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RENAL FAILURE IN EXPERIMENTAL SEPSIS: ROLE OF ENDOTHELIN AND THE TOLL-LIKE RECEPTOR 4

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Abstract

Sepsis is the leading cause of renal failure in critically ill patients, but the pathogenesis of septic kidney dysfunction is poorly defined. The current paradigm states that hypoperfusion and excessive renal vasoconstriction results in renal ischemia. However, experimental data also exist indicating a direct immune-mediated basis of septic renal impairment. This thesis aimed to investigate the contribution of both a potent vasoconstrictor peptide, endothelin-1 (ET-1), and a receptor that activates the immune system in response to a bacterial infection, the Toll-like receptor 4 (TLR4), to the renal failure caused by sepsis.

The first part of this thesis investigated the role of the endothelin system in renal microcirculatory and functional impairment caused by experimental septic renal failure, by studying the effects of dual endothelin type A and B (ETA/ETB) antagonism and selective ETA-antagonism during porcine endotoxemia. ET-1 is a vasoconstrictor peptide that is a potent modulator of microcirculatory blood flow. It is released in high amounts during sepsis and experimental data have shown that ET-1 reduces renal blood flow. In paper I and II pigs were subjected to lipopolysaccharide (LPS) infusion and the renal microcirculatory effects of dual ETA/ETB or selective ETA antagonism were investigated. The main findings were that dual ETA/ETB blockade attenuated the endotoxemia induced reduction in renal cortical microcirculation, as well as the increase in plasma creatinine levels (paper I). In addition, selective ETA antagonism reduced the decline in renal medullary microcirculation, but had no significant effect on diuresis or creatinine clearance (paper II).

The second part of this thesis investigated the role of TLR4 activation in renal failure caused by hyperdynamic endotoxemia or sepsis. Conscious surgically prepared sheep were subjected to LPS or live Escherichia coli infusion and observed for 24-36 hours. A main finding was that pretreatment with a TLR4-inhibitor attenuated renal failure and hypotension caused by endotoxemia in sheep. This effect was greater compared to norepinephrine treatment, in a dose that prevented hypotension (paper III). Moreover, it was observed that septic renal failure developed without renal hypoperfusion and that treatment with a TLR4-inhibitor reversed renal failure when administered 12 hours into sepsis (paper IV). This effect was independent of changes in systemic or renal hemodynamics but was associated with a reduced renal neutrophil accumulation.

In conclusion, despite no reduction in renal perfusion or arterial blood pressure, septic renal failure may still develop. During hyperdynamic sepsis, stimulation of the innate immune system, via TLR4 activation, may contribute to the development of renal failure. In addition, TLR4-inhibition is an effective treatment to improve renal function in ovine sepsis induced by E.coli. In hypodynamic endotoxemia, ET-1 contributes to renal vasoconstriction. By acting on ETA, ET-1 reduces renal medullary blood flow causing ischemia but has no short-term effect on renal function.

List of Publications

I. The endothelin receptor antagonist tezosentan improves renal microcirculation in a porcine model of endotoxemic shock.

Fenhammar J, Andersson A, Frithiof R, Forestier J, Weitzberg E, Sollevi A, Hjelmqvist H.

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II. Endothelin receptor A antagonism prevents renal medullary blood flow impairment in endotoxemic pigs.

Fenhammar J, Andersson A, Forestier J, Weitzberg E, Sollevi A, Hjelmqvist H, Frithiof R. Submitted

III. Toll-Like Receptor 4 Inhibitor TAK-242 Attenuates Acute Kidney Injury in Endotoxemic Sheep.

Fenhammar J, Rundgren M, Forestier J, Kalman S, Eriksson S, Frithiof R.

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IV. Renal effects of treatment with a TLR4 inhibitor in conscious septic sheep. **Fenhammar J,** Rundgren M, Forestier J, Eriksson S, Özenci V, Hultenby K, Wernerson A, Frithiof R. Manuscript

Publications by the author, which are not included in the thesis:

The effects of isoflurane anesthesia and mechanical ventilation on renal function during endotoxemia.

Frithiof R, Soehnlein O, Eriksson S, **Fenhammar J,** Hjelmqvist H, Lindbom L, Rundgren M. Acta Anaesthesiol Scand. 2011 Apr;55(4):401-10.

Endothelin-mediated gut microcirculatory dysfunction during porcine endotoxaemia.

Andersson A, **Fenhammar J,** Weitzberg E, Sollevi A, Hjelmqvist H, Frithiof R.

Br J Anaesth. 2010 Nov;105(5):640-7.

Mixed endothelin receptor antagonism with tezosentan improves intestinal microcirculation in endotoxemic shock.

Andersson A, **Fenhammar J,** Frithiof R, Weitzberg E, Sollevi A, Hjelmqvist H. J Surg Res. 2008 Sep;149(1):138-47.

Haemodynamic and metabolic effects of resuscitation with Ringer's ethyl pyruvate in the acute phase of porcine endotoxaemic shock.

Andersson A**, Fenhammar J,** Frithiof R, Sollevi A, Hjelmqvist H. Acta Anaesthesiol Scand. 2006 Nov;50(10):1198-206.

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List of abbreviations

AKI	Acute Kidney Injury
ANG II	Angiotensin II
AVR	Ascending vasa recta
BUN	Blood urea nitrogen
CD	Cluster of differentiation
CLP	Cecal ligation and puncture
CO	Cardiac output
DVR	Descending vasa recta
E.coli	Escherichia coli
ET-1	Endothelin-1
ETA	Endothelin receptor type A
ETB	Endothelin receptor type B
GFR	Glomerular filtration rate
IL	Interleukin
LD	Laser doppler
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
NAG	N-acetyl-β-D-glucosaminidase
NF	Nuclear factor
NO	Nitric oxide
PMN	Polymorphonuclear leukocyte
TGF	Tubuloglomerular feedback
TLR	Toll-like receptor
TNF	Tumor necrosis factor
RBF	Renal blood flow

INTRODUCTION

Acute kidney injury (AKI) is one of the most important forms of organ dysfunction in sepsis. Renal failure triggered by sepsis is common and associated with high mortality among critically ill patients, despite advanced treatment. This might be related to inadequate knowledge of the underlying mechanisms causing septic AKI.

Reduced renal blood flow and impaired microcirculation resulting in ischemia is often considered to be the main basis for septic renal dysfunction, at least when this thesis was initiated. This is based on the notion that in sepsis a decrease in cardiac output and hypotension results in renal hypoperfusion. Additionally, increased sympathetic nerve activity and increased production of vasoconstrictive substances such as angiotensin II and ET-1 acts on the renal microcirculation and generates renal vasoconstriction, thereby worsening renal ischemia. Infusion of the potent vasoconstrictor peptide, ET-1 has been shown to reduce renal microcirculatory blood flow experimentally, indicating a role in the renal circulation. ET-1 also has inflammatory properties, and increasing plasma levels had been correlated to the severity of sepsis in patients. Taken together, this gave a theoretical rationale to further investigate the role of the renal microcirculation and ET-1 in septic renal failure.

During the course of this thesis, alternative views on the pathological mechanisms that underlie the development of septic AKI gained more attention. The paradigm of renal hypoperfusion was challenged by experimental data reporting preserved or even increased renal artery blood flow in experimental sepsis, thus suggesting the involvement of other mechanism than renal ischemia to septic renal impairment. As a result, focus in the second part of this thesis was slightly changed in order to investigate direct immune-mediated modulation of renal function. More specifically, the inhibition of Toll-like receptor 4 (TLR4) during experimental sepsis was studied.

The Toll-like receptors recognize specific structures of pathogens. The Toll-like receptors recognize specific structures of pathogens and will, upon activation, induce an innate immune response through production and release of inflammatory mediators, such as cytokines, and stimulation of inflammatory cells

This thesis is comprised of four papers. It describes *in vivo* studies performed in two separate models of experimental septic renal failure, with the aim to further our understanding of the mechanisms underlying kidney dysfunction caused by the inflammatory response to a severe infection.

BACKGROUND

THE KIDNEY

The kidney is responsible for several important functions in the body. It is, for example, a vital organ for regulation of water and electrolyte balance, excretion of metabolic waste and hormones, regulation of blood pressure and gluconeogenesis. The kidney can be divided into two regions: the cortex and the medulla. These two regions are structurally and functionally different, and both are essential for the formation of urine. The basic unit of the kidney is the nephron, and each kidney contains of approximately one million nephrons. These nephrons are built up by a filtering unit called the renal corpuscle and a reabsorption unit named the tubules. In the cortex of the kidney, blood enters the glomeruli and filtrates over the vessel wall, creating primary urine. The filtrate thereafter enters the renal tubules where most of the filtrate is reabsorbed. A network of blood vessels surrounds the nephron and recovers the fluid and solutes reabsorbed from the different segments. The urine is further concentrated in the medulla (Thomas, 2009;Eaton, 2009).

The Glomerulus

A portion of the blood that enters the kidney is filtered under pressure across the capillary walls in the glomerulus over the so-called glomerular filtration barrier. This barrier is built up by the glomerular endothelial cell surface layer, a fenestrated glomerular endothelium, the glomerular basement membrane and the slit-membranes between podocytes. The volume of filtrate formed in the glomerulus per unit time is known as the glomerular filtration rate (GFR). Under normal physiological condition, this value is approximately 180l/day (Eaton, 2009;Patrakka & Tryggvason, 2009). The renal microcirculation is essential in the preservation of GFR.

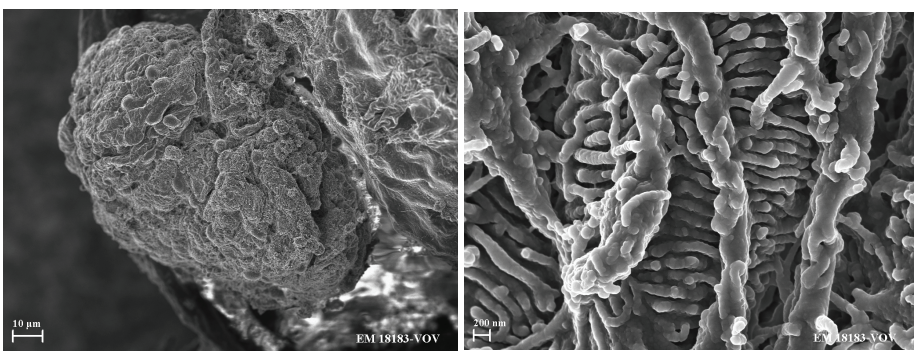

Figure 1. Scanning electron microscopy images. On the left, a glomerulus from sheep. The blood reaches the glomeruli through the afferent arteriole and enters the capillaries where filtration takes place. Podocyte cells, with foot processes, make up the surface of the glomerulus. On the right, podocytes and several foot processes lining the glomerular capillaries. The podocyte has an elongated cell body, and the foot processes radiate out and cover the capillaries in the glomerulus.

Microcirculation in the renal cortex

Autoregulation

The renal artery enters the kidney and branches to interlobar arteries. These interlobar arteries divide up to the arcuate arteries, which branch out to interlobular arteries that give rise to afferent arterioles. These afferent arterioles give blood supply to a glomerulus in the cortex. After filtration in the glomerulus, the blood continues in the efferent arteriole, which branch out and becomes the peritubular capillaries. The relationship between the afferent and efferent arterioles facilitates the pressure that drives glomerular filtration and the peritubular capillaries built up from efferent arterioles surrounds the proximal and distal tubules, and reabsorbs the large amount of glomerular filtrate. In this vascular setting RBF and GFR are highly controlled, mainly through autoregulation in the renal cortex. This control occurs in the pre-glomerular microcirculation, the afferent arteriole, and is mediated by two mechanisms, the faster myogenic mechanism and the slower tubuloglomerular feedback. This is very important because autoregulation preserves the regulation of body salt content and fluid balance and preserves the glomerular structure. Renal blood flow is very large. The kidney is approximately 2% of the body mass and receives as much as 20-25% of the cardiac output. This high blood flow is, however, essential to maintain adequate filtration of the glomerulus. Renal blood flow is driven by arterial pressure, and autoregulation prevents fluctuations when a change in blood pressure occurs. In addition, the kidneys' metabolic rate is a function of renal blood flow, increased blood flow will generate more filtration and reabsorption, which is energy demanding (Evans *et al.*, 2004).

The myogenic mechanism

The underlying mechanism for myogenic control is presumably pressure (probably wall tension in the afferent arteriole) and not renal blood flow *per se*. This myogenic response will generate a contraction or a relaxation of the smooth muscle cells in the vessel wall to prevent changes in renal blood flow when arterial pressure is altered. This myogenic response is very fast and occurs within 0.3-1 second upon stimuli (Cupples & Braam, 2007).

Tubuloglomerular feedback.

The primary site for this autoregulation of GFR is also the afferent arteriole. The structural anatomy of the nephron includes that the distal tubule loops back to the glomerulus and the afferent arteriole of the same nephron. This way information of tubular fluid content is sensed (Cl^- and /or osmolality of the tubular fluid) and can use this to regulate GFR and RBF. If the tubular fluid concentration is increased, the afferent arteriole will be constricted, and as a result a reduction of GFR will occur. This mechanism of vasoconstriction by TGF is suggested to be mediated by adenosine or

ATP. The contribution of the TGF mechanism to the renal autoregulation is approximately 20-50% (Cupples & Braam, 2007).

Microcirculation in the renal medulla

The renal medulla is built up by tubule segment called the loop of Henle, collecting duct segments and small blood vessels, *vasa recta*. The blood supply for the renal medulla originates from the efferent arteriole of some juxtamedullary nephrons deep in the cortex, but vessels that do not enter any glomerulus, so-called peri-glomerular shunts may also contribute to the medullary blood supply. The medullary blood flow is, in total approximately 10% of total RBF (Evans *et al.*, 2004). The first part of the descending vasa recta (DVR) is similar to arterioles with pericytes and smooth muscle in the vessel wall. The DVR change in structure and become more like capillaries but with no fenestrations as they descend deeper into the medulla. These vessels will give to a small capillary network that builds up the ascending vasa recta (AVR). These AVR rise up through the medulla and are characterized by a high degree of fenestrations of their vessel wall (Pallone *et al.*, 2003). The medullary blood flow is in part controlled by the flow in juxtamedullary efferent arteriole and DVR, but AVR may also be a site for regulation (Pallone *et al.*, 2003). The medullary blood flow is also influenced by vasodilatory stimuli from NO and prostaglandins. These mediators have a prominent local production within the medulla and supports to maintain a controlled flow of the medulla and attenuates the influence of vasoconstrictors such as ANG II and ET-1 (Evans *et al.*, 2004).

Renal metabolism

In the kidney reabsorption of sodium by active pumps, Na/K ATPase, is highly oxygen-dependent. Approximately 80-90% of the oxygen consumed in the kidney is used by mechanisms facilitating reabsorption. This gives a close relationship between oxygen consumption and GFR. However, the kidneys' ability to efficiently use oxygen for reabsorption is also an important factor, and both NO and vasoconstrictors such as ANG II may influence the efficiency of sodium reabsorption. Large parts of the medulla are dependent on oxidative metabolism, making this region susceptible to hypoxia. Oxygen delivery to the medulla relies not only on medullary perfusion but also on extraction of oxygen in the cortex (Evans *et al.*, 2008). The oxygen delivery to the medulla is low and this is in part related to the countercurrent functions of DVR and AVR. Oxygen delivered via the DVR diffuses to AVR and is shunted back to the cortex. This feature in combination with high oxygen consumption in the process of sodium handling makes this region vulnerable for ischemia (Pallone *et al.*, 2003). Large amount of glucose from the systemic circulation is filtered and reabsorbed in the kidney; but the kidney also plays an important role in the formation of glucose by gluconeogenesis. Glucose is produced in the gluconeogenesis from several precursors such as lactate, glycerol and amino acids. Glucose metabolism is different in the cortex

and medulla. The medulla is mainly fueled by glycolysis, an energy-producing metabolic pathway that converts glucose to pyruvate. The cortex is on the other hand the major contributor of the energy consuming process that forms glucose out of precursors, the gluconeogenesis. This gives a relationship were the cortex is a net producer of glucose and the medulla is a consumer and the sum of these processes is a net production of glucose in the kidney. The major precursor for gluconeogenesis in the human kidney is lactate. Hypoglycemia will increase renal gluconeogenesis by increased renal uptake of precursors and thereby increase gluconeogenesis (Mather & Pollock, 2011). Based on findings from animal experiment, the removal of lactate through renal gluconeogenesis is suggested to be increased during acidosis and the net removal of lactate is intact despite severe reductions of renal blood flow (Bellomo, 2002).

