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FACTORS INFLUENCING SPLANCHNIC MICROCIRCULATION IN ANIMAL MODELS OF ENDOTOXAEMIA

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ABSTRACT

Even though severe sepsis and septic shock therapy has improved in recent years, mortality remains high (22-50%). Disturbances in splanchnic organ homeostasis and increases in gut permeability have long been presumed to contribute to systemic inflammation and multiple organ dysfunction syndrome in critical illness and septic shock. In recent years, microcirculatory dysfunction has been highlighted as an important player in the development of sepsis-induced organ failure. This thesis investigated splanchnic microcirculatory changes during endotoxaemia, and the microcirculatory effects of ethyl pyruvate, endothelin (ET) receptor antagonists, and a norepinephrine-induced increase in perfusion pressure. Laser Doppler flowmetry (papers I-IV) and sidestream dark field microscopy (paper IV) were used to evaluate the microcirculation.

In papers I-III a 5-hour model of porcine endotoxaemia was used. In this model, the systemic haemodynamic response to endotoxin was hypodynamic, with decreasing cardiac index (CI), hypotension, and systemic acidosis. Splanchnic regional blood flow and microcirculatory perfusion deteriorated, and ileal mucosal acidosis measured with air tonometry developed in parallel.
Although intervention with the resuscitation fluid Ringer's ethyl pyruvate solution (REPS) temporarily improved systemic haemodynamics, no major differences in haemodynamic parameters or splanchnic perfusion were found compared to standard therapy with Ringer's acetate (RA).
The mixed ET_A/ET_B receptor antagonist tezosentan did not increase superior mesenteric artery flow (SMAF), but microcirculatory perfusion in the ileal mucosa and ileal mucosal acidosis was still improved. Tezosentan also increased portal vein flow compared to controls, but no significant improvement of hepatic microcirculatory perfusion could be demonstrated. Selective $endothelin_A$ receptor antagonism with TBC3711 failed to improve splanchnic regional blood flow, splanchnic microcirculatory perfusion or ileal mucosal acidosis.

A 25-hour model of endotoxaemic shock in sheep, mimicking the hyperdynamic circulation seen in septic patients, was used in paper IV. After 24 hours of endotoxaemia, CI was increased and systemic hypotension had developed. Although SMAF also increased, microcirculation in the ileal mucosa and muscularis was disturbed, ileal mitochondrial complex I activity decreased, and ileal mucosal acidosis developed. Increasing perfusion pressure with norepinephrine after 24 hours of endotoxaemia did not significantly alter SMAF, ileal microcirculation, ileal mitochondrial enzyme activity or ileal mucosal acidosis.

In conclusion, gut microcirculatory alterations have a weak correlation to systemic and regional indices of flow and pressure in endotoxaemia, strengthening the hypothesis that monitoring and therapies directed towards the microcirculation could be of value in sepsis. The ET system is involved in the development of gut microcirculatory dysfunction in endotoxaemia, and mixed ET receptor antagonism is necessary to counteract the effects of ET in this context. Resuscitation with REPS does not appear to have initial positive haemodynamic or microcirculatory effects compared to RA.

Key words: Microcirculation, endotoxin, sepsis, pig, sheep, endothelin, ethyl pyruvate, gut, liver, splanchnic circulation, shock, tonometry, norepinephrine

