blood to vital organs such as the brain and the heart in order to maintain perfusion pressure (9). However, the capacity of compensatory mechanisms is limited. Further deprivation of delivery of oxygenated blood to organs during ongoing HS can lead to cellular injury, and may end with MODS and/or death (10). Treatment strategies for HS are to stop the bleeding as soon as possible and to rapidly conduct a fluid resuscitation protocol to restore circulating blood volume and tissue perfusion. This will allow recovery of the oxygen supply and termination of tissue ischemia and hypoxia (11). However, after the initial survival of HS and resuscitation (HS/R) episode, the risk of developing multiple organ failure is still high (12). Every year, in the United States, more than 60,000 people die due to hemorrhage, and an estimated 1.9 million deaths worldwide (13). Trauma is one of the leading causes of death worldwide, with around 40% of trauma mortality resulting from hemorrhage and HS. 33 to 56% of these deaths occur during the prehospital period (14).

Resuscitation after HS is conceived as a global ischemia/reperfusion injury insult. The resulting tissue ischemia and systemic inflammatory response can be fatal to the patient (15). In HS/R, a strong inflammatory response prevails in vital organs, via the activation and the transmigration of leukocytes into these organs. The kidney is one of the vulnerable and most frequently damaged organs after HS/R (16), with Acute Kidney Injury (AKI), a sudden loss of kidney function within a very short time, frequently occurring. In rats it was shown that HS decreased the microvascular pO₂ in the kidney at a much earlier time point than in other organs (17) and that fluid resuscitation could not fully restore renal microvascular oxygenation (18).

In recent years, many experimental animal studies have been performed to explore the pathophysiology of multiple organ failure during or after HS/R and to investigate potential treatment strategies. The most commonly used animal models of HS are fixed-pressure hemorrhage, fixed-volume hemorrhage, and uncontrolled hemorrhage (9). In our research, we use a fixed-pressure hemorrhagic shock model in mice to study
systemic inflammatory responses in HS/R and the influence of drug intervention. In this standardized model, animals are bled until the mean arterial pressure (MAP) reaches a pre-established level of 30 mmHg for 90 minutes. This low MAP level is maintained by additional blood withdrawal or by blood restitution if necessary during the hemorrhagic shock period (9).

Sepsis

Sepsis is defined as the host's dysregulated systemic response to an infection that injures its own organs and tissues, leading to severe life-threatening organ dysfunction (19, 20). Septic shock is a subtype of sepsis complicated by persistent hypotension and abnormal circulatory and cellular metabolism, which are severe enough to enhance the risk of death (20). Sepsis is the leading cause of mortality in in-hospital patients worldwide and in-hospital mortality is around 25–33% (21). An estimated 31.5 million sepsis patients and 19.4 million severe sepsis patients are treated in hospitals worldwide each year (21). Sepsis and sepsis related symptoms are a huge global health problem and represent a major economic burden in the world (22).

During the development of sepsis, an infection triggers a host reaction manifested as an exaggerated pro-inflammatory response (called systemic inflammatory response syndrome, SIRS) and an anti-inflammatory response (immunosuppression) (23). The aim of the host response is to clear invading infection and protect tissues and organs. However, the exaggerated pro-inflammatory reaction can lead to cell death and tissue damage, while the immunosuppressive response leaves the host more susceptible to secondary infections (24). Lipopolysaccharide (LPS), also known as endotoxin, is the main component in the outer cell wall of Gram-negative bacteria and functions as a key mediator of sepsis (25, 26). These excessive, and often prolonged immune responses induce microvascular thrombosis, microcirculatory alteration, increased endothelial permeability, and leukocyte recruitment, which will give rise to tissue damage, MODS, and finally to death (5). Many organs can be affected in sepsis, and the kidney is one of the failing organs (24).

A proper animal model mimicking most of the aspects of human sepsis is a prerequisite for studying the development of sepsis and exploring effective therapeutic targets.
Currently, three categories of sepsis models are extensively used: the cecal ligation and puncture (CLP) model, bacterial infection models, and the LPS-induced endotoxemia model (27). In the CLP model, sepsis is induced by disruption of the endogenous protective gut barrier in animals, while bacterial infection models consist of exogenous infusion of live bacteria as a viable pathogen (28). Endotoxemia models are induced by intravenous or intraperitoneal injection with an exogenous bacterial toxin such as LPS. This latter model is widely used due to the reproducibility in sepsis associated-systemic inflammatory response development and other physiological reactions (27, 28). In addition, intravenous injection of LPS into healthy volunteers can be employed as human sepsis model to mimic and study some of the pathophysiological and clinical processes of sepsis in humans (29). In our studies, we employed systemic i.p. administration of LPS as endotoxemia mouse model.