ACUTE KIDNEY INJURY

The term acute kidney injury (AKI) is used to describe the full range of renal impairment, from small changes to complete organ failure, and the degrees of AKI are determined by changes in functional parameters. Two measures of renal function have been standardized for defining AKI: changes in serum creatinine level or urine output. These variables have been chosen for their ability to identify patients with increased risk for an adverse clinical outcomes (death or requirement for renal replacement therapy) and not for their ability to accurately detect changes in renal function or structural damage in the kidney (Murugan & Kellum, 2011). A consensus definition for AKI was suggested from the Acute Dialysis Quality Initiative (ADQI) group in 2005. They proposed the RIFLE classification system for grading the severity of AKI. This classification is based on relative changes in plasma-creatinine and/or changes in urine output (Bellomo *et al.*, 2004). The RIFLE definition and classification of AKI by ADQI have been further refined by the Acute Kidney Injury Network (AKIN) (Mehta *et al.*, 2007). They recommend that an absolute change in plasma-creatinine (25 µmol/l) should be used to indentify AKI among some other adjustment. The definition of AKI has been introduced to standardize the research of AKI, both for prevention and treatment. The classification of AKI by RIFLE criteria is at present a widely accepted system for identifying and staging AKI in the clinical setting as well as in science.

However both creatinine and urine output are variables that manifest relatively late after injury has occurred. Both these systems also use the baseline value of creatinine, which is most often not known for patients in the clinical setting, making classification difficult in the clinical setting (Ricci *et al.*, 2011a). To overcome some of the limitations with creatinine and urine output, incorporation of other biomarkers of AKI into these classification systems may be beneficial. The RFILE classification is not directly adaptable to the experimental setting, and a classification of AKI to be used in animal research is being considered.

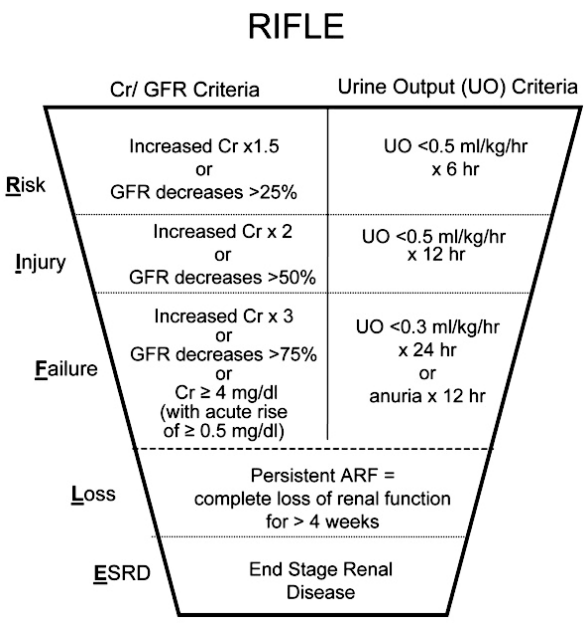

Figure 2. Classification of AKI by RIFLE criteria. To meet the criteria of each stage creatinine or urine output criteria need to be fulfilled (Mehta et al., 2007).

Sepsis

The word sepsis has been used for more than 2700 years. One of the earliest references are from Homer´s poems, were sepsis was generated from the verb, sepo [σηπω], "I rot." The ancient Greek physician, Hippocrates (460 BC – 370 BC) also made use of this term: the word sepidon [σηπεδ´ ων] ("the decay of webs") can be found in the medical collection, Corpus Hippocraticum (Geroulanos & Douka, 2006). Today, sepsis is a clinical syndrome defined by the presence of an *infection* and a *systemic inflammatory response* (Levy *et al.*, 2003). The definitions of sepsis have been changing over the years and a recent attempt to make a clear definition was made in 2001 at the International Sepsis Definitions Conference (Levy *et al.*, 2003). The term *infection* was then defined as a pathological process by the invasion of normally sterile tissue or fluid or body cavity by pathogemic or potentially pathogenic microorganisms. Defining a *Systemic inflammatory response* was on the other hand somewhat more difficult since no purely biochemical and/or immunological test is available in the clinical setting. Therefore a list of possible signs of systemic inflammation in response to infection is defined. Some of the signs are: fever, hypothermia, tachypnea, tachycardia, leukocytosis/penia, hypotension, increased cardiac output, oliguria and an increase in plasma creatinine. The disease is also staged in order of severity; sepsis, severe sepsis and septic shock. *Severe sepsis* refers to when an organ dysfunction has developed that worsens sepsis. *Septic shock* is a state of acute circulatory failure characterized by hypotension that persists despite adequate volume resuscitation. Sepsis is a very common disease, and the total number of deaths is increasing. The

incidence of sepsis has been reported to increase over the past decades and was in 2001 240 cases per 100,000 based on surveys from the United States (Martin *et al.*, 2003). Mortality among patients with sepsis is high, ranging from 20-70% for in-hospital mortality, and these patients suffer increased risk of organ failure (Winters *et al.*, 2010). The most common sites for infection are the lungs, the abdomen and the urinary tract. Among the patients with severe sepsis and septic shock, Gram-negative bacteria are the cause in approximately 40% of the cases. E.coli is the most common pathogen among the Gram-negative bacteria to cause sepsis (Bochud *et al.*, 2001).

Sepsis and the kidney

One of the most important forms of organ dysfunction in sepsis is AKI. Sepsis is the major cause of AKI in critically ill patients (Parmar *et al.*, 2009). Though it for a long time was considered merely as a result of aggravating sepsis, it is now recognized that AKI is an independent risk factor associated with both increased mortality and morbidity (Bagshaw *et al.*, 2007;Cruz & Ronco, 2007;Hoste *et al.*, 2006;Joannidis & Metnitz, 2005;Levy *et al.*, 1996). It has been suggested that a possible explanation to why mortality remains high in septic AKI despite today's advanced treatment is that the pathophysiology of septic AKI is not fully understood (Wan *et al.*, 2008). Current pharmacological therapies and technologies are still inadequate for the treatment of septic AKI. The treatment of sepsis and septic AKI aims mainly at supporting the failing organs and the use of renal replacement therapy, diuretics, and different fluid resuscitation protocols are often not sufficient to improve renal function (Ricci *et al.*, 2011b).

PATHOPHYSIOLOGY OF SEPTIC AKI

Current paradigm: hypoperfusion and ischemia

There are several views on the pathological mechanisms that underlie the development of septic AKI. The traditional explanation for renal impairment during sepsis is based in part on findings from animal experiment and also from patients. This hypothesis suggests that renal hypoperfusion secondary to decreased cardiac output and hypotension is important for the development of septic AKI. The renal hypoperfusion will eventually lead to ischemia in the susceptible kidney, and acute tubular necrosis and apoptosis will develop. Furthermore, increased sympathetic nerve activity, ET-1 and catecholamine production as well as activation of the renin-angiotensin-aldosterone system will be triggered to reduce the systemic arterial vasodilatation. These hormones are suggested to even further worsen the vasoconstriction in the renal microcirculation, aggravating ischemia (Schrier & Wang, 2004). Endothelial damage in sepsis in combination with apoptosis, necrosis and disturbances in the coagulation in the renal microcirculation also contribute to the ischemic damage of the kidney. Apoptosis and

necrosis are major mechanisms of cell death that have important roles in ischemic AKI. (Schrier & Wang, 2004;Shimamura *et al.*, 1983;Sharfuddin & Molitoris, 2011).

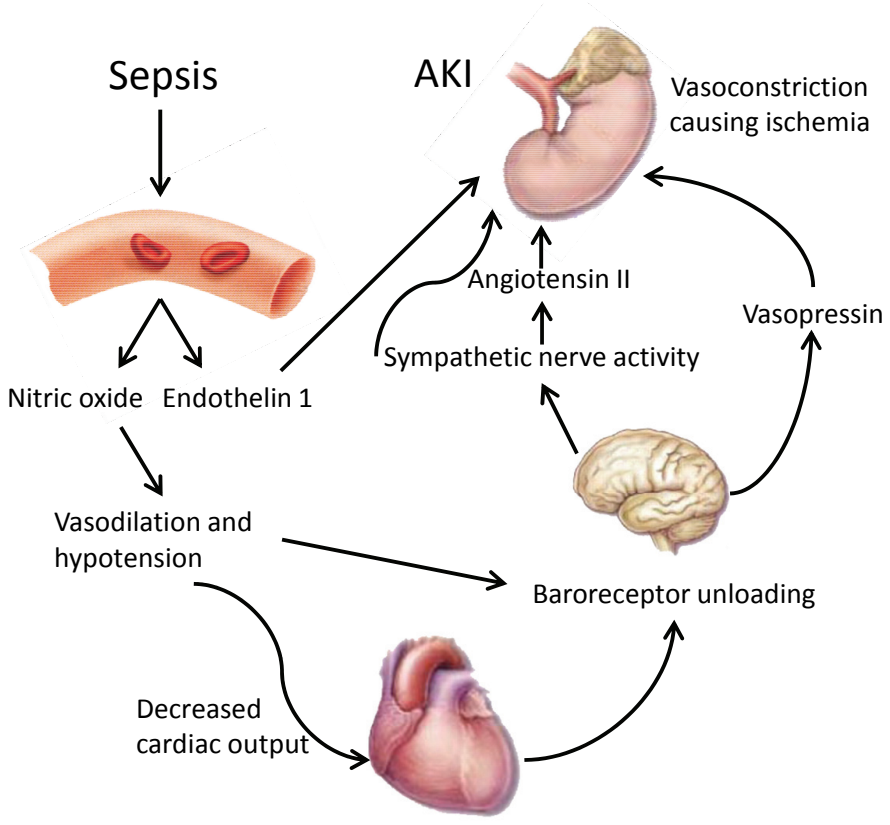

Figure 3. Schematic illustration of pathophysiological mechanism for septic AKI according to the current paradigm. This view suggests that increased sympathetic nerve activity and vasoactive hormones worsen renal vasoconstriction causing renal salt and water retention and subsequently ischemia.

Alternative concepts

Renal hyperperfusion

In the past years a different view of the pathophysiology in septic AKI has gained more focus in the literature. Increased perfusion of the kidney during sepsis has been observed in large animal experiments with hyperdynamic sepsis (Langenberg *et al.*, 2006b). Data from the continuous measurement of renal blood flow in human sepsis is however sparse, and the data supporting the hypothesis that renal blood flow is increased during sepsis are therefore mainly derived from animal experiments, but there are some evidence from human studies (Brenner *et al.*, 1990). An early observation in 1977 by Ravikant and Lucas showed in a porcine model of sepsis (live bacteria

intramuscular injection in the hind-limb) that renal blood flow increased as well as medullary blood flow (Ravikant & Lucas, 1977). Some of these results have later been reproduced in other large animal experiments, and the concept of increased or maintained renal blood flow in sepsis is gaining more attention (Langenberg *et al.*, 2006b). It is hypothesized that vasodilatation in the renal microcirculation causes a reduction in glomerular filtration pressure as GFR, in part, is a function of the relationship between the afferent and efferent arterioles. If the afferent arteriole dilates and the efferent arteriole dilates even more, RBF will increase, but the pressure within the glomerulus will fall and result in a reduced GFR.

Immune mediated dysfunction

Septic AKI is suggested by some to be more of a local inflammatory mediated process rather than a hemodynamic problem. This hypothesis is in line with a study in critically ill patients with severe sepsis, where blood pressure levels did not correlate with the severity of AKI (Chawla *et al.*, 2007). The production of cytokines, oxygen radicals and infiltration of inflammatory cells may cause direct inflammatory injury to the kidney. Systemic inflammation and local renal inflammatory pathways are suggested to be combined mechanisms inducing impairment of endothelial and microvascular function worsening renal injury (Murugan & Kellum, 2011;Majumdar, 2010;Murugan *et al.*, 2010). The role of proinflammatory cytokines in AKI is not well understood, but several experimental studies have implicated TNF-α, a pro-inflammatory cytokine, to be involved in the development of renal dysfunction in sepsis (Pelte & Chawla, 2009). TNF-α injures the kidney directly through specific receptors (TNF-R) and can cause glomerular inflammation and tubular damage (Ishikawa *et al.*, 2010). Apoptosis is an energy-requiring process of programmed cell death and in experimental septic AKI both TNF-α and LPS have shown to result in apoptotic cell death (Pelte & Chawla, 2009). Apoptosis have also been confirmed to be present in kidney biopsies from patients in septic shock combined with capillary leukocyte infiltration (Lerolle *et al.*, 2010). In addition, invading microbes during sepsis are identified by Toll-like receptors and upon stimuli they will initiate production of cytokines and activate inflammatory cells. These TLRs have been suggested to participate in the development of renal dysfunction in sepsis (El Achkar *et al.*, 2008), but the exact mechanisms were elusive prior to this thesis.

CURRENT TREATMENT OF SEPTIC AKI

Optimization of hemodynamic variables such as blood pressure and administration of fluids is often essential in the critically ill patient with sepsis. In the treatment of sepsis the use of drugs with a nephrotoxic effect, such as antibiotics and contrast agents is regularly the case in the clinical setting making prevention of AKI of clinical importance (Ricci *et al.*, 2011b). The clinical management of sepsis is often based on the recommendations from The Surviving Sepsis Campaign, an organization developed

to improve the management, diagnosis, and treatment of sepsis. They recommend, among several things, that urine output should reach ≥0.5ml/kg/h and mean arterial blood pressure should be supported to reach 65mmHg (Dellinger *et al.*, 2008). As fluid administration alone often is inadequate to restore optimal hemodynamic stability, infusion of vasoactive drugs such as norepinephrine is often used. Norepinephrine mediates vasoconstriction via α_1-receptors on vascular smooth muscle cells and increases heart rate and contractility of the heart via β_1-receptors. Loop diuretics, such as furosemide, is frequently used in the clinical setting to increase diuresis. Increased urine production by loop diuretics is mediated through inhibition of the $Na^+/K^+/Cl^-$ pump in the medullary thick ascending loop of Henle. By inhibiting this pump, the loop diuretics reduce the reabsorption of NaCl, which leads to more urine. This will also give a reduced oxygen demand in the kidney. The use of loop diuretics could therefore reduce the effect of ischemia, given that ischemia is involved in the pathophysiology of septic AKI. In the clinical setting aggressive fluid resuscitation to restore hemodynamic stability may lead to disturbed fluid balance, and loop diuretics can be used to reduce this by increasing urine output. If renal function is further reduced, extracorporeal blood purification with so-called renal replacement therapy is used as a last resort to restore homeostasis. The use of mechanical strategies to reduce fluid overload, correct electrolyte disturbance and metabolic disturbances is however more of a support rather than a curative measure in the failing kidney, but more advanced technologies are under development for removal of endotoxin and inflammatory mediators. Current pharmacological therapies and technologies are still inadequate for the treatment of AKI in sepsis and especially in septic shock, and mortality remains high (Ricci *et al.*, 2011b;Ricci *et al.*, 2011a).