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I. Andersson A, Fenhammar J, Frithiof R, Sollevi A, Hjelmqvist H.
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II. Andersson A, Fenhammar J, Frithiof R, Weitzberg E, Sollevi A, Hjelmqvist H.
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III. Andersson A, Fenhammar J, Weitzberg E, Sollevi A, Hjelmqvist H, Frithiof R.
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IV. Andersson A, Rundgren M, Kalman S, Rooyackers O, Brattström O, Oldner A, Eriksson S, Frithiof R.
Gut microcirculatory and mitochondrial effects of hyperdynamic endotoxaemic shock and norepinephrine treatment.
In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BE	Base excess
CO	Cardiac output
CI	Cardiac index
CS	Citrate synthase
CVP	Central venous pressure
DAMP	Damage-associated molecular pattern
DO_2I	Systemic oxygen delivery index
EGDT	Early goal directed therapy
EP	Ethyl pyruvate
ET	Endothelin
ET-1	Endothelin-1
ET_A receptor	Endothelin receptor subtype A
ET_B receptor	Endothelin receptor subtype B
GALT	Gut-associated lymphatic tissue
HR	Heart rate
ICU	Intensive care unit
iNOS	Inducible nitric oxide synthase
LD	Laser Doppler flowmetry
LEDs	Light-emitting diodes
LPS	Lipopolysaccharide
MAP	Mean arterial pressure
MFI	Microvascular flow index
MPAP	Mean pulmonary artery pressure
NO	Nitric oxide
PAMP	Pathogen-associated molecular pattern
$PCO_{2muc\text{-}art}$	Mucosal-arterial PCO_2-gap
PCWP	Pulmonary capillary wedge pressure
PRR	Pattern recognition receptor
PU	Perfusion units
PVFI	Portal vein flow index
RA	Ringer´s acetate
REPS	Ringer´s ethyl pyruvate solution
SDF	Sidestream dark field imaging
SSC	Surviving sepsis campaign
SMAFI	Superior mesenteric artery flow index
SVI	Stroke volume index
SvO_2	Mixed venous oxygen saturation
SVR	Systemic vascular resistance
SVRI	Systemic vascular resistance index
VO_2I	Systemic oxygen consumption index

INTRODUCTION

BACKGROUND

The treatment of severe sepsis is a challenge to the intensive care unit (ICU) physician, and severe sepsis and septic shock remain leading causes of death in non-coronary ICU patients. Even though therapy has improved with the introduction of protocols mainly guided by systemic haemodynamic variables, mortality remains high (22-50%), and the pathophysiology of septic shock remains to be fully elucidated[1]. In recent years, microcirculatory dysfunction has been suggested to play an important role in the development of sepsis-induced organ failure[2]. In septic patients with comparable systemic haemodynamics, persistent microcirculatory alterations have been correlated to patient outcome[3], and therapies targeting the microcirculation have been proposed as a new therapeutic strategy in sepsis. Presumably, a deeper understanding of the factors contributing to this microcirculatory failure could contribute to the development of better therapies for patients with severe sepsis.

Perfusion derangements in the splanchnic organs may compromise the barrier function of the intestinal mucosa, facilitating the translocation of bacteria and toxins to the blood and lymph, with subsequent activation of systemic inflammation. Moreover, the gut and the liver have important immunologic functions, and therapies aimed at restoring microcirculatory blood flow and preventing splanchnic organ dysfunction could potentially be of major importance in the treatment of sepsis. This thesis investigated splanchnic microcirculatory changes during endotoxaemia, and the microcirculatory effects of ethyl pyruvate, endothelin receptor antagonists, and a norepinephrine-induced increase in perfusion pressure.

THE CIRCULATORY SYSTEM

The main functions of the circulatory system are the maintenance of tissue homeostasis and the convective transport of nutrients, cells, gases, waste products and hormones. The heart serves as the central pump circulating blood through a continuous system of blood vessels. Cardiac output (CO) is the volume of blood being pumped by the heart per minute, a function of heart rate (HR) and stroke volume. The main determinants of stroke volume are preload, afterload and cardiac contractility. Cardiac output is continuously regulated to meet the demands of the tissues.

The driving pressure for blood in the systemic circulation is the difference between mean arterial pressure (MAP) and central venous pressure (CVP). Correspondingly, the perfusion pressure for a specific organ is calculated as the difference between the mean arterial pressure and the venous pressure in that specific tissue. MAP is primarily determined by cardiac output and systemic vascular resistance (SVR). The relationship between CO, MAP, CVP and SVR is given by the equation: CO = (MAP-CVP)/SVR.