**The vasculature and the vascular endothelium**

The vasculature belongs to the blood circulatory system that transports blood throughout the whole body. Arteries, arterioles, capillaries, venules, and veins are the five major structural components of the blood vessels. All blood is carried in these vessels, each of them possessing specific structures and functions in maintaining organ and whole body homeostasis (30).

The vascular endothelium lines the luminal surface of all blood vessels in the whole circulatory system that delivers blood to all organs and tissues of the body, from the largest arteries and veins to the smallest capillaries (31). The endothelium is highly active and functions as a barrier between the vessel lumen and the underlying tissue. Additionally, the endothelium is a major player in the regulation of thrombosis and thrombolysis, involving the interaction of leukocytes with inflamed tissues, and controlling vasomotor tone (32). The endothelium can also modulate the function of the vessel wall via actively engaging in dynamics of blood flow and inflammation responses (33). The smallest blood vessels called capillaries, are particularly involved in disease-related pathophysiological processes such as angiogenesis that for example accompanies wound healing and tissue repair, and vascular leakage and leukocyte recruitment in inflammation and conditions of shock (34).
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Endothelial cells in shock

Endothelial cells (ECs) form a barrier between the circulating blood and the rest of the vessel wall, and the underlying tissue. Due to their anatomical position, endothelial cells are the first cells exposed to circulating inflammatory stimuli, invading pathogens, their metabolites, and microbial toxins. Endothelial cells will become pro-inflammatory activated when exposed to hemorrhagic shock or sepsis associated stimuli, such as LPS, TNF-α and other pro-inflammatory cytokines (35). The glycocalyx barrier is located on the luminal surface of ECs and has protective functions in the vasculature. During sepsis, activated endothelial cells shed the glycocalyx, shift from hemostasis to a prothrombotic and antifibrinolytic state, facilitate enhanced leukocyte adhesion, and show increased permeability (36).

Because of their crucial roles in triggering of and retaining the host response to invading pathogens, ECs are increasingly recognized as a contributor to sepsis associated mortality, with loss of endothelial barrier integrity and exaggerated endothelial activation being a hallmark of the processes occurring (37). Bacterial LPS directly triggers inflammatory activation in ECs during shock via the induction of the secretion of the pro-inflammatory cytokines (IL-6), chemokines (including IL-8 and MCP-1), and enhanced expression of adhesion molecules (P-selectin, E-selectin, VCAM-1 and ICAM-1) (6). These activated vascular endothelial cells initiate a multistep adhesion cascade, in which circulating leukocytes recognize and interact with the endothelium via sequential steps that encompass capture, rolling, and firm adhesion, and finally extravasation through the vessel wall into the inflamed tissues (38). Leukocyte capture and rolling are regulated by the endothelially expressed selectins (P-selectin and E-selectin) (39). Following rolling, leukocyte integrins including very late antigen 4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) become activated and bind to adhesion molecules VCAM-1 and ICAM-1 expressed on the surface of activated endothelial cells, respectively (40). This interaction induces firm adhesion of the leukocytes to the vascular endothelium, and facilitates leukocytes crawling on the surface of endothelium and transmigration through the endothelial layer into the underlying tissue (38).
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This increased endothelial mediated leukocyte trafficking into the inflamed tissue can lead to impaired organ function during the pathogenesis of sepsis (6). When the endothelium is excessively activated and unable to restore the homeostatic state, the ECs are considered to have become dysfunctional (41). Understanding the molecular mechanisms of excessive endothelial pro-inflammatory activation and dysfunction during the initiation and progression of shock will enable us to identify new therapeutic targets for therapeutic intervention of shock and sepsis related endothelial functional derailment.