ANIMAL MODELS OF SEPSIS

Several animal models of sepsis are used in research, and they posses different advantages and restrictions. The ideal model would be able to reproduce human sepsis in all of its complexity. This model should have similar hemodynamics, inflammatory response and show the same histological findings as in human sepsis. The most common models include administration of exogenous toxins (e.g. LPS), rupture of the animals endogenous barrier to intestinal bacterial flora (e.g., cecal ligation and puncture) or administration of live pathogens. These three types of models are used in both small animals (e.g. rodents such as mice and rat) and large animals (e.g. pigs, sheep and dogs). The use of large animals has some advantages, such as the possibility to perform: repeated blood sampling, extensive hemodynamic monitoring -- both systemic and organ-specific -- and to administer continuous infusion of fluids and/or therapeutic agents (Doi et al., 2009). The advantage of small animals such as mice is the availability of genetically modified animals or knockouts. Knockout mice are animals in which a specific gene has been deleted, and have proven useful in studying specific mechanisms of disease (Zanotti-Cavazzoni & Goldfarb, 2009). In this thesis

we used the LPS model in both pig and sheep (paper I-III). Infusion of live pathogens was used in sheep (paper IV).

Toxin administration

Models with exogenous toxin administration often use a LPS from Gram-negative bacteria such as E.coli. Administration of LPS results in an inflammatory response with production and release of cytokines such as TNF-α without the presence of bacteria (Michie *et al.*, 1988). However, the use of a single mediator might not reproduce the complex immunological responses seen in sepsis caused by viable pathogens. The cytokine response in human sepsis is usually less intense, if measured by plasma concentration of cytokines. The immune response is also prolonged over time compared to endotoxemia in animals (Fink & Heard, 1990a). Endotoxin administration does however result in the development of AKI, as indicated by a decline in GFR, reduced urine production, increase in blood urea nitrogen and creatinine (Cunningham *et al.*, 2004;Doi *et al.*, 2009). The dose of endotoxin can be adjusted from a lethal dose to a gradual decline in GFR but with stable hemodynamics. Many small laboratory animals, including mice, are relatively resistant to endotoxin, and this model may therefore be of limited use for some species. As seen in this thesis, the difference between anaesthetized pigs with mechanical ventilation and conscious sheep is significant regarding their responsiveness to a similar dose of LPS, paper I-II vs. paper III.

Rupture of the intestines

Another model often used in experimental sepsis research is cecal ligation and puncture (CLP). This model mimics the clinical scenario of sepsis after surgery, such as leakage from intestinal anastomoses after bowel surgery. Another example is a patient with a ruptured appendix. In the CLP model, the cecum is ligated and the ligated part is perforated with a needle. This perforation of the intestinal wall will result in a spread of feces with a poly-microbial content into the peritoneum (Buras *et al.*, 2005). This model can be adjusted in severity by changing the size and number of perforations in the caecum or by increasing the area of ligation. The advantage of this model is that the cytokine response is closer to human sepsis compared to endotoxemia. However, with the exception of some studies, in this model AKI seldom develops (Doi *et al.*, 2009). The poly-microbial flora in this model also makes it more difficult to elucidate the specific pathophysiological mechanism involved in the study. However, this model with spread of feces with a poly-microbial content into the peritoneum is by many considered very useful, because of the hemodynamic response with an early hyperdynamic phase as well as the typical cytokine profile (Doi *et al.*, 2009;Buras *et al.*, 2005).

Infusion of live pathogens

Bacterial infusion models allow the study of a single pathogen in a controlled experiment. These models have been used to study several aspects of sepsis, but of particular interest for this thesis is the response in renal hemodynamics and function in large animals of sepsis (Doi *et al.*, 2009;Langenberg *et al.*, 2006a;Langenberg *et al.*, 2007). The dose of bacteria is often high compared to the amount of bacteria found in blood cultures in patients, and the infused bacteria usually do not colonize the laboratory animal, which is the scenario in the clinical setting. The route of administration is commonly intravenous or intra-peritoneal, but administrations into the lungs to mimic pneumonia-induced sepsis have also been reported (Buras *et al.*, 2005).

THE ENDOTHELIN SYSTEM

The endothelin system was first described in 1988, by Yanagisawa et al. They discovered a very potent vasoconstrictor peptide from the culture supernatant of endothelial cells isolated from porcine aorta. They named this peptide endothelin (Yanagisawa *et al.*, 1988). The endothelins are produced in a variety of tissues including the kidney and mediate their function through a group of receptors, so-called endothelin receptors. Early after the discovery of the endothelins, a link between LPS and endothelin production was reported (Sugiura *et al.*, 1989). Elevated levels of endothelins was also demonstrated in septic patients (Weitzberg *et al.*, 1991b) and the connection to local effects in the kidney was later suggested (Weitzberg *et al.*, 1991a). The endothelins were initially described as modulators of vascular tone, cell proliferation and hormone production. Development of specific endothelin receptor antagonists has also increased our understanding for the endothelin system and its importance in vascular physiology (Levin, 1995). Endothelins are suggested to be involved in the development of hypertension, pulmonary disease, renal disease as well as numerous other diseases.

Production

Three endothelins, ET-1, ET-2 and ET-3 have been found to be encoded by human genes in chromosome 6, 1 and 20, respectively (Kawanabe & Nauli, 2011). These endothelins are produced by initial synthesis to pre-pro-endothelins, which is reduced in number of amino acids by intracellular peptidases to form big-endothelins. In this form, big-endothelin is further reduced in amino acids through cleaving by a group of enzymes named endothelin converting enzymes to form ET-1. ET-1 is suggested to be the most important isoform of the endothelins in the endothelium. In addition, the vast majority of studies regarding endothelins have focused on ET-1. The secretion of endothelins is maintained by two mechanisms: first, secretion of granule whit stored endothelin and second, constitutive secretion, which is believed to be the most important pathway (Khimji & Rockey, 2010). In vascular endothelial cells, production

of ET-1 is secreted toward the baso-lateral surface of the cells and acts directly on smooth muscle cells in an autocirne/paracrine pathway. Plasma levels of ET-1 is therefore much lower than within the tissue (Haynes & Webb, 1994). Production of new ET-1 is the main pathway for regulation of ET-1 levels, since granule secretion is of limited importance. The release of ET-1 is stimulated by several mediators such as cytokines, angiotensin II, LPS and insulin. ET-1 synthesis is inhibited by several other mediators such as NO, ANP, prostaglandin E_2 and prostacyklines (Piechota *et al.*, 2010).

Endothelin receptors

ET-1 mediates its effect through a group of G-protein coupled receptors. Two major types of receptors have been described, ETA and ETB receptors. The ETA is mainly found on smooth muscle cells and ETB primarily on endothelial cells. However, both have been localized on a variety of cell types in different organs. In general, ETA mediates vasoconstriction and cell proliferation. ETB, on the other hand mediates vasodilatation via the release and production of NO and prostaglandins. ETB is also suggested to be responsible for the clearance of endothelins from the circulation. Results from animal experiments have shown that the vascular endothelium in the lungs has a high concentration of ETB and these receptors are suggested to extract about 50-70% of plasma ET-1 during pulmonary passage (Dupuis *et al.*, 1996a;Dupuis *et al.*, 1996b). When ETB is located on vascular smooth muscle cells the effect can also be vasoconstriction (Levin, 1995). When ET-1 binds ETA an intracellular reaction occurs which leads to the activation of phospholipase C and the subsequent formation of inositol 1,4,5 triphosphate (IP3). Specific receptors on the endoplasmic reticulum are then opened by IP3, and a release of Ca^{2+} into the cytosol occurs. This elevation in intracellular Ca^{2+} causes cellular contraction by activation of actin–myosin cross-bridge cycling, which leads to vasoconstriction. In addition to this increase in Ca^{2+} ETA activation have been seen to induce cell growth and mitogenesis (Simonson & Dunn, 1990). ET-1 may also through activation of ETB stimulate NO production in endothelial cells by activation of endothelial cell NO synthase. Increased NO levels will lead to the production of cyclic GMP. This will reduce the intracellular levels of Ca^{2+} as well as activate of protein kinase G. As a result actin–myosin cross-bridge cycling is reduced leading to vasodilatation (Ito *et al.*, 2004).

Endothelin and the kidney

The endothelin system is a modulator of several mechanisms in the kidney including renal blood flow, GFR, reabsorption of water and sodium, and of the kidneys contribution to acid-base balance. ET-1 production in the kidney is increased by several factors including shear stress, inflammatory mediators, oxidative stress, by the renin-ANG II system (Kohan *et al.*, 2011). The major isoform of endothelins within the human kidney is ET-1, and ET-1 is suggested to mediate most of the renal effects

(Karet, 1996). A very high production of ET-1 is localized to the medulla and the collecting ducts. The inner medullary collecting ducts produces 10 times more ET-1 than any other tubular segment in the kidney (Karet, 1996;Kitamura *et al.*, 1989). ET-1 have however been shown to be produced in a variety of locations within the kidney including the endothelial cells lining intrarenal blood vessels, intra-glomerular endothelial cells, mesangial cells, podocytes and the tubular epithelium (Neuhofer & Pittrow, 2009). In the porcine kidney, both ETA and ETB have been identified in the glomerulus, but only ETB is found in papilla preparations (medulla) (Backer *et al.*, 2001). In comparison, human kidneys show ETA receptors in both the cortex and medulla (30% ETA of total number receptors in both medulla and cortex) (Nambi *et al.*, 1992). The vascular role of endogenous ET-1 in normal physiology may be to primarily act on ETB and mediate vasodilatation in the kidney, but selective ETA-blockade have shown to increase cortical and medullary blood flow (Evans *et al.*, 2004;Kohan *et al.*, 2011). On the other hand, exogenous infusion of ET-1, in systemic or renal circulation, reduces renal blood flow and GFR (Kohan *et al.*, 2011). ET-1 is suggested to constrict the vasculature in the kidney primarily via ETA. This effect is seen in larger renal arteries, such as the inter-lobar and arcuate arteries. The vasoconstrictive effect of ETA is also seen in the smaller vessels such as the afferent and efferent arterioles. The blood flow of descending vasa recta in the medulla have also been shown to be reduced by ETA activation within the kidney, in part by reducing arteriole diameter in juxtamedullary nephron. In the renal vasculature ETB acts on the endothelium to induce vasodilatation. Thus, ET-1 is able to induce both vasoconstriction and vasodilatation to regulate the vasculature an modulate renal circulation (Neuhofer & Pittrow, 2009). ET-1 can also induce remodeling in podocytes (the function of podocytes in the filtration barrier is closely related with their morphology, which features interactions of foot processes from adjacent podocytes). However, it is unclear if this effect can alter GFR (Kohan *et al.*, 2011). Renal water transport is modulated by the endothelin system within the kidney, and especially within the collecting duct. Arginine vasopressin (AVP) is an anti-diuretic hormone produced in the hypothalamus and secreted into the circulation. The renal effect of AVP includes enhanced water permeability in the distal tubule and collecting duct. This will result in increased water reabsorption and a subsequent increase in urine concentration. The endothelin system modulates this AVP-mediated effect and reduces the responsiveness of the collecting duct to AVP. It is suggested that this effect is carried out by ETB in the collecting duct, but the exact mechanism remains unclear (Edwards *et al.*, 1993;Kohan, 2009). ET-1 may also alter diuresis through the modulation of sodium reabsorption. Epithelial sodium channels in the collecting duct of the kidney are highly selective ion channels, performing active sodium reabsorption in the collecting duct. ET-1 rapidly decreases the opening of the epithelial sodium channels in the collecting duct through ETB activation (Bugaj *et al.*, 2008). ET-1 has also been suggested to induce an inhibitory effect on Na/K ATPase activity in the

medullary collecting duct though ETB. This mechanism is suggested to be in part mediated via increased prostaglandin E_2 (PGE_2) production in the collecting duct (Zeidel *et al.*, 1989).

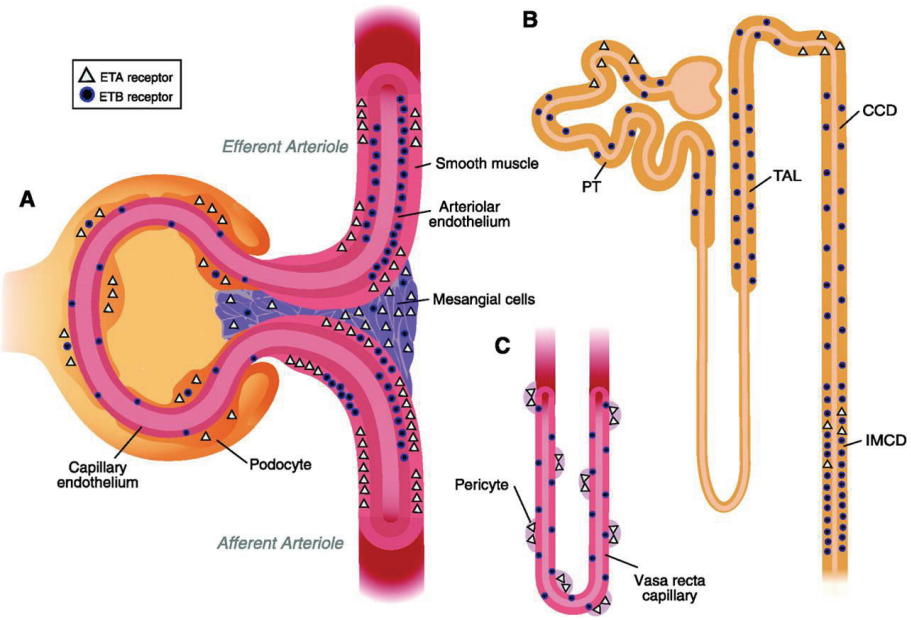

Figure 4. ET receptors in the glomerulus and renal arterioles (A), nephron (B), and vasa recta (C). The amount of ET receptor shown in a given area is representative of the level of ET receptor activity in that region. Afferent arteriolar smooth muscle has more vasoconstrictive ET receptors than do efferent arterioles, while efferent arteriole endothelium has more vasodilatory ETB than does afferent arteriole (A). The inner medullary collecting duct (IMCD) has the greatest density of natriuretic ET receptors, although natriuretic ET receptors exist in the cortical collecting duct (CCD), thick ascending limb (TAL), and proximal tubule (PT) (B). Vasa recta express contractile ETA on pericytes and vasodilatory ETB on endothelial cells (C). Kohan D E et al. Physiol Rev 2011;91:1-77. Re-produced with permission from the American Physiological Society

Endothelin and the kidney in sepsis

The endothelin system has been investigated with different antagonists in several experimental sepsis studies. These studies have been performed in rodents, dogs and pigs. The most common sepsis model is endotoxemia, but CLP and fecal peritonitis (a variation of CLP, but instead of rupture of the intestines, feces is simply put inside the peritoneal cavity) have also been used. The approaches have been administration of the antagonist prior to sepsis, so-called pre-treatment or after the onset of sepsis. The results from pre-treatment studies are not univocal. Pre-treatment with a dual ETA/ETB antagonist attenuated the rise in creatinine in endotoxemic rats (Ruetten *et al.*, 1996).