The microcirculation

The microcirculation is usually defined as blood vessels with a diameter <100 µm, and includes the arterioles, capillaries and venules. The main tasks of the microcirculation are to ensure adequate oxygen delivery to meet the oxygen demands of cells in the tissue, and to regulate tissue perfusion to maintain tissue homeostasis. The microcirculation also has important functions in regulating the distribution of fluid between the intravascular and extravascular compartments, and in temperature regulation[4].

The cardiac output is intermittently ejected from the heart, but this is converted to a continuous, pulsatile flow by the elastic nature of the aorta and the large arteries. The arterioles have abundant smooth muscle in their walls, and are the main site where SVR is regulated. Moreover, the arterioles regulate the distribution of CO between different organs and contribute to the control of capillary hydrostatic pressure. The pulsatile flow seen in large arteries is dampened and intravascular pressure is reduced in the arterioles, making blood flow continuous when it reaches the capillaries. The capillary wall consists of a single layer of endothelial cells, and capillaries provide a large surface area for the exchange of oxygen and nutrients between tissues and blood. The capillaries are also the main site for regulation of the distribution of fluid between the intravascular and extravascular compartments. The main determinants of capillary blood flow are arteriolar tone, driving pressure, haemorheology, and capillary patency. The small veins and larger venules serve as elastic capacitance vessels, maintaining cardiac filling pressures. Postcapillary resistance in venules also contributes to the regulation of capillary hydrostatic pressure.

THE GUT

In man, the small intestine is divided into three structural parts; duodenum, jejunum and ileum. Besides being the main site for food digestion and nutrient absorption, the small intestine also has important immunological functions, and contains the gut-associated lymphatic tissue (GALT), the largest lymphatic organ in the body. The large intestine, the colon, is the main site for water absorption in the intestines.

In the small intestinal wall, the mucosa is mainly responsible for the absorption of nutrients and for maintaining gut barrier function. The absorptive surface is increased through circular folds of mucosa and submucosa, called plica circulares, with finger-like mucosal projections, called villi. The villi epithelial cells also display numerous small protrusions, called microvilli, further increasing the area available for absorption. Between the villi are small openings of tubular glands in the mucosa, called the intestinal crypts. The small intestinal mucosa also contains a layer of loose connective tissue, the lamina propria, and a thin muscular layer, the muscularis mucosae. The submucosa is located beneath the muscularis mucosae, and contains large arterial and venous plexuses. Below the submucosa is the muscularis, containing an inner circular

and an outer longitudinal smooth muscle layer. The muscularis is responsible for intestinal propulsive motion. A thin layer of loose connective tissue, the serosa, covers the muscular layer.

In man, the duodenum is supplied with blood from the coeliac trunk and the superior mesenteric artery supplies the jejunum, ileum and proximal and transversal parts of the colon, while the inferior mesenteric artery supplies the distal part of the colon and the rectum. Blood vessels supplying the small intestinal wall penetrate the muscularis and form a large vascular plexus in the submucosa. Branches from the submucosal plexus, together with serosal arterioles, supply the muscularis layer. From the submucosa, arterioles also extend though the muscularis mucosae and the lamina propria into the villi where a capillary network is formed. Venules arise from the capillary network at the top of the villi and run in the opposite direction from the arteriole, reaching the venous plexus of the submucosa. Venous blood from the small intestine is collected in the superior mesenteric vein, which drains into the portal vein.

The particular vascular arrangement in the small intestinal mucosa, with each villus being perfused by a central arteriole in close proximity to the returning venule, results in a counter-current exchange where oxygen diffuses from the arteriole to the venous side. Due to this counter-current mechanism, PO_2 is progressively lowered towards the tip of the villus. In normal conditions, the PO_2 at the tip of the villus is as low as 2.0-3.3 kPa, decreasing even further during low-flow states[5]. This anatomical arrangement makes the villi particularly vulnerable to hypoperfusion[6].