Heterogeneity of vascular endothelium

The endothelial lining of blood vessels shows a remarkable heterogeneity at the level of morphology, molecular components, and functional output (42). At the structural level, endothelial cells are elongated and oriented along the direction of the blood flow in straight vascular beds in arteries, while ECs show a rounded shape in venules and are irregularly shaped in capillaries. Morphologically endothelium is divided into continuous endothelium, fenestrated endothelium, and discontinuous endothelium, which relates to its functions (43). For example, the blood-brain barrier in brain microvasculature is composed of continuous endothelium, assuring a strict control of permeability. Discontinuous endothelium lines the sinusoidal vessels in the liver where highly fenestrated sinusoidal ECs act as scavengers and clear soluble waste (macro)molecules from the circulation (44, 45). Endothelial cells in the arterioles primarily control vascular tone, while postcapillary vein ECs are mainly involved in regulating leukocyte-endothelial interactions, these processes may also happen in other vascular beds such as capillaries and veins (44, 45).

Microvascular endothelial heterogeneity is also observed in different microvascular compartments within one organ (31, 45). In the kidneys, blood flows into the glomerulus via the afferent arteriole and is filtered in the glomerular capillaries. The endothelium in renal arterioles is primarily associated with controlling glomerular blood flow and filtration rate, while the glomerular endothelial cells function as a semi-permeable filtration barrier for filtering water and small molecules into the (pre)urine. Efferent arterioles control glomerular outflow and feed into peritubular...
capillaries, which supply the renal tubules and interstitial cells with oxygen and nutrients (46). Leukocyte trafficking and permeability changes in response to inflammation mainly take place in postcapillary venules (45).

Endothelial heterogeneity in expression of adhesion molecules in different quiescent microvascular segments has been known for some time, while the heterogenic responses of endothelial cells to inflammatory stimuli are only recently being revealed (30). In our previous hemorrhagic shock studies, an upregulation of VCAM-1 protein was observed in renal extraglomerular endothelial segments, while its expression in glomerular endothelium was limited (7). The microvascular segment-restricted VCAM-1 protein expression during inflammatory insults is likely explained by heterogenic post-transcriptional control in endothelial cells in glomeruli. While VCAM-1 was transcriptionally induced in both arteriolar and glomerular endothelial cells exposed to acute inflammatory stimuli, its translation to protein was significantly reduced in glomeruli. This coincided with high miR-126 levels in glomerular segments that acts as a negative regulator of VCAM-1 protein expression (47). Understanding the molecular control of heterogeneity in endothelial phenotype and endothelial responsiveness in different renal microvascular segments is an important first step in understanding the pathogenesis of HS and sepsis induced AKI. This knowledge may provide crucial insights for future microvascular bed-specific treatment strategies for patients.

Molecular controls of shock and therapeutic intervention options

Microbes express certain molecular motifs termed pathogen-associated molecular patterns (PAMPs), such as LPS, lipopeptides, and peptidoglycans. As key constituents of the host’s immune system, pathogen recognition receptors (PRRs) recognize these dangerous PAMPs. As well as endogenous damage-associated molecular patterns (DAMPs), such as HMGB-1, heat shock protein, and DNA, triggering innate and adaptive immune responses (48). During HS, the initial ischemic insult can lead to systemic inflammatory responses and the release of DAMPs (49). The pathogen recognition receptor families include the subfamilies Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and Nod-like receptors (NLRs). All
three PRR subfamilies are involved in recognizing invading pathogens and initiating pro-inflammatory responses (50).

The role of TLR4 and RIG-I in LPS-mediated inflammation activation in the development of sepsis has been studied by our group (51) and other research groups (52, 53). The exposure of ECs to LPS leads to endothelial activation via TLR4 and RIG-I (Figure 1). LPS first interacts with LPS-binding protein (LBP), which catalyzes the formation of the LPS-CD14 complex (54). The uptake of LPS into cells can be facilitated via scavenger receptors or TLR4 receptors. In the TLR4 signaling pathway, the LPS-CD14 complex binds to the LPS receptor TLR4-myeloid differentiation protein (MD2) complex and activates TLR4 signaling through several adaptor proteins (55). The endothelial TLR4 signaling activates mitogen activated protein kinase (MAPK) signaling and IκB kinase (IKK), which in turn regulate the activation of transcription factors activator protein (AP)-1, and NF-κB, respectively (56, 57). Furthermore, the TLR4 pathway regulates phosphatidylinositol 3-kinase (PI3K)/AKT activation, which can modulate NF-kB activation. Recent data from our group showed that RIG-I functions independent of TLR4 to mediate LPS induced endothelial activation. In this situation, intracellular LPS likely binds to RIG-I, which recruits its adaptor protein MAVS and activates NF-κB signaling (51). NF-κB activation functions as a major contributor to the upregulation of adhesion molecules and the release of pro-inflammatory cytokines and chemokines, and thus leukocyte recruitment (58, 59).