In another porcine study, pre-treatment with bosentan, also a dual ETA/ETB antagonist improved diuresis, without any effect on renal artery blood flow (Weitzberg *et al.*, 1996). In contrast, another porcine study reported that pre-treatment with a dual ETA/ETB antagonist in combination with diclofenac (a non-steroidal anti-inflammatory drug that inhibits prostaglandin synthesis by inhibition of cyclooxygenase) improved renal artery blood flow but not diuresis (Wanecek *et al.*, 1997). Positive renal effects with improved renal artery blood flow, urine output and creatinine have been reported in endotoxemic dogs pre-treated with another dual ETA/ETB antagonist, TAK-004 (Mitaka *et al.*, 1999). Pre-treatment with selective ETA or ETB antagonists report no effect on renal artery blood flow or creatinine levels in some rodent studies (Ruetten & Thiemermann, 1996;Gardiner *et al.*, 2001), but reduced creatinine was observed in another (Albertini *et al.*, 2003). In contrast, rats pre-treated with selective ETB or dual ETA/ETB antagonists reduced total renal blood flow and cortical microcirculation, but no effect was seen on GFR (Nitescu *et al.*, 2008). In summary, pre-treatment with selective or dual ETA/ETB antagonists in sepsis/endotoxemia report conflicting renal effects, but results from evaluation of renal microcirculation is sparse. There are a few studies that administer the ET-antagonists after the onset of experimental sepsis, and these effects are also diverse. In a porcine endotoxemia study, dual ETA/ETB antagonism did not show any effect on renal artery blood flow (Oldner *et al.*, 1998), but renal blood flow (microsphere technique) and GFR increased in another porcine endotoxemic study (Chin *et al.*, 2002). In addition, a porcine study reported some effect on renal cortical blood flow in fecal peritonitis, but this effect was abolished after resuscitation (Krejci *et al.*, 2003). Dual ETA/ETB antagonism have recently reported reduced renal inflammation after sepsis induced by CLP in rats (He *et al.*, 2006). The effects of endothelin receptor antagonists in experimental sepsis are not clear, and contradictory effects on renal hemodynamics have been reported. Prior to the work of this thesis, conflicting results about renal artery blood flow was reported (Chin *et al.*, 2002;Weitzberg *et al.*, 1996;Wanecek *et al.*, 1997;Mitaka *et al.*, 1999;Oldner *et al.*, 1998;Ruetten & Thiemermann, 1996;Gardiner *et al.*, 2001) which urged further studies, especially on the renal microcirculatory level (paper I). During the work with this thesis (paper II), the effect of selective endothelin antagonists on renal microcirculation have been reported in pre-treated rats (Nitescu *et al.*, 2008), and in a porcine model with treatment after the onset of fecal peritonitis (Krejci *et al.*, 2003).

TOLL-LIKE RECEPTORS

The discovery of the Toll-like receptors (TLR) introduced a new link between several pathogens and the actions of the immune system. The first mammalian TLR to be discovered was the Toll-like receptor 4 (TLR4). The recognition of LPS was linked to TLR4, and further studies revealed more information of this signaling pathway (Poltorak *et al.*, 1998a;Qureshi *et al.*, 1999a;Hoshino *et al.*, 1999a). This family of

receptors was first described in the drosophila as important genes in the embryonic development of the fly. The discovery of these signaling pathways and their regulation has increased our understanding of sepsis. Toll-like receptors act as pattern recognition receptors for a range of molecules, such as bacterial components, fungi, viral components but also for host specific molecules. Their response includes the activation of immune cells and production of inflammatory substances (e.g. cytokines) (Takeda & Akira, 2007). Until present date, 10 members of the Toll-like receptor family have been identified in humans, but up to 13 in other species. The Toll-like receptor family in humans can be divided into two groups depending on their location. The first group TLR1, TLR2, TLR4, TLR5 and TLR6 is located on the cell surface for detection of microbial cell wall components. The other group is TLR3, TLR7, TLR8 and TLR9 and they are located within the cell in vesicles. This intra-cellular group mainly recognizes microbial nucleic acids (Kawai & Akira, 2010).

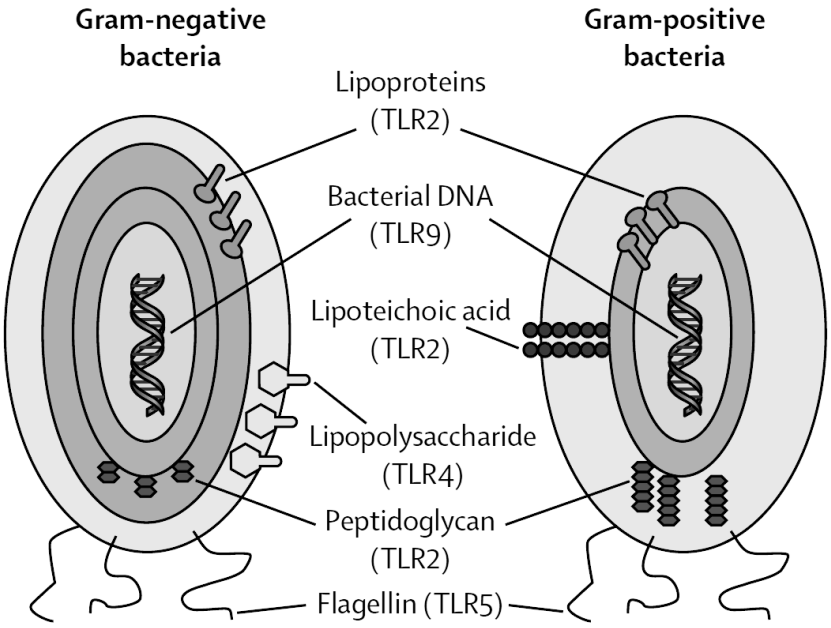

Figure 5. Gram-positive and Gram-negative bacteria are recognized by overlapping and distinct repertoire of TLRs. Gram-positive pathogens express lipoteichoic acid, Gram-negative pathogens express lipopolysaccharide; common pathogen include peptidoglycan, lipoproteins, flagellin, and bacterial DNA. Reproduced with permission from the Lancet.(van der & Opal, 2008)

Toll-like receptor 4

The first receptor in this family to be identified in mammalians was the Toll-like receptor 4 (TLR4). This receptor was shown to be involved in the recognition of LPS, the major component in Gram-negative bacteria (such as E.coli) (Poltorak *et al.*, 1998b;Hoshino *et al.*, 1999b;Qureshi *et al.*, 1999b). However other molecules, besides TLR4 are also necessary for the recognition of LPS. The first step involves the binding of LPS to LPS-binding protein. This complex is identified by CD14 (surface protein mainly on macrophages and neutrophils), a co-receptor for TLR4. Myeloid differentiation 2 (MD-2) has also been identified as a co-receptor for TLR4. MD-2 forms a complex with TLR4 and enhances the receptor's responsiveness for LPS. Other molecules than LPS have been suggested to act as ligands for TLR4. These molecules are often a result of cell death and/or injury. Heat shock proteins and extracellular matrix components such as fibronectin, hyaluronic acid, and heparan sulfate have been implicated as ligands in TLR4 signaling (Triantafilou *et al.*, 2001). HMGB1 is a pro-inflammatory mediator in septic shock that is released by inflammation or necrotic cells and is recognized by TLR4 (also by other TLRs such as TLR2 and TLR9) (Yang & Tracey, 2010;Yang *et al.*, 2010). TLR4 is the main receptor for LPS, but other receptors may also be involved in the recognition of this molecule. For example, when LPS is transported into the cells after recognition by TLR4, other intracellular recognition mechanisms may be important, such as NOD1 receptors (Inohara *et al.*, 1999). The expression of Toll-like receptors is widespread in several types of cells. Inflammatory cells such as monocytes, macrophages, dendritic cells and mast cells have all been reported to express different Toll-like receptors (Takeda & Akira, 2007). However, TLR4 is expressed not only on circulating immune cells but also within the kidney itself, and the expression of renal TLR4 in tubules, glomeruli and peri-tubular capillaries are increased after sepsis (El Achkar *et al.*, 2006;Samuelsson *et al.*, 2004).

Toll-like receptor 4 signaling

Toll-like receptors are trans-membrane proteins with domains containing leucine-rich repeats that mediate the recognition of pathogens. They consist of trans-membrane domains and an intra-cellular domain, named the Toll–interleukin 1 (IL-1) receptor (TIR) domains. These TIR are required for the downstream intracellular signal transduction (Kawai & Akira, 2010). Individual Toll-like receptors activate different inflammatory responses. The response from an activated TLR4 includes both a cytokine response and an interferon response. These different responses are mediated by different adaptor molecules binding the TIR-domain in the intracellular space. TLR4 uses at least four adaptor molecules in its response. MyD88, the first identified member of this TIR family adaptor molecule activates the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokine production (Akira *et al.*, 2006). TRIF another TIR family adaptor molecule induces a pathways for activation of the transcription factors IRF3 and NF-κB as well as

induction of type I interferon and inflammatory cytokines. TRAM and TIRAP, TIR family adaptor molecules functions as sorting adaptors that recruit TRIF to TLR4 and MyD88 to TLR4, respectively. TLR4 binds TIRAP at the plasma membrane; thereafter this complex binds MyD88 and starts the initial activation of NF-κB. The TLR4 receptor complex is moved into the endosome and forms a new complex with TRAM and TRIF, instead of TIRAP and MyD88, which starts the TRIF-dependent pathway that leads to the late-phase activation of NF-κB. TLR4 activates both the MyD88-dependent pathway and the TRIF-dependent pathway and activation of both these pathways results in the induction of inflammatory cytokines by TLR4 signaling, This signaling pathway is specific for TLR4, whereas the other known Toll-like receptors need only to activate one of these pathways to result in an inflammatory response (Kawai & Akira, 2010).

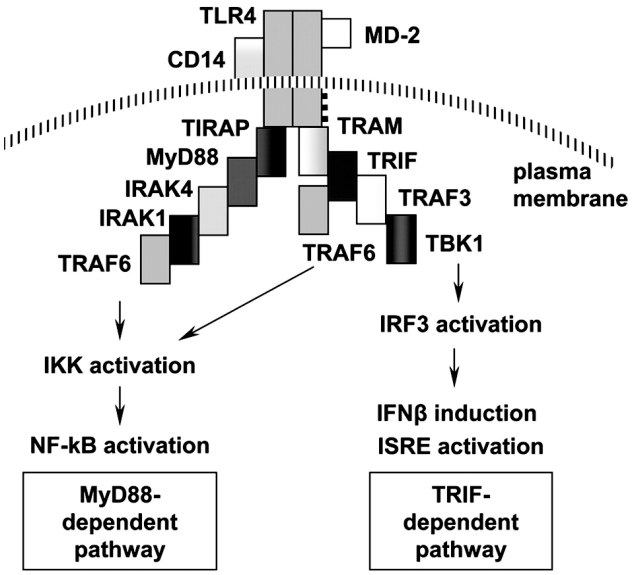

Figure 6. TLR4 signaling illustrating MyD88- and TRIF-dependent pathways reproduced with permission (Matsunaga et al., 2011).

Activation of the Myd88-dependent pathway results in increased production of many inflammatory genes for inflammatory mediators and also for co-factors of NF-κB subunits, which will induce specific production of cytokines such as IL-6, IL-10 and TNF-α but also co-factors that will restrict NF-κB activity (Kawai & Akira, 2010). In the other pathway, TRIF activates co-factors that lead to NF-κB activation, through mechanisms similar to those of the MyD88-dependent pathway. In addition to NF-κB activation, the TRIF-dependent pathway leads to IRF3 activation and interferon-β transcription. The TRIF-dependent pathway also inhibits the activation of the MyD88-dependent pathway (Kawai & Akira, 2010). Toll-like receptor signaling also involves other negative-feedback loops, thus preventing overwhelming activation of their

signaling. These negative-feedback loops acts on several levels and includes splice variants of their different adaptor molecules and transcription regulator molecules. Alternative splicing is a process by which RNA are reconnected in multiple ways during RNA splicing. The resulting different mRNA may be translated into different protein isoforms, which can lead to altered activity of the protein.

TLR4 and the kidney in sepsis

The kidney is susceptible to infections by bacterial invasion through the urinary tract system which could lead to urinary tract infection and following pyelonephritis, but also by acute kidney injury caused by sepsis. First renal TLR4 mRNA was discovered in human kidneys, and then the full TLR4 receptor was located in the renal tubules of the human kidney. TLR4 in renal tissue is found in both renal cortex and medulla, predominantly at the tubules and collecting ducts (El Achkar *et al.*, 2008). The expression of TLR4 is increased in tubules, glomeruli, and the renal vasculature in experimental sepsis. In septic rats CD14, the essential co-factor for LPS binding to TLR4, is also increased (El Achkar *et al.*, 2006). In addition, systemic infusion of radio-labeled LPS in rats reaches the renal tissue, indicating that uptake of LPS occurs within the kidney. However, if this uptake of LPS in renal tissue is TLR4-dependent remains unclear, though indications from experimental data suggest such a pathway (El Achkar *et al.*, 2008;Musson *et al.*, 1978). This hypothesis, that systemic LPS reach the tubule where both TLR4 and CD14 are expressed is supported by experimental findings in rodents. LPS is probably filtered in the glomeruli and reabsorbed by proximal tubular cells through the apical membrane (El Achkar *et al.*, 2008;Musson *et al.*, 1978). The importance of TLRs in mediating septic AKI has been implicated by experimental studies, including an important observation in mice with a mutation in TLR4 (Cunningham *et al.*, 2004). These mice shown reduced BUN levels (BUN levels are used as an estimation of renal function), renal neutrophil infiltration and renal cell apoptosis after LPS injection compared to mice with normal TLR4 (wild-type).The investigators then performed a cross-transplantation of kidneys between wild type and TLR4 knockout mice. These mice were then subjected to an injection of LPS. When TLR4 was present systemically but not in transplanted kidneys, the mice developed severe AKI. When TLR4was present only in the transplanted kidney and not systemically, less severe AKI developed. However, no observations of systemic circulation or renal hemodynamics were performed in these mice.

AIMS

The overall aim of this thesis was to describe the pathogenesis of septic renal failure, with special emphasis on the endothelin system and the Toll-like receptor 4.

More specifically, the studies aimed to:

- Establish experimental models for studies of septic renal failure
- Characterize renal microcirculatory changes in hypo- and hyperdynamic experimental sepsis
- Investigate the role of the endothelin system in causing renal microcirculatory and functional impairment in experimental septic renal failure
- Investigate whether TLR4 activation during experimental sepsis is beneficial or detrimental for kidney function and, in case of the latter, treatment with a TLR4-inhibitor has favorable effects once renal impairment is already present

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The experiments were conducted in accordance with "The European Convention for Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (Council of Europe No 123, Strasbourg 1985). The Regional Ethics Committee for Experiments in Animals, Stockholm, Sweden approved the studies in advance.

	Species	Model	Observation time	
Paper I	Pig	LPS	5 hours	Anaesthetized
Paper II	Pig	LPS	5 hours	Anaesthetized
Paper III	Sheep	LPS	24 hours	Conscious
Paper IV	Sheep	Live E.coli	36 hours	Conscious

The pig

In this thesis two different species were used, the pig and the sheep. In paper I and II we used crossbred (Landrace/Yorkshire/Hampshire) female anaesthetized pigs. Pigs are omnivores by nature and share many similarities with humans in their metabolism of fat, proteins and glucose. They are very sensitive to stress (Van Zuphen *et al.*, 2006a) and are therefore not suitable to be used in conscious models of sepsis. The pig is often used in renal research because there are many similarities in both anatomy and physiology of the porcine and human kidney (Dalmose *et al.*, 2000). Differences of interest include lower urine concentration ability and higher GFR in pigs compared to the human kidney (Ibrahim *et al.*, 2006). Diuresis is larger in the pig mainly due to its reduced ability to concentrate urine (Hammer & Thein, 2002), and a degree of proteinuria is often seen in pig as compared to none in humans (Ibrahim *et al.*, 2006). The renal artery and its intrarenal branches also share many similarities with those of the human kidney but are not identical to the human kidney. The renal veins in the porcine kidney have many anastomoses, and the arrangement of the venous system cannot be completely transposed between the species, but the likeness is considered to be large. Also the constitution of the collecting duct system and the size of the kidney share many similarities between the porcine and human kidney (Dalmose *et al.*, 2000;Bagetti Filho *et al.*, 2008;Pereira-Sampaio *et al.*, 2004;Sampaio *et al.*, 1998). The porcine model used in this thesis was adapted, with slight modifications from previous

work by Somell et al, who showed that treatment with tezosentan improved cardiac and pulmonary function in endotoxemia (Somell *et al.*, 2007).