Intestinal microcirculation

The mucosa and submucosa receive most of the small intestinal blood flow, 60-90%[7,8]. The villi receive approximately 30% and the crypts approximately 25% of total intestinal blood flow in resting conditions[8]. The arterial supply to the mucosa and muscularis layers is arranged in parallel, allowing for independent control of flow to these two layers. A decrease in perfusion pressure normally leads to a redistribution of intestinal blood flow towards the mucosa, and particularly the villi[9], presumably a defence mechanism preserving mucosal barrier function. On the contrary, an increase in venous pressure redistributes blood flow towards the muscularis layer[10].

Blood flow in the small intestine is regulated by several different mechanisms:

- When oxygen supply does not meet oxygen demand, vasodilating metabolites are released to the interstitium, increasing blood flow and oxygen supply. K^+, H^+, adenosine and osmolality are among the factors proposed as mediators of this local metabolic control of flow.
- The arterioles exert a myogenic control of flow through their ability to constrict as a response to an increase in transmural pressure, and dilate when transmural pressure decreases (the Bayliss effect). This is the main mechanism behind the pressure-flow autoregulation seen in the intestine. However, this pressure-flow

autoregulation is a considerably weaker phenomenon than the autoregulation of renal blood flow.

- Activation of the sympathetic nervous system reduces intestinal blood flow through α_1-receptor stimulation inducing arteriolar constriction. However, when nerve activation is continued, flow is gradually recovered, a pattern called *the autoregulatory escape from sympathetic stimulation*. When the sympathetic nerve stimuli cease, there is a short period of hyperaemia before flow returns to normal. Local release of adenosine is an important factor contributing to the autoregulatory escape[11]. The autoregulatory escape is strongest in the villous part of the mucosa[12].
- Circulating vasoactive substances influence the intestinal microcirculation, for instance, both angiotensin II and vasopressin are potent vasoconstrictors in the intestinal wall. Circulating norepinephrine also induces vasoconstriction followed by an autoregulatory escape phenomena.
- Intestinal capillary pressure and transcapillary fluid flux are regulated through a complex interplay between arterioles, precapillary sphincters, and venules.

Capillary haematocrit is normally lower than systemic haematocrit. In small vessels, red cells tend to move away from the vessel wall and occupy the axial stream, leaving a relatively cell-free layer close to the vessel wall. At vessel branching points, the distribution of haematocrit between the two branches can be uneven, with the smaller branch receiving blood with a relatively lower haematocrit, a phenomenon called plasma skimming[13,14]. The vascular arrangement in the intestinal mucosa, with arterioles branching of from the submucosal plexus at a right angle, increases the degree of plasma skimming. This results in a relatively lower capillary haematocrit in the intestinal mucosa compared to the muscularis layer[13].

THE LIVER

The liver serves a wide range of functions in the human body, including important metabolic functions, immunological functions, the synthesis of a variety of proteins, drug metabolism, and filtration of venous blood from the splanchnic area.
The blood supply to the liver is somewhat unique in its dual supply from the portal vein and hepatic artery. The liver receives approximately 25% of cardiac output, 75-80% of this through the portal vein and 20-25% through the hepatic artery. However, due to lower oxygen saturation, the portal vein only delivers 50-60% of basal oxygen supply, 40-50% being supplied by the hepatic artery.

The pressure-flow autoregulation of the hepatic artery is weak, making the relationship between pressure and flow approximately linear below systolic arterial pressures of 80 mmHg[15]. Flow in the portal vein is determined by the outflow from the extrahepatic splanchnic organs. In normal conditions, the liver is to some extent protected from hypoperfusion through the hepatic arterial buffer response[16]. When portal vein flow is

reduced, liver perfusion is preserved through an adenosine-dependent hepatic artery vasodilation increasing hepatic artery blood flow. Conversely, when portal vein flow increases, hepatic artery flow decreases.