NF-κB activation in endothelial cells induces the expression of pro-inflammatory cytokines, which can lead to further activation of NF-κB pathway, thereby amplifying the inflammatory responses (50). This positive feedback loop may cause more serious harm than the initial insult. This activation of endothelial NF-κB pathway likely contributes in a major way to the impairment of vascular function during endotoxemia and the occurrence of septic shock (60). Thus, the IKK/NF-κB pathway is considered an important therapeutic target for treatment of shock associated microvascular inflammation and MODS (61). Treatment with an IKK inhibitor during the resuscitation phase after a period of shock inhibited shock induced NF-κB activation. A previous study showed that blockade of NF-κB activation during resuscitation reduced HS induced lung, liver and kidney damage in rats (62). Furthermore, IKK inhibition inhibited nuclear translocation of NF-κB p65 which was associated with multiple organ dysfunction.
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during sepsis in mice (63). BAY11-7082 is an anti-inflammatory drug that selectively and nonreversibly inhibits IκB and therewith reduces nuclear translocation of NF-κB p65. In endothelial cells, BAY11-7082 inhibits activated IKK induced phosphorylation of the IκBα protein, thereby inhibiting the activation and nuclear translocation of NF-κB. This resulted in the decreased transcription and translation of pro-inflammatory molecules (64). Thus, in our study the endothelial NF-κB signaling pathway was chosen as a potential treatment target to counteract HS induced endothelial pro-inflammatory activation in mice.

Figure 1. Schematic overview of signaling pathways in endothelial cells that are known to be activated by LPS.

LPS interacts with LBP to form LPS-LBP complex, which can be taken up into endothelial cells via scavenger receptors or TLR4 receptors. In the latter case, the LPS complex binds to CD14 and
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then colocalizes with TLR4-MD2 complex and activates TLR4 signaling. This signaling depends on the recruitment and activation of downstream adaptor proteins (not shown in figures). In the LPS induced TLR4 signaling pathway, downstream IKK, MAPKs, and PI3K/AKT are activated. IKK activation leads to the phosphorylation and degradation of IκBα protein, allowing NF-κB to translocate into the nucleus to act as transcription factor. The activation of MAPK pathways in turn activates p38 MAPK, ERK, and JNK pathways, causing the activation of AP-1 transcription factor. Furthermore, PI3K/AKT can modulate NF-κB activation. In addition to these known pathways, our group more recently discovered that intracellular LPS-LBP complex also activates RIG-I, which then recruits and activates its adaptor MAVS. RIG-I-MAVS signaling specifically regulates downstream NF-κB activation. Together the TLR4 and RIG-I pathways control expression of endothelial adhesion molecules, pro-inflammatory cytokines, chemokines, and other endothelial related molecules implicated in the pathogenesis of sepsis.

Abbreviations: LBP, LPS binding protein; MD2: myeloid differentiation protein 2; MyD88, myeloid differentiation primary response protein 88; IKK, IκB kinase; PI3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; MAP3K, MAP kinase kinase kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; AP-1, activator protein 1; RIG-I, retinoic acid-inducible gene I; MAVS, mitochondrial anti-viral signaling protein; E-selectin, CD62 antigen-like family member E (CD62E); VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule 1; IL-6/8, Interleukin 6/8; MCP-1, Monocyte chemoattractant protein 1.
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Besides LPS-induced NF-κB activation via TLR4 and RIG-I, LPS can also directly induce IRF-1 expression (65). Interferon regulatory factor 1 (IRF-1) is a transcription factor and firstly recognized as a transcriptional regulator of the interferon system (66). It has been reported that IRF-1 is involved in the host innate and adaptive immune system and plays essential roles in the response to viral infections and the control of expression of pro-inflammatory molecules (67-69). IRF-1 shows low constitutive expression levels in almost all cell types and can be induced by types I and II interferon, pro-inflammatory cytokines TNFα, IL-1β, and IL-6, as well as a viral infection (70). IRF-1 knockout mice were significantly protected against LPS mediated induction of pro-inflammatory cytokines, thereby being protected from a lethal dose of LPS injection compared to control mice (71). However, the role of IRF-1 and the underlying molecular mechanisms of IRF-1 controlling endothelial cell activation in LPS mediated endothelial inflammatory responses in sepsis remain unclear.