The sheep

Paper III and IV in this thesis describe conscious experiments performed in crossbred ewes. Both the endotoxin and the live E.coli model were used to study renal effects of a Toll like receptor 4 antagonist. Sheep are ruminants, and in addition to their stomach they have three pro-ventriculi: the rumen, the reticulum and the omasum. Sheep feed only on vegetable material, and the process of bacterial fermentation in the rumen is a very important step in the digestion and uptake of nutrients. Sheep spend 10-15 hours per day on rumination. The main source of energy in the sheep is volatile free fatty acids formatted in the rumination by bacterial fermentation. Glucose metabolism is therefore of less importance in these animals compared to humans. In addition, the normal physiological pH in the sheep is around 7.50. They are by nature calm, which makes them suitable to conscious experiments when they have adapted to the laboratory environment (Van Zuphen *et al.*, 2006b).

SURGICAL PREPARATIONS AND PROTOCOLS

All surgical preparations were performed under general anesthesia. However, insertion of intravascular catheters and urinary retention catheters in paper III were performed after administration of local anesthesia in conscious animals.

Paper I and II (porcine endotoxemia)

Anesthesia

Anesthesia was initiated by a pre-medication with ketamine hydrochloride (10 mg/kg intramuscular injection), midazolam (0.5 mg/kg i.m.), and atropine sulfate (0.05mg/kg i.m). This first step of pre-medication was performed with one pig separated from the group in a separate boxed area but with visual contact with the other pigs. The procedure was performed by injection with an 18 gauge (G) needle connected to an extension line and a syringe. The animals were then transported from the housing quarters to the laboratory room with their eyes covered to minimize visual stimuli. Induction of anesthesia was then performed via one of the veins of the pigs' ear with intravenous administration of propofol (60±20mg). The animals were orally intubated and ventilated with oxygen in air (FiO2 0.44) and a PEEP of 4 cm H2O. Ventilation was adjusted to reach a PCO_2 of 4.7–5.3 kPa at baseline and was kept constant throughout the experiment. During surgical preparation and the following experiment, anesthesia was maintained with sevoflurane [2.6% endtidal concentration (Et%), followed by 1.0 Et% throughout the experiment] and an infusion of fentanyl (10 mg/kg/h) and midazolam (0.15 mg/kg/h) throughout the experiment. If needed, additional doses of fentanyl and midazolam were given. Pancuronium bromide (0.5

mg/kg/h) was given for muscle paralysis after control of the depth of anesthesia. The urinary bladder was catheterized with a Foley catheter No. 8 for urine collection. A separate monitor was used for monitoring of the electrocardiogram and airway gas parameters.

Fluid resuscitation

During the surgical preparation of paper I and II, a continuous infusion of hydroxyethyl starch 130/0.4 60 mg/ml was given at a rate of 10 ml/kg/h to all animals. In paper I, all animals received a continuous infusion of saline with glucose 25 mg/ml at a rate of 20 ml/ kg/h throughout the experiment. In paper II, a continuous infusion of Ringer´s Acetate (15 ml/kg/h) and saline with glucose 25 mg/ml (5 ml/ kg/h) was started directly after the surgery and kept constant throughout the five-hour experiment.

Intravascular catheters

Measurements of MAP and blood sampling were achieved with a single lumen catheter inserted into the left carotid artery. Fluid infusions were administered through a triple lumen catheter inserted into the left jugular vein. A balloon-tipped pulmonary artery catheter was connected to a Vigilance Monitor system and inserted through the right jugular vein. The position in the pulmonary artery was decided by pressure guidance on a monitor. The pressure transducers used in this thesis were calibrated to atmospheric pressure at the level of the right atrium and to 100mmHg using a saline column.

Measurement of renal macro- and microcirculation, paper I and II

The animals were positioned in a supine position, and a midline laparotomy was performed after control of the depth of anesthesia. An ultrasonic flow probe for continuous registration of regional blood flow was placed around the left renal artery (size 3 or 4 mm) and connected to a recorder. A laser doppler probe was sutured to the surface of the left kidney for measurements of cortical microcirculation.

Measurements in the medulla were also included in paper II, in addition to the cortex probe. A needle laser doppler probe was inserted 10-12 mm into the kidney for medullary measurements. Position was confirmed visually by opening the kidney post mortem. Calibration was performed according to the manufacturer's instructions before inserting the laser doppler probes. The quality of the laser doppler signal was confirmed before closing the abdomen.

Microdialysis (paper II)

In paper II, two microdialysis catheters were inserted in the kidney. A small opening of the capsule was made with a needle, thereafter one probe was inserted in the cortical region of the kidney. The second microdialysis catheter was inserted in a right angle in a separate opening of the capsule and placed in the renal medulla. Position was

confirmed visually by opening the kidney post mortem. The two probes were continuously perfused at a speed of 1µl/min with a standard perfusion solution. A stabilization period of 60 minutes was allowed before baseline measurements. Samples were collected for 10 minutes at baseline, two and five hours after start of endotoxemia. All samples were analyzed immediately on a bench top analyzer.

Experimental protocols

Porcine endotoxemia (paper I and II)

All animals received a continuous infusion of endotoxin (Escherichia coli lipopolysaccharide, serotype 0111:B4, 900 000 units/µg endotoxin, Sigma-Aldrich Sweden AB, Stockholm, Sweden). Endotoxin infusion was started at 0.3125 µg/kg/h and was increased stepwise until reaching 2.5 µg/kg/h after 30 min. The rate was then kept constant throughout the experiment (5 hours).

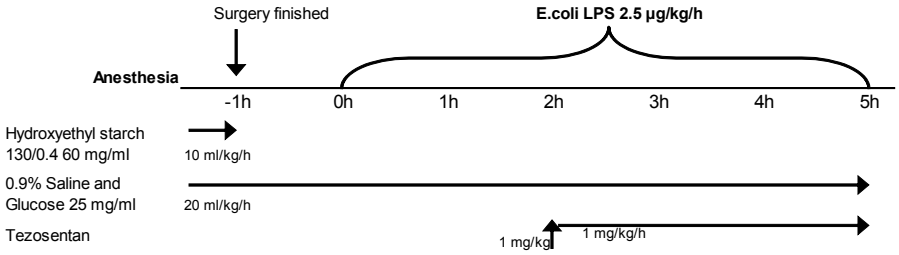

Figure 7. Schematic illustration of the experimental protocol in paper I. Pigs were surgically instrumented and subjected to 5h Escherichia coli (E.coli) lipopolysaccharide (LPS) infusion. After 2h of endotoxemia they were randomized to receive a bolus dose followed by a continuous infusion of either Tezosentan, a dual ETA/ETB antagonist (n=8), or to serve as controls (n=8).

Paper I Dual Eta/ETB antagonism

The animals were randomized to receive treatment with tezosentan (n=8) or to serve as controls (n=8). After two hours, the animals in the tezosentan group were treated with tezosentan 1 mg/kg administered over 10 min and then, a continuous infusion with tezosentan 1 mg/kg/h were started. After five hours of endotoxemia, the animals were terminated by a lethal dose of sodium pentobarbital injected into a central vein.

Paper II selective ETA antagonism

After two hours of endotoxemia animals were randomized to receive treatment with the selective ETA antagonist, TBC 3711, 2 mg/kg (n=8) or to serve as controls (n=8). The dose of TBC 3711, with no ETB effect, was based on the results from pilot experiments described by Andersson in (Andersson *et al.*, 2010). At the end of the experiment, the animals were terminated by a lethal dose of sodium pentobarbital injected into a central vein.

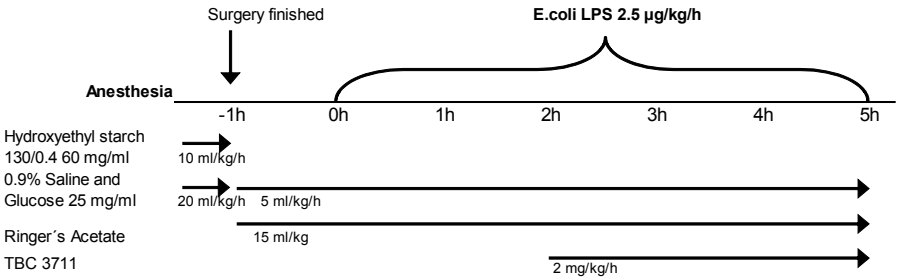

Figure 8. Schematic illustration of the experimental protocol in paper II. Pigs were surgically instrumented and subjected to 5h Escherichia coli (E.coli) lipopolysaccharide (LPS) infusion. After 2h of endotoxemia they were randomized to receive a bolus dose of the selective endothelin receptor type A antagonist TBC 3711 (n=8) or to serve as controls (n=8.)

Paper III (ovine endotoxemia)

In view of our findings that anesthesia with isoflurane aggravates the renal impairment caused by LPS, conscious animals were used for further investigations in paper III and IV (Frithiof *et al.*, 2011). The porcine model previously used by our group was not suitable for this purpose since pigs are easily stressed in conscious experiments. Prior to the experiments one carotid artery was surgical exteriorized and placed in a cervical skin loop that were closed around the artery. This procedure makes the arteries accessible for easy cannulation. This surgical procedure was performed under general anesthesia with isoflurane (2.1–2.3% end-tidal concentration). Induction of anesthesia was made by intravenous sodium thiopental (10 mg/kg). All animals were intubated and mechanically ventilated. Post-operative treatment for 2 days with buprenorphine (0.002 mg/kg) and benzylpenicillin (20 000 IU/kg)–dihydrostreptomycin (0.0025 g/kg) were made routinely to reduce post-operative pain and risk of infection. The animals were allowed a recovery period of 7-14 days to allow wound-healing before the conscious endotoxemia experiments.

Paper III Pre-treatment with a TLR4 inhibitor

The animals were randomized to receive a bolus dose (2 mg/kg), followed by a continuous infusion (4 mg/kg/24 h) of either the selective TLR4 inhibitor, TAK-242 (n =7), or vehicle (n=7). Randomization was blinded. Endotoxemia was started by intravenous infusion of E.coli LPS after the bolus dose of TAK-242 or vehicle. The infusion rate was slowly increased during the first 30 min to reach 3µg/kg/h. This rate was then maintained for the remainder of the experiment (24 hours). In an additional group, LPS was administered as previously described. However, to prevent hypotension, norepinephrine was titrated to maintain MAP at baseline levels. Fluid volume support was administered as Ringer's acetate solution at 0.5 ml/kg/h in all three groups. In the norepinephrine group, additional volume support was given as bolus doses (200–250 ml) of hydroxyethyl starch if MAP did not increase as expected in

response to norepinephrine. Blood samples were drawn at baseline and at 6, 12, 18, and 24 hours after the start of endotoxemia. Urinary output was measured, and urine samples were collected every second hour.

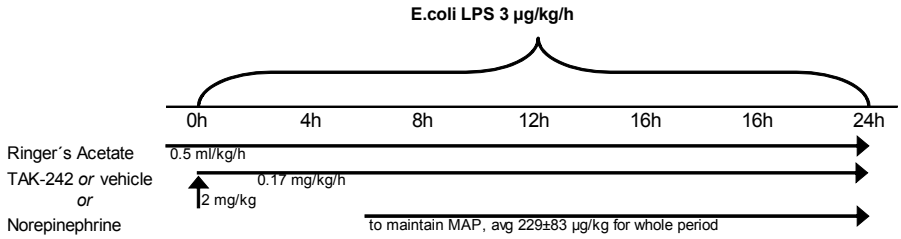

Figure 9. Schematic illustration of the experimental protocol in paper III. Before an Escherichia coli (E.coli) lipopolysaccharide (LPS) infusion for 24 h, sheep were randomized to receive a bolus dose, followed by a continuous infusion, of either TAK-242 (n= 7) or vehicle (n= 7). A third group of LPS-treated sheep (n = 6) received norepinephrine, titrated to maintain baseline arterial blood pressure (MAP). In the norepinephrine group, additional volume support was given as bolus doses (200 –250 ml) of hydroxyethyl starch if MAP did not increase as expected in response to norepinephrine.

Paper IV (ovine live E.coli infusion)

In this paper, the animals were subjected to one surgical preparation and were then allowed to recover for 12-18 hours before the experiments commenced.

Anesthesia

Surgical anesthesia included premedication with acepromazin (0.3 mg/kg), in order to reduce stress and discomfort for the animals. Anesthesia was conducted by the same protocol as in paper III.

Intravascular catheters

A small incision was made and the carotid artery was visualized and cannulated. Intravascular catheters were inserted in the jugular veins and a flow-directed thermodilution catheter was fed into the pulmonary artery via an introducer in the right jugular vein. The position of the catheter was determined by pressure guidance on a monitor. All catheters were sutured to the skin and the incision above the carotid artery closed. Proper fixation of these catheters was essential for a successful experiment. The catheter was secured and positioned in way that discomfort for the animals was minimal.

Measurement of renal macro- and microcirculation, paper IV

After a flank incision the left kidney was visualized and an ultrasonic flow probe was placed around the renal artery. Thereafter a laser Doppler probe (0.25mm fiber

separation, 780nm wavelength) was sutured to the surface of the kidney for cortical measurements and a needle laser Doppler probe (0.15mm fiber separation, 780nm wavelength) was inserted 10-12 mm into the kidney for medullary measurements. Two microdialysis catheters (as in paper II) were inserted into the renal cortex and medulla. After surgical preparation, the flank incision was carefully closed. Connecting tubes and their connectors placed at a paralumbar position. Postoperative fluid support was given of hydroxyethyl starch, 10 ml/kg, iv (130/0.4, 60 mg/ml, Voluven). Peri- and postoperative analgesics were administered as buprenorphine (0.002 mg/kg im) and fentanyl (1 µg/kg iv).

Figure 10. Schematic illustration of the experimental protocol in paper IV. Sheep were surgically instrumented and subjected to 36h infusion of live E.coli. After 12h of sepsis they were randomized to receive a bolus dose followed by a continuous infusion of either TAK-242, a selective TLR4 inhibitor, (n=7) or vehicle (n=7).