The portal venules and the hepatic arterioles run parallel to each other in the liver, forming the portal triads together with bile ducts, and supplying the blood to the sinusoids. The sinusoids correspond to the capillary bed of the liver, and constitute a low-pressure system where the supply of nutrients and removal of metabolic products takes place. The endothelium of the sinusoids is highly fenestrated, and separated from the hepatocytes by the space of Disse. After flowing through the sinusoids, the blood drains into central hepatic venules, and is finally collected in the hepatic veins[16]. The hepatic sinusoidal microcirculation is locally regulated by a critical balance between vasodilating agents, like nitric oxide and carbon monoxide, and vasoconstrictive agents, like endothelin-1[17].

SEPSIS

Definitions and epidemiology

The definitions of sepsis, severe sepsis and septic shock have been under debate over the years. In 1992 a consensus was reached by a panel of experts at the Consensus Conference of American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM)[18]. Sepsis was defined as a systemic inflammatory response to a confirmed or suspected infection; severe sepsis as sepsis with organ dysfunction; and septic shock as sepsis with hypotension, despite adequate fluid resuscitation (Table 1).

Table 1.

Definitions of sepsis (simplified from Bone et al [18])

Systemic inflammatory response syndrome (SIRS)	A systemic inflammatory response elicited by a variety of clinical insults. Includes ≥ 2 of the following findings: ▪ Body temperature >38 or <36 ºC ▪ Heart rate >90 beats per minute ▪ Respiratory rate >20 breaths per minute or PaCO2 <4.3 kPa ▪ Leukocytes >12 or $<4 \cdot 10^{9} \cdot L^{-1}$, or >10% immature cells
Sepsis	SIRS and confirmed or suspected infection.
Severe sepsis	Sepsis and signs of organ dysfunction, hypoperfusion or hypotension.
Septic shock	Severe sepsis and persistent hypotension despite adequate fluid resuscitation.

The incidence of severe sepsis ranges from 0.13[19] to 3.0[20] per 1000 between studies using different methods and from different geographic areas. In Finland, the incidence of ICU-treated patients with severe sepsis was found to be 0.38 per 1000[21], and in a

recent Swedish study the incidence of severe sepsis in 2005 varied from 0.13 to 0.43 per 1000 depending on the criteria used for the diagnosis[19]. The incidence of severe sepsis also seems to be increasing over time[19]. Severe sepsis and septic shock are common in patients admitted to the ICU; the prevalence of severe sepsis in the ICU has been estimated to be 11% in Germany[22] and 10.5% in Finland[21]. Despite intense efforts to improve outcome mortality remains high, hospital mortality ranging from 22.1 to 55.2% in European conditions[19,21,23]. Among factors that have been associated with increased mortality are age, renal failure, number of organ failures, comorbidities and race.

During the past decades, gram-negative sepsis has decreased, while the incidence of gram-positive bacteria, polymicrobial infections and fungal infections has increased.

Moreover, infection with multi-resistant bacteria constitutes a growing problem worldwide.

Pathophysiology

Septic shock primarily reflects a dysregulated immune response by the body to a microbial pathogenic insult, but the pathophysiology of sepsis is complex and not yet fully understood.

The innate immune system constitutes a non-specific, early response system, detecting and reacting to pathogens in a rapid and powerful way. Innate immunity is considered to play a crucial role in the development of severe sepsis, and also has important functions in the activation and modulation of later antigen-specific, adaptive immune responses. The adaptive immunity has a slower onset, but the memory function of the adaptive immune system triggers faster and more powerful reactions after every new exposure to a particular pathogen. Recent studies indicate that the cholinergic nervous system, and in particular the vagus nerve, also is involved in the regulation and limitation of the septic inflammatory response through activation of α7cholinergic receptors on macrophages[24].