Aim of the thesis

As outlined in the preceding sections, patients suffering from shock and sepsis often develop multiple organ dysfunction syndrome (MODS), which is the leading cause of death in critically ill patients. Although numerous studies on the pathophysiology of shock have been reported, no effective drug intervention has been found so far to stop or mitigate the development of MODS. Previous studies showed that hemorrhagic shock/resuscitation (HS/R) and sepsis induce endothelial pro-inflammatory responses in an organ and vascular bed specific manner (7, 72). This thesis therefore aims to investigate the molecular mechanisms of endothelial activation during the pathogenesis of shock and explore recently identified potential molecular targets for the treatment or prevention of MODS.

I addressed the following research issues. The first dealt with the effects of two types of drugs (an NF-κB inhibitor and an inhibitor of histone deacetylase, see below) on microvascular endothelial inflammatory responses in mouse kidney, lung and liver during HS/R. I further investigated the responses of three renal microvascular segments
to HS/R and the effects of drug intervention with NF-κB inhibitor on these microvascular responses. Furthermore, to unravel one of the underlying pathophysiological molecular mechanisms, I explored the role of endothelial IRF-1 in the regulation of LPS-induced inflammatory activation of endothelial cells and the nature and kinetics of LPS-induced kinase signaling in endothelial cells in vitro.

As described above in the introduction, NF-κB signaling plays an essential role in the onset of inflammation and the induction of pro-inflammatory molecules during hemorrhagic shock (HS) and resuscitation. In addition, previous studies found that HS/R leads to an imbalance in histone acetyltransferase (HAT) and histone deacetylase (HDAC) activity, thereby affecting the posttranslational modification status of cells. HDAC inhibitors lead to enhanced acetylation of proteins and restore this balance (73) and have anti-inflammatory effects which in an HS rat model resulted in markedly improved survival following lethal hemorrhage (74, 75). To investigate microvascular endothelial behavior during HS and subsequent resuscitation and effects of NF-κB and HDAC inhibition, we applied a fixed-pressure hemorrhage and resuscitation mouse model in chapter 2. We treated mice with the IκB inhibitor BAY11-7082 and the HDAC inhibitor valproic acid (VPA) during resuscitation phase and studied microvascular EC inflammatory responses. In addition, we investigated the effects of the two drugs on TNFα-mediated endothelial pro-inflammatory activation in vitro.

Based on the knowledge generated in chapter 2 and our notion that endothelial cells in the different renal microvascular segments show remarkable basic functional heterogenic properties, we hypothesized that these different microvascular segments will respond differently to HS/R induced inflammatory stimuli and drug inhibition. Therefore, in chapter 3 of this thesis, we examined the responses of three microvascular segments in the kidney, i.e., arterioles, glomeruli, and postcapillary venules, to HS/R. In addition, we investigated the effects of intervention with BAY11-7082 during resuscitation on the endothelial pro-inflammatory responses in these microvascular beds. We applied laser microdissection of the microvascular segments of the kidney before gene expression analysis to enable zooming in on the different segments.

As explained above, activation of endothelial cells plays a pivotal role in the pathogenesis of sepsis. Therefore, we combined in vivo and in vitro studies to further
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examine the signaling pathways in endothelial inflammatory activation in the context of sepsis. As explained above, RIG-I is a receptor that regulates LPS-mediated endothelial activation independent of TLR4 signaling. It has been shown that IRF-1 regulates RIG-I basal transcription and dsRNA-mediated RIG-I upregulation in different cell types (76). In addition, the role of IRF-1 in regulating the expression of pro-inflammatory molecules in endothelial cells and animals has been reported (71, 77). In chapter 4, we investigated whether IRF-1 has a role in the regulation of LPS-mediated inflammatory activation in endothelial cells, and studied the associated signaling pathways using endothelial cells in vitro.

An increasing number of protein kinases have been shown to engage in the regulation of LPS-mediated endothelial inflammatory activation in endothelial cells (78). Also, in vivo studies found that multiple kinase pathways play a critical role in regulating LPS-induced EC activation and acute inflammatory responses (79, 80). In chapter 5 of this thesis, we explored in vitro the nature and kinetics of activation of series of protein kinases in endothelial cells induced by LPS using kinase array technology.

Finally, in Chapter 6, the outcomes of the experimental research presented in this thesis are summarized and discussed, and implications of the data generated and the knowledge gained for future study put in perspective.
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