Paper IV, treatment with a TLR4 inhibitor in manifest septic AKI

The experiments were performed with conscious animals placed in individual pens with access to water and hay ad libitum. A bacterial infusion was started with a bolus ($3.9 \cdot 10^9$ CFU) followed by an infusion ($6.0 \cdot 10^9$ CFU/), starting at a rate of 0.2ml/h. The infusion rate was then increased stepwise every 6 hours until reaching 4 ml/h after 30 hours and then kept constant until the end of the experiment. After 12 hours of sepsis sheep were randomized to receive a bolus dose (2 mg/kg) followed by a continuous infusion (4 mg/kg/24hours) of either the selective TLR4 inhibitor, TAK-242 (n=7) or vehicle (n=7). To exclude that the surgery *per se* had a major impact on the results obtained in the TAK-242 and vehicle groups, an additional sheep served as time-control. This included surgical preparation, recovery and monitoring for 36 hours, but no E.coli infusion or treatment. Fluid volume support was administered as Ringer´s Acetate solution iv at 1 ml/kg/h and started six hours before the infusion of live E.coli bacteria. After 36 hours of sepsis animals were deeply anaesthetized with sodium thiopental and terminated by an overdose of potassium chloride. The kidney was rapidly harvested and prepared for histological evaluation. If an animal showed signs of great discomfort during the experiment they were anaesthetized with sodium thiopental and terminated by an overdose of potassium chloride. This was the case for nine out of 23 sheep subjected to live E.coli. These animals were euthanized before completion of the protocol. Five of these nine animals were euthanized before any treatment was started. In addition, between 12 and 14 hours of sepsis two TAK-242 treated animals

and two vehicle-treated animals were also euthanized. These nine sheep were not included in the data.

HEMODYNAMIC AND METABOLIC MONITORING

In all four papers of this thesis hemodynamic recordings were performed. In paper I, II and IV carotid artery was surgically visualized and cannulated for measurement of arterial pressure. In paper III the carotid artery was put in a skin loop on the neck of the sheep for easy access. From the obtained arterial pressure curve mean arterial pressure and heart rate was calculated. The pressure transducers were calibrated to atmospheric pressure at the level of the heart and to 100 mmHg or 25 mmHg using a saline column. These transducers have a continuously active flush of 3 ml/h.

Thermodilution

In paper I-IV of this thesis cardiac output was measured with thermodilution technique. A flow-directed thermodilution catheter was fed into the pulmonary artery via an introducer in the right jugular vein and the position was determined by pressure guidance on a monitor. The pulmonary artery catheter was connected to a Vigilance Monitor system where the cardiac output was calculated every 30-60 seconds and core temperature was measured continuously. When the body temperature exceeded the upper range for automatic measurements (i.e. 41 degrees Celsius), manual cardiac output measurements were performed by three consecutive injections (10 ml) of isotonic saline. In brief, manual cardiac output measurements were performed as follows:

1. A 10 ml of saline (indicator) with a known temperature was injected into the right atrium through the catheter.

2. The circulation carried the saline through the heart, where it was mixed and temperature in the diluted blood lowered.

3. This diluted blood was carried by the circulation and the temperature was measured by the catheter after the heart, in the pulmonary artery.

4. These changes generated a time-temperature curve. The area under the curve is inversely proportional to cardiac output.

Some of the known errors from the manual cardiac output measurements are variations in temperature of the indicator, variations in volume (i.e. if 9 ml of saline is injected instead of 10 ml, resulting in an overestimation of cardiac output) and dead space volume of saline within the pulmonary catheter (Reuter *et al.*, 2010). Repeated measurements (three injections) were performed to increase reproducibility in the manual measurements. The continuous measurements of cardiac output by the Vigilance Monitor system also rely on the thermodilution principles. But, instead of

injecting cool saline in a bolus, blood in the superior vena cava is heated intermittently by an electric filament. The filament is heated for 1 to 4 seconds in a random sequence. These measurements are put together into a single thermodilution curve (Yelderman, 1990;Yelderman *et al.*, 1992). Continuous measurements may have limited accuracy during periods of rapid changes in cardiac output, due to time delay (5-15 minutes to detect changes in cardiac output from 20-80%) (Reuter *et al.*, 2010).

Ultrasonic flow probes

An ultrasonic flow probe was used for the measurement of renal artery blood flow in study I, II and IV. The probe was positioned around the left renal artery either after a midline laparotomy (paper I and II, size 3 or 4 mm probe) or after a flank incision (paper IV, 4 mm probe). The ultrasonic flow probes consist of a probe with an ultrasonic transducer and an acoustic reflector. The transducer sends out an ultrasonic wave through the vessel (renal artery for this thesis) in the upstream direction, and then hits the acoustic reflector. The probe then sends out a wave in the opposite direction (down-stream) and hits a reflector. After being received by the transducer the ultrasonic waves are converted into electrical signals. From these signals, the flowmeter derives an accurate measure of the time for the wave to pass the vessel (transit time). The ultrasonic beam is measured at several points, the receiving transducer sum these measurements over the renal artery, which can then be calculated to a volume flow.

Laser Doppler flowmetry

This technique is used in paper I, II and IV, and gives an estimate of the blood flow in the renal microcirculation. The main principle of this technique is the so-called doppler effect (Stern, 1985;Stern *et al.*, 1979;Stern *et al.*, 1977). The doppler effect states that a wave undergoes a change in frequency when the source of the wave or the observer of the wave is moving in position. This effect is sometimes exemplified by the high pitch of a siren when an ambulance is moving towards you and the following drop in pitch from the siren when the ambulance passes by. The laser doppler probe sends out a ray of light (laser), which hits the area of interest, in this thesis the renal cortex or medulla. This light will either be absorbed by the renal tissue itself, or scattered after hitting moving objects (e.g. red blood cells) or static objects (e.g. renal cells). The scattered light from moving objects will undergo a shift in frequency and scattered light from static object will not undergo a shift. All reflected light will be recorded by a photo-detector within the probe and carried out to the main unit. This signal is transformed to an estimate of the changes in velocity and often called Flux unit or Perfusion unit. This technique does not allow a *true* measurement of flow but instead an estimate of the flow measured in arbitrary units (Stern, 1985). One reason for this is that the volume of tissue being reflected is not static (maximum of $1mm^3$ with the probes used in this thesis). The depth of the signal in the tissue is limited by factors such as, the optical qualities of the tissue (darker tissue = less depth of the signal) and the distance between

sender and receiver in the probe (Stern, 1985;Stern *et al.*, 1979;Stern *et al.*, 1977). To reduce variations in measurement each probe is calibrated in a solution with a known inherent movement, often called a motility standard. Another limitation of this technique is that movement of the laser doppler probe or its base unit also interferes with the signal, therefore in paper IV, when the probes are used in conscious animals extra care was made for securing the probes to the surface of the kidney. However, any

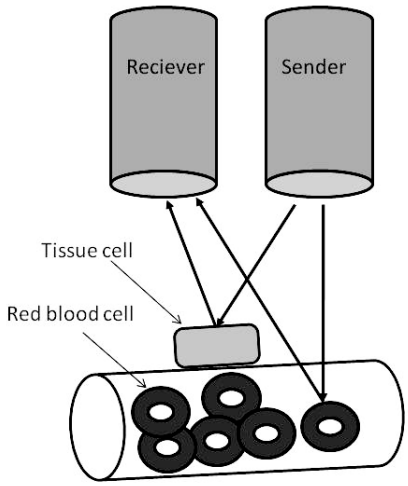

Figure 11. Schematic drawing of a laser doppler probe. The source sends out a beam of light. Both tissue cells and red blood cells will reflect the light to the detector. Light hitting moving objects will undergo a shift in frequency, the doppler effect. This signal is transformed to an estimate of red blood cell velocity.

movement of the animals will reduce the quality of the laser doppler signal. The individual flow in a specific micro-vessel, heterogeneity of the microcirculation or number of vessels with active flow cannot be measured with this technique.

Microdialysis

The interstitium is defined as the area between the capillary wall and the surrounding cells. As the interstitial fluid surrounds the cells, its composition is often interpreted as the local metabolic status of the cells. Regional monitoring have the advantage of revealing local disturbance that may be difficult to detect in systemic plasma samples. We therefore used the microdialysis technique which enables repeated measurements of the interstitial fluid. The microdialysis catheter is constructed by a membrane, permeable for water and small solutes, such as glucose, lactate and pyruvate. This catheter is introduced in the tissue of interest, in this thesis the renal cortex and medulla. The catheter is continuously perfused with a fluid that will pass by the membrane and then be collected for analysis. When the perfusion fluid reaches the membrane a diffusion of molecules between the extra-cellular fluid and the perfusion fluid will take place. The perfusion fluid is often called dialysate after this diffusion of molecules. The content of the dialysate is then analyzed. This technique gives an estimate of the interstitial fluids that surrounds the cells in the tissue of interest. However, the collected dialysate will reflect only a fraction of the true concentration in

the interstitial fluid. This proportion is often referred to as the *recovery* of a substance in the dialysate. The recovery of a substance is dependent of several factors including flow rate of the perfusion fluid, area of the semi-permeable membrane and temperature. The highly vascularized tissue in the kidney increases the risk for bleeding when inserting the probes, however only small amounts of blood was seen during the surgical preparations in both the pig and sheep model. In addition local damage and necrosis at the probe site may have an influence on the measurements. In paper II, a stabilization period of 60 minutes was allowed before baseline measurements. The perfusion speed was set to 1µl/min and samples were collected for 10 minutes at baseline, 120 and 300 minutes after start of endotoxemia in anaesthetized pigs. In paper IV, a recovery period of 12-18 hours was allowed before baseline measurements was performed for six hours. The perfusion speed was set to 0.3 µl/min. Every sample period was then six hours and continued during 36 hours of sepsis in conscious sheep.

Interventional substances and procedures

Endotoxin

LPS from E.coli bacteria was administered intravenously in paper I, II and III to create an experimental model of sepsis. LPS is a component of the cell wall of the Gram-negative bacteria and stimulates the release of inflammatory mediators, such as cytokines (Fink & Heard, 1990b). LPS accounts for approximately 70% of the outer membrane of Gram-negative bacteria and is essential for cell viability for Gram-negative bacteria (except for *Neisseria meningitides*, in which some strains do not produce LPS) (Opal & Gluck, 2003). The toxicity of LPS is related to the host response to this microbial mediator, because LPS presumably have no toxic properties by itself (Beutler & Rietschel, 2003). This model is reproducible and often used in animal models, but is not considered to be identical of sepsis in humans (Poli-de-Figueiredo *et al.*, 2008). LPS is built up by a polysaccharide antigen (O-antigen) which is connected to an oligosaccharide and a glycolipid structure (lipid A). Lipid A is the portion of the molecule that LPS is anchored to the cell membrane. The O-antigen is composed of repeating oligosaccharide extending out from the bacteria, and this structure and composition vary between serotypes of bacteria. This structure can protect the bacteria against complement complex and protect from several antibiotics. Lipid A is the region of LPS that activate TLR4. The number and the length of lipid A's fatty acids vary among different bacteria. The length of these fatty acids has a major influence on the biological activity of lipid A (Banoub *et al.*, 2010).

Because endotoxin forms micelles and binds to glass surfaces, solutions of endotoxin were vortexed for 30 minutes or longer before dilution or direct use. Endotoxins are highly heat-stable and resistant to extreme pH values and are not destroyed under regular sterilizing conditions. Endotoxin can be inactivated when exposed at temperature of 250ºC for more than 30 minutes or 180º C for more than 3 hours. The

term endotoxin units (EU) describe the biological activity of an endotoxin and make comparison between different batches of LPS easier.

Live Escherichia Coli

A live E.coli infusion was used in paper IV to induce experimental sepsis. In all animals the same strain of bacteria was used. The bacteria in the study were isolated from a positive blood culture acquired from a septic patient and identified as E.coli. The isolate was sub-cultured in broth in conical bottles and incubated at 30°C over night on a rotary shaker. The broth was centrifuged and the pellet was re-suspended in phosphate buffered saline. The bacteria were counted, batched in $2 \cdot 10^{11}$ colony forming unit (CFU) bacteria per tube and frozen at −70 °C for further use. LPS is, as mentioned before, a component of the cell wall of the bacteria and continuously released into the surroundings. The release does not only occur with cell death but also during growth and division of the bacteria. A single E.coli bacterium contains about two million LPS molecules. In addition to LPS, E.coli bacteria consist of several products that can be recognized by the immune system such as, bacterial DNA which can be recognized by TLR9, lipoproteins detected by TLR2, peptidoglycan detected by TLR2 and flagellin detected by TLR5, as mentioned in previous chapters. Thus the E.coli bacteria may be identified by the immune system through several different TLRs.

Tezosentan

Tezosentan is a potent and water soluble endothelin receptor antagonist. This drug has a high affinity to ETA receptors, but also to the ETB receptor. The potency against ETA over ETB is about 30 times. This is a competitive antagonist with a short half-life, about 10 minutes in humans (Clozel *et al.*, 1999). The primary elimination mechanism is biliary excretion of unchanged compound. However, some metabolism occur by hydroxylation, but the impact of this metabolite is suggested to be low, and no accumulation has been reported (Dingemanse *et al.*, 2002). In paper I, animals received tezosentan as a bolus injection 1mg/kg followed by a continuous infusion at 1 mg/kg/h. The dose of tezosentan was selected in accordance to findings by Chin et al who showed beneficial effects on renal function at this dose in neonatal piglets (Chin *et al.*, 2002).

TBC 3711

This drug was used in Paper II due to its high potency and selectivity for ETA over ETB (approximately 400000-fold for ETA over ETB). TBC3711 have a half-life of about 7 hours in humans. (Wu *et al.*, 2004).

Norepinephrine

In order to explore if any effect of TAK-242 was due to preventing hypotension, a group of LPS-treated animals received norepinephrine titrated to maintain baseline blood pressure in paper III. Norepinephrine mediates several effects on the circulation. Vasoconstriction is mediated via α_1-receptors on vascular smooth muscle cells. Norepinephrine also increases heart rate and contractility of the heart via B_1-receptors.

TAK-242

TAK-242, a cyclohexene derivative, is a small-molecule compound that selectively inhibits TLR4 signaling. TAK-242 binds to the intracellular TIR domain of TLR4 and by doing so inhibits TLR4 signaling by disrupting the interactions of TLR4 with its adaptor molecules As a result NF-κB activation and cytokine gene expression in response to LPS are inhibited (Matsunaga *et al.*, 2011).

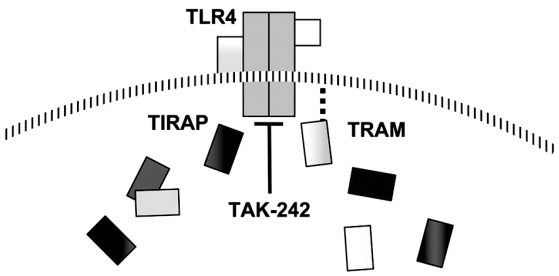

Figure 12. TAK-242 a selective TLR4 inhibitor interferes with interactions between TLR4 and its adaptor molecules, TIRAP and TRAM. Reproduced with permission (Matsunaga et al., 2011).

Enzyme-linked immunosorbent assay

In paper I, enzyme-linked immunosorbent assay (ELISA) manufactured kits were used for the determination of porcine plasma levels of three cytokines, porcine TNF-a, porcine IL-10, and porcine IL-6. All ELISA-kits were specific for the respective porcine cytokine. In brief, in each kit antibodies against the respective cytokine was pre-coated in wells of a plate. Samples as well as control and standard were put into the wells. If the sample contains the cytokine, it is bound to the pre-coated antibody in the well. After washing the wells, a monoclonal antibody with an enzyme linked to it is added and bounds to the antibody/cytokine complex. After additional wash the substrate for the enzyme is added in a solution. The reaction by the enzyme and substrate solution will result in a blue product that will change color to yellow when a stop-solution is added. The intensity of the color, measured in on a plate-reader, is in proportion to the amount of the respective cytokine bound in the initial step of the assay.

Radioimmunoassay

In paper I, plasma concentrations of aldosterone and angiotensin II were measured by radioimmunoassay (RIA) using commercially available kits. In paper I and II of this

thesis, plasma concentrations of ET-1 was also measured by RIA as previously described by Hemsen (Hemsen, 1991). In brief, radioimmunoassay is a sensitive method for measuring very small amounts of a substance.