The initial event in severe sepsis and septic shock is the interaction between an invading pathogenic threat and the innate immune system. Cells of the innate immune system detect and react to invading microbes through specific receptors called pattern-recognition receptors (PRRs). These PRRs can recognize both pathogen-associated molecules (pathogen-associated molecular patterns, PAMPs), and endogenous, intracellular proteins released following host tissue injury (damage-associated molecular patterns, DAMPs). Lipopolysaccharide (LPS), also called endotoxin, is a component of the gram-negative bacterial cell wall considered to be an important PAMP. Endotoxin activates the innate immune system through binding to toll-like receptor 4, an important PRR. The activation of PRRs triggers the production and release of inflammatory mediators, mainly through activation of the intracellular transcription factor NF-κB, and thereby promotes systemic and local inflammation[25,26].

A prominent feature of septic shock is the upregulation of inducible nitric oxide synthase (iNOS) with increased production of nitric oxide (NO), resulting in generalized vasodilation. Plasma levels of the potent vasoconstrictor endothelin-1 (ET-1) are also increased in septic shock. Moreover, plasma levels of ET-1 have been correlated to mortality and morbidity in septic patients[27,28], indicating a role for the endothelin system in the pathophysiology of septic shock.

The initial activation of the immune system results in an early, hyperactive inflammatory response with activation of endothelial cells and leukocytes, increased capillary permeability, and activation of coagulation and the complement system. In the tissues, the septic inflammatory response induces both microcirculatory and mitochondrial dysfunction, hypothesized to be the two main factors behind septic organ failure[29]. Besides being the energy suppliers of cells, mitochondria are also involved in oxygen sensing and cell death-signalling pathways (necrosis and apoptosis). Disturbances of oxidative phosphorylation within the mitochondria have been observed in both septic patients and animal models of sepsis[30].