N-acetyl-β-D-glucosaminidase

N-acetyl-β-D-glucosaminidase (NAG) is a proximal tubular brush border lysosomal enzyme, which is released into the urine after renal proximal tubule injury. The relatively high molecular weight (140 kDa) prevents filtration of the enzyme by glomeruli. Small alterations in the epithelial cells in the brush border of the proximal tubules result in release of NAG into the urine and the amount of enzyme have been directly correlated to tubular injury. The use of urinary NAG activity as a marker of acute and chronic kidney injury was described in 1975 by Wellwood and colleagues (Wellwood *et al.*, 1975;Liangos *et al.*, 2007).

Statistical analysis

The results are presented as means and standard error of the mean (SEM) for paper I-III. In paper IV results are presented as means and standard deviation of the mean (SD) or as mean and 95% confidence interval. Main effects were analyzed using a two-way analysis of variance with time as a repeating variable as within effects and treatment as between effects. In paper III the study investigated the effect of three different treatments (TAK-242, Vehicle or Norepinephrine) and if the ANOVA resulted in a significant main effect, pairwise comparisons were performed. To address the problem of multiple comparison Bonferroni correction was used. Transformation of the data was performed by taking the logarithm of the raw data, if it did not follow a normal distribution.

RESULTS AND COMMENTS

The present thesis is based on four separate papers, of which two are published and two are presented in manuscript form. Detailed information about results, methods and discussion are found in the respective paper. This section will highlight some specific results and in brief put them in perspective.

ETA/ETB IN PORCINE ENDOTOXEMIA

Paper I

The aim in paper I of this thesis was to evaluate the role of ET-1 in endotoxemia. The hypothesis was that inhibition of ETA and ETB with tezosentan would attenuate the deterioration in renal cortical microcirculatory blood flow. The rationale for this hypothesis was that previous experimental results had shown that exogenous administration of ET-1 reduced renal cortical blood flow and constricted cortical and juxtamedullary arterioles (Denton *et al.*, 2004). In addition to this vascular effect under physiological conditions, previous studies had demonstrated beneficial effects of tezosentan on total renal blood flow in endotoxemic shock (Chin *et al.*, 2002). However, the results of previous studies investigating the effects of endothelin receptor blockers where not univocal, as another endothelin receptor blocker, bosentan, had shown contradictory effects on renal hemodynamics in animal models of sepsis (Krejci *et al.*, 2003;Heyman *et al.*, 2000;Wanecek *et al.*, 1997;Mitaka *et al.*, 1999;Oldner *et al.*, 1998). In addition to the renal effects, dual ETA/ETB antagonism had been reported to improve cardiac function, reduce pulmonary hypertension, and reduce the inflammatory response in animal studies, prior to our investigations (Konrad *et al.*, 2004;Rossi *et al.*, 2004;Urbanowicz *et al.*, 2004). ET-1 had also been reported to induce inflammation by stimulating the release and production of pro-inflammatory cytokines and activation of the transcription factor nuclear factor κB (NF-κB), all important mediators in the septic inflammatory response (Cunningham *et al.*, 1997;Browatzki *et al.*, 2000).

In paper I, animals were randomized to treatment with tezosentan or to serve as control and the main finding in this investigation was that treatment with tezosentan attenuated the acute reduction of renal artery blood flow as well as the perfusion in the cortical microcirculation in response to endotoxemia. In contrast to these results in paper I, bosentan, a dual ETA7ETB antagonist with less pronounced ETA affinity over ETB compared to tezosentan, had failed to alter renal cortical blood flow during septic shock (fecal peritonitis) in pigs (Krejci *et al.*, 2003). Different experimental models (fecal peritonitis vs. endotoxemia) as well as differences in pharmacological properties between tezosentan and bosentan could account for the discrepancies in effect on the renal vasculature. The results from Krejci *et al* were also difficult to interpret. In their

study, bosentan was given after two hours of shock induced by fecal peritonitis, and in the control group renal cortical microcirculation was significantly lower compared to the bosentan-treated group before any treatment was given. This makes statistical interpretation of their renal results difficult (Krejci et al., 2003).

As the decline in cardiac output was attenuated by tezosentan, one could argue that the improved renal artery blood flow and cortical microcirculation seen in Paper I only reflects the increase in cardiac output. However, earlier studies including those of Krejci and colleagues have failed to demonstrate renal improvements, despite positive changes in cardiac output (Krejci *et al.*, 2003;Heyman *et al.*, 2000;Wanecek *et al.*, 1997;Mitaka *et al.*, 1999). Thus, changes in systemic circulation could not be translated into a correspondent change in renal perfusion, at least in the aforementioned experimental studies.

As mentioned earlier, an additional aim was to evaluate the role of ET-1 in the early inflammatory response in endotoxemia. Interleukin (IL)-6 is a cytokine produced by both inflammatory and non-inflammatory cells and its production is induced by acute inflammatory reactions such as sepsis. The release of IL-6 is stimulated by TNF-α and other pro-inflammatory cytokines and is therefore often used as a marker of proinflammatory cytokine activation. IL-6 has also been suggested as mediator of thrombosis in sepsis (Song & Kellum, 2005). Another cytokine IL-10 is on the other hand has anti-inflammatory properties and reduces the production of both IL-6 and TNF-α. It was described prior to the initiation of paper I, that ET-1 had inflammatory properties (Cunningham *et al.*, 1997;Browatzki *et al.*, 2000;Sasser *et al.*, 2007), but in Paper I, tezosentan could not attenuate the plasma release of IL-6, IL-10, or TNF-α. The effects of tezosentan on renal perfusion, at least in this early phase of endotoxemia, appeared not to be mediated by interference with these cytokines. However, measuring plasma levels of these cytokines was not sufficient to rule out any changes in local production of these cytokines in the kidney.

The clearance of ET-1 from the circulation had been suggested to mainly be mediated by ETB receptors and inhibition of ETB leads to increasing levels of ET-1 in the circulation (Fukuroda *et al.*, 1994). This effect was demonstrated in paper I, and treatment with tezosentan resulted in a significantly higher arterial plasma level of ET-1 compared with the control group. Since a close interaction between endothelin and the renin–angiotensin system had been described prior to this thesis, plasma levels of ANGII and aldosterone were examined in paper I. ET-1 had been shown to mediate some of the renal vasoconstriction and cardiovascular effects of angiotensin II (Alexander *et al.*, 2001;Herizi *et al.*, 1998). In addition, it was reported that ET-1 stimulate the synthesis and release of aldosterone in the adrenal cortex , via both ETA and ETB receptors (Rossi *et al.*, 2000). This made it reasonably to propose that tezosentan exerts some of its effects through the renin–angiotensin–aldosterone systems. This could, however, not be confirmed in paper I, at least not by changes in

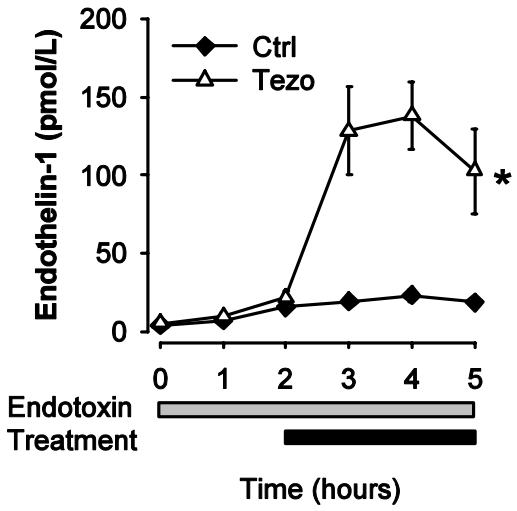

Figure 13. Changes in plasma levels of endothelin-1 in response to endotoxin infusion and subsequent tezosentan (1 mg/kg/h) or control. Treatment was started at 2 hours of endotoxemia. Symbols show mean and SEM. *p<0.05 for the time–treatment interaction between 2 and 5 hours

plasma levels of aldosterone or ANG II between the groups. Given that ET-1 had been shown to contribute to some of the renal vasoconstrictor actions of ANG II (Riggleman *et al.*, 2001). Reduced vasoconstrictor effect of ANGII in the renal circulation by tezosentan in endotoxemia could however not be ruled out.

These results in Paper I showed that treatment with a dual ETA/ETB antagonist attenuated the acute reduction of renal perfusion in response to endotoxemia. A more preserved diuresis was expected as well. This was however not the fact, and a suggestion was that this was related to the ETB blockade by tezosentan as activation of ETB stimulates diuresis and natriuresis. Thus, a possible diuretic effect by the increase in renal blood flow could have been offset by the ETB inhibition. As the increase in plasma creatinine was significantly attenuated, a better preserved GFR by tezosentan could be suggested. However, urine production was very low, and no clearance measures were performed, which could have gained a more accurate estimation of GFR compared to merely measuring plasma creatinine. It was suggested that the effect of dual ETA/ETB-antagonism on cortical microcirculation seen in paper I were mainly mediated by the ETA inhibition. This made further study of selective ETA antagonism interesting, and paper II was initiated.

ETA IN PORCINE ENDOTOXEMIA

Paper II

As mentioned before, ET-1 had been described to be a modulator of the renal microcirculation by constricting afferent and efferent arterioles mainly through activation of ETA (Denton *et al.*, 2004). Furthermore, ET-1 had been shown to have tubular effects favoring diuresis and natriuresis described under normal physiological

conditions. The natriuretic effect of ET-1 was believed to be mediated through a reduction of Na/K ATPase activity in the collecting ducts (Zeidel *et al.*, 1989) and through an inhibitory effect on the opening of epithelial Na channels in the collecting duct by ETB (Bugaj *et al.*, 2008). The diuretic effect of ET-1 was suggested to primarily be due to the inhibition of arginine-vasopressin stimulated water permeability by activation of ETB (Edwards *et al.*, 1993;Kohan *et al.*, 2011). It was hypothesized in paper II that ET-1 mediates renal microcirculatory impairment in endotoxemia through activation of ETA and that treatment with TBC 3711, a highly selective ETA antagonist would improve renal microcirculation and function. The theoretical rationale for this was that the diuretic and vasodilatory effects of ETB would be preserved whereas the vasoconstrictive and pro-inflammatory properties of ETA would be inhibited. Pigs were subjected to LPS infusion for five hours and after two hours of endotoxemia they were treated with TBC 3711or served as endotoxin-treated controls. The main finding in paper II was that ET-1 reduced renal medullary perfusion due to activation of ETA-receptors. This medullary effect was independent of changes in renal artery blood flow, suggesting that the effect was mediated on a microcirculatory level. Furthermore, ETA-receptor antagonism also attenuated renal oxygen demand and the L/P-ratio measured in the renal cortex but had no significant effect on diuresis or creatinine clearance.

As described in paper I, dual ETA/ETB antagonism attenuated the decline in renal artery blood flow and cortical microcirculation. This was not reproduced in paper II with selective ETA antagonism. There are several factors that could have influenced this difference in results between paper I and paper II. First, the effect on cardiac output by the ETA-antagonist in this study was less pronounced than the effect of tezosentan in the previous investigation. Second, in this normotensive model of endotoxemia, selective ETA antagonism caused hypotension. The reduced arterial pressure in the TBC 3711 group may have reached the lower limit of the autoregulatory range for RBF in pigs and influenced any effect by ETA antagonism on renal hemodynamics (Buckley *et al.*, 1983). However, this hypotensive effect of ETA antagonism further indicates that the maintained medullary perfusion in the treatment group was not perfusion pressure-dependent. Moreover, no difference in renal artery blood flow was detected between groups indicating that the effect of TBC 3711 was on a microcirculatory level in the medulla. This effect may have derived from redistribution of blood flow between juxtamedullary and cortical glomeruli, which could have lead to more perfusion of the medulla. Another alternative was redistribution towards pre-glomerular shunts.

Hypoxia in the kidney is a matter of vigorous debate in the pathogenesis of septic acute kidney injury. In paper II, the early phase of endotoxemia renal oxygen delivery was increased, indicating that oxygen supply was not impaired at the onset of renal damage. Though renal artery blood flow was reduced by approximately 30%, oxygen delivery was maintained and a major reason for this was hemoconcentration. Endotoxemia often

results in a relative hypovolemia and reduced plasma volume. This leads to hemoconcentration and can be used as an indicator of hypovolemia. However, especially in pigs, hemoconcentration may be caused by extensive constriction of the spleen. This leads to increased amount of red blood cells in the system circulation, and spleen contraction have earlier been shown to contribute up to 20-25% of the red cell volume in pigs (Hannon *et al.*, 1985).

Reduced total renal oxygen demand was seen in the TBC 3711-treated group. As reabsorption of sodium is the most oxygen-demanding process in the kidney, changes in oxygen consumption may reflect a reduced work load of the tubules. However, no differences were seen in sodium excretion (estimated by FENa), renal artery blood flow or creatinine clearance. The control animals developed a more significant acidosis compared to TBC 3711-treated pigs and acidosis have been reported to influence renal oxygen handling (Evans *et al.*, 2010). Lower pH (or higher carbon dioxide) reduces the binding affinity of oxygen to hemoglobin (Bohr *et al.*, 1904), and this will result in more oxygen being released in the kidney when metabolic demand increases. This effect was highlighted in a rat model (Chen *et al.*, 2010), but the relative impact in the porcine kidney remains to be explored.

The limitations of endogenous creatinine-clearance for determination of GFR may have influenced the results in paper II. When GFR is reduced, overestimation by creatinine-clearance may occur in pigs due to the tubular secretion of creatinine. It could therefore not be ruled out that the reduced oxygen-consumption in the TBC 3711-treated group was a result of reduced GFR.

Our hypothesis that selective ETA antagonism would improve diuresis by preserving ETB function was not supported by the findings in paper II. This might have been due to an inadequate observation period, but it may also suggest that the relative importance of using a specific ETA-antagonist for maintaining diuresis during endotoxemia is of limited use.

Paper I and paper II of the first part of this thesis evaluated both selective ETA and dual ETA/ETB antagonism in porcine endotoxemia. First, the results from paper I confirmed previous investigations that mixed ETA/ETB antagonism with tezosentan may attenuate the decline in renal blood flow induced by endotoxemic shock. Second, selective ETA antagonism attenuated the decline in medullary blow flow in normotensive endotoxemia. However neither selective nor dual ETA/ETB antagonism could improve diuresis.

TLR4 IN OVINE ENDOTOXEMIA

Paper III

Study III was conducted to investigate the role of TLR4 in the development of LPS-induced AKI in sheep. To do this, conscious animals were pre-treated with the selective TLR4 inhibitor TAK-242 or vehicle and subjected to 24 hours of endotoxemia during which renal function was monitored with measurements of creatinine clearance, urine output and plasma levels of BUN and creatinine. The main finding was that pre-treatment with a TLR4-inhibitor reduced the renal dysfunction caused by endotoxemia. In order to explore if any effect of TAK-242 was due to preventing hypotension, a third group of LPS-treated animals receiving norepinephrine titrated to maintain baseline blood pressure was added. However, preserving MAP with norepinephrine and

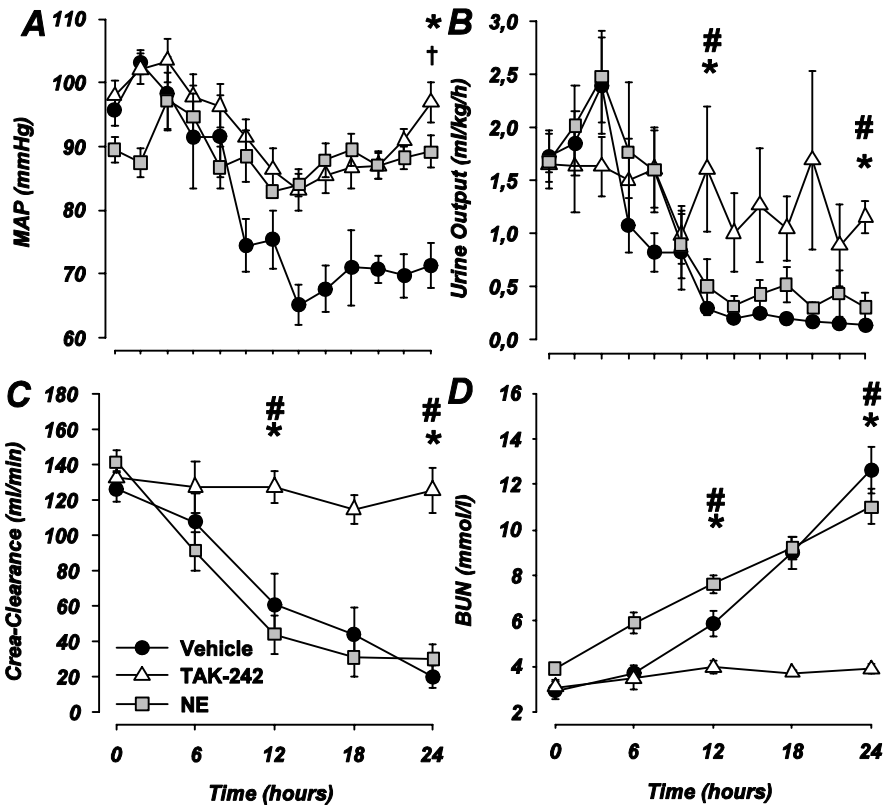

Figure 14. Changes in MAP (A), urine output (B) creatinine clearance (C) and BUN in response to LPS-infusion and treatment with either the selective TLR4 antagonist, TAK-242 (6 mg/24 hours, n=7) or vehicle (n=7). Data are expressed as mean ± SEM. * indicates a significant difference between TAK-242 and vehicle in response to LPS. †, indicates a significant difference between norepinephrine (NE) and vehicle in response to LPS.

additional fluid did not prevent the development of AKI. Thus, the renal protective effect of TLR4 inhibition was not dependent of prevention of hypotension. It was proposed that hypotension was of less importance for renal dysfunction in paper III. However, as no measurement of renal blood flow was performed in paper III, the question of any participation of renal hypoperfusion and ischemia warranted further research and renal hemodynamics was later investigated more thoroughly in paper IV.

In paper III, TAK-242 did not reduce the fever-response to LPS. In earlier studies performed in rodents, TAK-242 had prevented some changes in body temperature (i.e. hypothermia). The lack of effect on body temperature could have been a consequence of incomplete inhibition of TLR4. The pharmacological TLR4 inhibitor that was used, TAK-242, binds directly to the amino acid Cys747 in the intracellular domain of the receptor (Takashima et al., 2009) and inhibits the activation of the downstream signaling pathway of the receptor (see figure 12 in the Materials and Methods section). Experiments to confirm the specificity of TAK-242 for TLR4 had been reported prior to paper II, mainly in rodents and humans. However, sheep TLR4 corresponds well to human TLR4 (Menzies & Ingham, 2006) but if this also reflects the same binding affinity for TAK-242 in not clear. Another proposed explanation for the remaining fever response was that the commercially LPS used in our studies was not perfectly pure and may contain pyrogens activating receptors other than TLR4.

In paper III, TLR4 inhibition was administered prior to endotoxemia and the subsequent development of AKI. Several questions were generated after performing this study. First, paper III did not elucidate if TLR4 inhibition could reverse an already present AKI. Second, are changes in renal hemodynamics by TLR4 inhibition responsible for the beneficial effect on renal function? Third, is TLR4 inhibition effective if renal dysfunction were to be induced by live bacterial infection with a broad variety of pathogen patterns instead of a single toxin model such as LPS infusion? These questions urged further experiments, which were performed in paper IV.

TLR4-INHIBITION AS A TREATMENT OF SEPTIC AKI

Paper IV

One of the main objectives of paper IV was to investigate if TLR4 inhibition is effective in attenuating or reversing renal dysfunction even after sepsis has developed. Therefore, TAK-242 was administered 12 hours after the onset of sepsis and the observation time was extended to 36 hours. Live E.coli infusion resulted in sepsis with a normotensive circulation for the entire experiment. To further explore the hypothesis that septic AKI was due to regional blood flow restriction, total renal blood flow as well as cortical and medullary microcirculation was measured.

The laser doppler technique had previously been used in both paper I and II, but the use in conscious animals added some complexity. Optical fibers chronically implanted in

conscious rats for detection of renal cortical and medullary blood flow changes by laser-doppler flowmetry had previously been used and validated in chronic studies (Mattson *et al.*, 1994;Lu *et al.*, 1993). However, in paper IV the experiments started 12-18 hours after the probes were surgically implanted. This was a limitation, and may have influenced the result. To address this issue a sham-experiment was performed with a sheep subjected to surgery and the following protocol without treatment or sepsis. However, a chronic implantation of laser doppler probes with a following recovery period of several weeks is indeed an alternative that is of great interest for further investigations.

The renal histopathology was not clear in septic AKI and studies investigating structural damage to the kidney in long-term animal model of sepsis were sparse, (Langenberg *et al.*, 2008). In paper IV, both light and electron microscopy were used to analyze renal samples taken at the end of the 36 hour study period with regards to possible morphological changes causing the renal dysfunction. An additional objective to this study was to explore potential local immunological pathways causing septic AKI. In order to do this, histological sections were stained for infiltrating leukocytes. Anti-myeloperoxidase antibodies were used to stain leukocytes and helped the investigator to identify neutrophils.

The main finding in paper IV was that TAK-242 reversed a progressive decline in renal function when administered therapeutically after 12 hours of hyperdynamic E.coli sepsis. This was associated with a reduction in renal neutrophil infiltration as well as an attenuation of arterial and renal hyperlactemia. Signs of decreased swelling in the endothelium of the glomerular capillaries were also observed. In addition to this, no major effects on systemic or local renal hemodynamics by TAK-242 were seen. As this ovine model of sepsis, resulted in a normotensive circulation combined with severe renal dysfunction, it was suggested that hypotension was not a necessary attribute for septic AKI. This was in line with earlier studies in critically ill patients with severe sepsis, where blood pressure levels did not correlate with the severity of AKI (Chawla *et al.*, 2007). Previous investigations had shown that experimental septic AKI developed although total renal blood flow was increased (Langenberg *et al.*, 2006b;Frithiof *et al.*, 2011) and when hypotension was counteracted by fluid and norepinephrine (paper III). The findings in this thesis (paper I, II and IV) show diversity in the renal blood flow response to endotoxemia and sepsis, but they are not the only ones reporting contradictory results in this matter. Findings on renal blood flow in experimental sepsis have been reviewed by Langenberg and coworkers. Their systematic review of 159 experimental studies showed that 99 studies reported decreased RBF whereas 60 reported unchanged or increased renal perfusion (Langenberg *et al.*, 2005). They also tried to investigate variables that could influence RBF in these studies. Several variables were chosen including animal size, small animals (rats, mice, rabbits and piglets) versus large animals (dogs, pigs and sheep).

Technique for measuring RBF, direct measurement of RBF (ultrasonic or electromagnetic flow probes) was compared to indirect measurement (microspheres, para-aminohippurate (PAH), video microscopy). Other investigated variables were recovery period between surgical preparation and actual experiment, duration of sepsis, animal model of sepsis (e.g. LPS, live bacteria and CLP), fluid resuscitation, consciousness of animals and cardiac output. They concluded that the available experimental data was extraordinarily heterogeneous. The sole variable that was significant after a multivariate logistic analysis was cardiac output (CO). Low CO predicted a decrease in RBF and an increased or preserved CO predicted increased or preserved RBF (Langenberg *et al.*, 2005). In view of the papers included in this thesis, data show both decreased (paper I and II) and increased renal artery blood flow (paper IV). Indeed a difference in cardiac output between the different papers was found, but if other variables, such as consciousness also may have influenced renal blood flow response is not clear. With regards to the effects of TAK-242, no major effects on systemic or local renal hemodynamics by were seen in paper IV.

Regarding measurements of metabolism in paper IV, both cortical and medullary L/P ratios were significantly increased by sepsis but subsequently reduced by TAK-242. In view of the preserved renal circulation the renal hyperlactemia cannot easily be explained by reduced renal oxygen supply. Though, as mentioned before, one of the limitations of the laser doppler technique is that heterogeneity in the renal microcirculation cannot be detected. In addition, direct measurement of renal tissue oxygenation or the utilization of oxygen by renal mitochondria was not performed in our study. In both paper II and IV renal hyperlactemia developed as a result of experimental sepsis. However, hyperlactemia is not only an indicator of ischemia in sepsis. Aerobic glycolysis, liver failure, decreased pyruvate dehydrogenas function, elevated catecholamine concentrations and mitochondrial dysfunction have all been suggested to participate in septic hyperlactemia (Venkatesh et al., 2010). It is proposed that, when cells are subjected to ischemia the hydrolysis of ATP will generate an accumulation of hydrogen ions in the cytosol of the cells. This will result in increased acidosis. However, under aerobic conditions the hydrogen ions produced by hydrolysis of ATP will be used in the metabolism of glucose. The later reaction will therefore not result in a net acidosis. During sepsis both anaerobic and aerobic metabolism may occur simultaneously, but the relative importance of each metabolic pathway is difficult to estimate. In other non-renal tissue, such as muscle, lactate may be produced under aerobic conditions in a process linking glycolytic ATP supply to stimulation of Na/KATPase (Levy *et al.*, 2005). Opposite to muscle, the kidney is, under normal conditions, important in the removal of lactate from the circulation (Bellomo, 2002). This is performed by glucose production and account for a large amount of the glucose created in sheep renal gluconeogenesis (Kaufman & Bergman, 1974). Sepsis reduces the capacity of this renal process, contributing to excessive levels of lactate (Ardawi *et al.*, 1990). Based on the data in paper IV it may be postulated that sepsis diminished

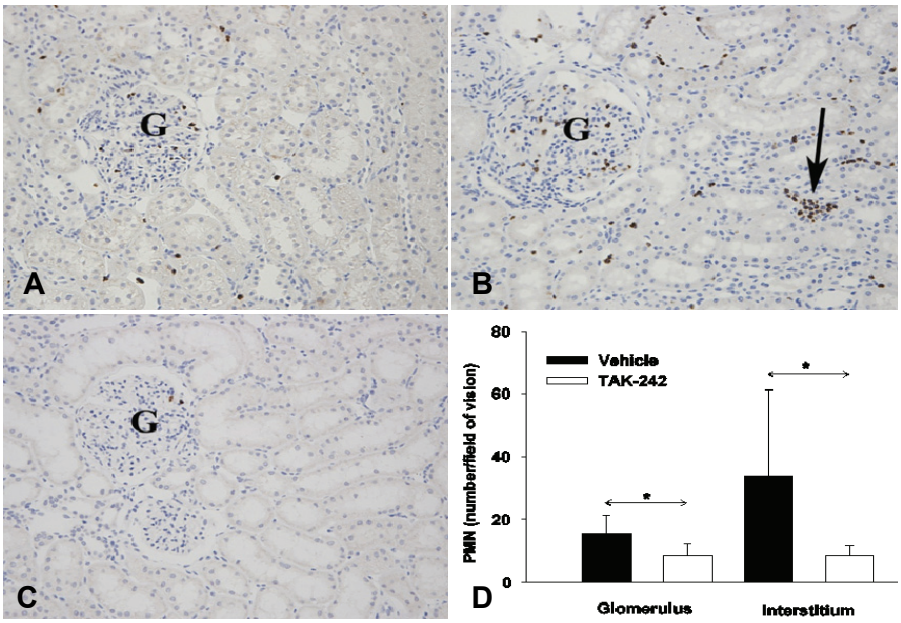

Figure 15. Immunohistochemistry of polymorphonuclear leukocytes (PMN) in sections with anti-myeloperoxidase antibodies show increased number of PMN in glomerular capillaries (G) and interstitium/peritubular capillaries (arrow) in vehicle (A) and TAK-242 treated group (B) in comparison to sham (C). Number of PMN in glomerulus and interstitium in vehicle and TAK-242 treated group (D). Data are expressed as mean and SD. * indicates a significant difference between TAK-242 and control in response to sepsis.

lactate removal by the kidney, and that TAK-242 inhibited this effect. Lower lactate levels within the kidney in paper IV, as compared to arterial levels, further support the concept of the kidney as a consumer rather than a net producer of lactate. The renal hyperlactemia seen in paper IV may be a reflection of systemic hyperlactemia rather than a sign of renal ischemia. The lactate that reaches the kidney from the systemic circulation is freely filtered in the glomerulus and reabsorbed in the proximal tubule (Barac-Nieto *et al.*, 1980). Therefore, the renal interstitial lactate levels in paper IV may presumably reflect production of both renal lactate and that of systemic origin.

With regards to other variables than merely hemodynamics, reduced TLR4-dependent infiltration of polymorphonuclear leukocytes (PMN) may have contributed to the improved renal function in the TAK-242 group of paper IV. Immunohistochemistry was performed on renal biopsies and revealed that TAK-242 treatment significantly reduced the number of PMNs in both the glomeruli and the interstitium. The endothelium is essential in maintaining vascular integrity but also important in the onset and preservation inflammation. Activation of renal endothelial TLR4 has been suggested to play a critical role in the up-regulation of adhesion molecules which can

promote the recruitment of leukocytes to areas of injury and aggravate damage and inflammation in the tissue (Andonegui *et al.*, 2003;Chen *et al.*, 2011). A study that examined renal biopsies taken immediately after death in 19 patients dying of septic shock with anuric AKI and these biopsies were compared with nine patients without septic shock or AKI and to eight trauma victims. This study showed intense infiltration of leucocytes in glomeruli and interstitial capillaries and apoptosis of tubular cells and glomerular cells did also occur (Lerolle *et al.*, 2010). Our histological findings in paper IV display several similarities, increased infiltration of PMN in both glomerulus and interstitium, limited signs of tubular injury and thrombi formation only in a few biopsies. The study in septic patients from Lerolle et al suggested that acute tubular necrosis may not be the main mechanism for septic AKI, other mechanism such as renal inflammation and apoptosis may large contributors to septic renal dysfunction.

In paper IV, examination of the glomerulus with electron microscopy could not detect any difference in the degree of foot process effacement, which is a sign of podocyte injury displayed as retraction, widening, and shortening of the processes of each podocyte. The thickness of the glomerular basement membrane between the vehicle- and TAK-242-treated groups displayed no differences. However, there was a difference in the ultrastructure of the endothelial cells in the glomerular capillaries. In general, the endothelial cells of the vehicle-treated animals were swollen and showed decreased fenestration compared to TAK-242-treated animals. Although these electron microscopy findings need further investigation, a TLR4-dependent pathway causing renal inflammation and severe renal impairment in sepsis is an exciting possibility.

CONCLUSIONS

Based on the experiments performed in this thesis the following conclusions were made:

- Neither hypotension nor renal hypoperfusion is mandatory for the development of septic renal failure
- Activation of TLR4 may contribute to the development of renal failure in ovine sepsis, but not by affecting systemic or renal hemodynamics
- Treatment with a TLR4-inhibitor effectively restores renal function in ovine sepsis
- Endothelin-1 contributes to renal vasoconstriction in hypodynamic porcine endotoxemic shock
- Activation of ETA by endothelin-1 reduces renal medullary blood flow causing ischemia but does not acutely impair kidney function in normotensive porcine endotoxemia

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