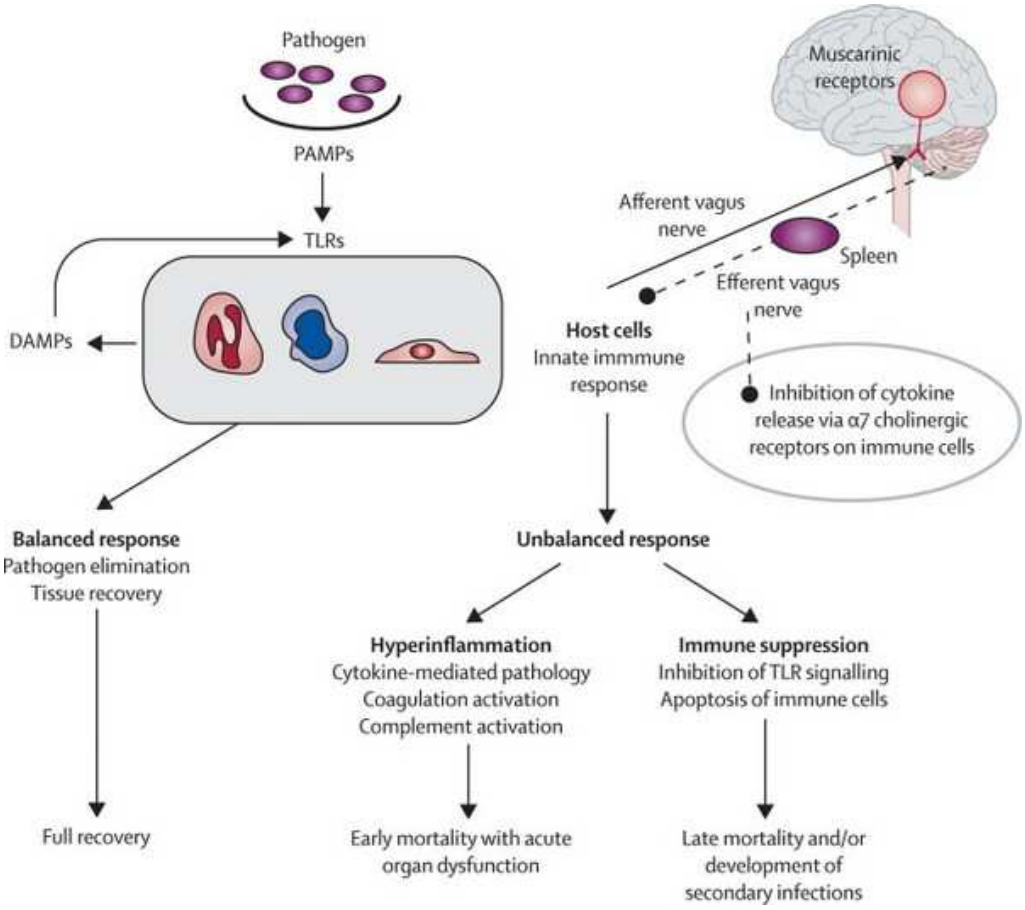

Figure 1. An overview of the host-pathogen interaction in sepsis. PAMPs, pathogen-associated molecular patterns; DAMPs, damage-associated molecular patterns; TLRs, toll-like receptors. Reproduced with permission from van der Poll and Opal, Lancet Infect Dis 2008; 8: 32–43.

After the initial proinflammatory response, an anti-inflammatory immune response with toll-like receptor suppression and an increased apoptosis of immune cells may follow. This phenomenon is considered to be the main factor behind the increased susceptibility to secondary infections seen in patients with septic shock.
An overview of the immune response in sepsis is presented in Figure 1.

Sepsis and the microcirculation

Currently, goal-directed septic shock therapy is mainly guided by systemic haemodynamic variables. Protocol-driven treatment of septic shock has improved mortality, but even though global haemodynamic goals are met, there is still a considerable mortality among septic shock patients[31]. In recent years, microcirculatory changes have been highlighted as an important part of the pathophysiology of severe sepsis and septic shock[2]. Several studies have confirmed the presence of severe microcirculatory disturbances in septic shock patients[3,32], and the presence and persistence of such abnormalities have been found to be associated with morbidity and mortality in patients[3,33]. These findings indicate that incorporating therapies targeting the microcirculation into goal-directed septic shock treatment could be of value.

The relationship between septic microcirculatory alterations and changes in systemic and regional indices of flow and pressure is still under debate, but there seems to be a poor correlation between systemic haemodynamic variables and microcirculatory changes in sepsis, and microcirculatory failure can occur in the presence of adequate values for central haemodynamics and oxygen delivery[34]. Results from both animal studies and patients also indicate that there is heterogeneity in microcirculatory alterations between different vascular beds[35,36].

During severe sepsis, all microcirculatory components are severely disturbed, leading to loss of the microcirculatory autoregulation of blood flow. The microcirculatory changes in sepsis are summarized in Figure 2. The inflammatory response induces systemic activation of the endothelium and endothelial cell swelling and dysfunction. The endothelium has an essential role in vasomotor regulation and integration in the microcirculation, and endothelial dysfunction with loss of the normally precise local control of microvascular flow is considered to be a pivotal factor in the development of sepsis-induced microcirculatory failure. Endothelial activation also results in the expression of proadhesive and procoagulant factors, and in increased vascular permeability[2]. Activated leukocytes adhere to the endothelium, penetrate the vascular barrier and release reactive oxygen species further damaging the microcirculation. Moreover, increased leukocyte rolling and aggregation in venules contributes to microcirculatory flow impairment[37]. Capillary clotting is increased due to an impaired ability of red blood cells to deform in the capillaries, leukocyte aggregation, and activation of coagulation with deposition of platelet/fibrin clots[37].
NO has a pivotal role in the local control of microcirculatory blood flow, but the NO system is severely disturbed in septic shock. iNOS is upregulated and total body NO

levels are increased, but iNOS is heterogeneously expressed within and between tissues, potentially creating localized areas of relative NO deficiency. Alterations in the NO system are thought to be a major factor contributing to the heterogeneity of tissue perfusion and microcirculatory shunting seen in septic shock[38]. The endothelial cell production of endothelin-1 is also greatly increased in sepsis, and ET-1-induced vasoconstriction in the microcirculation has been proposed as an important cause of microcirculatory failure in sepsis[39].

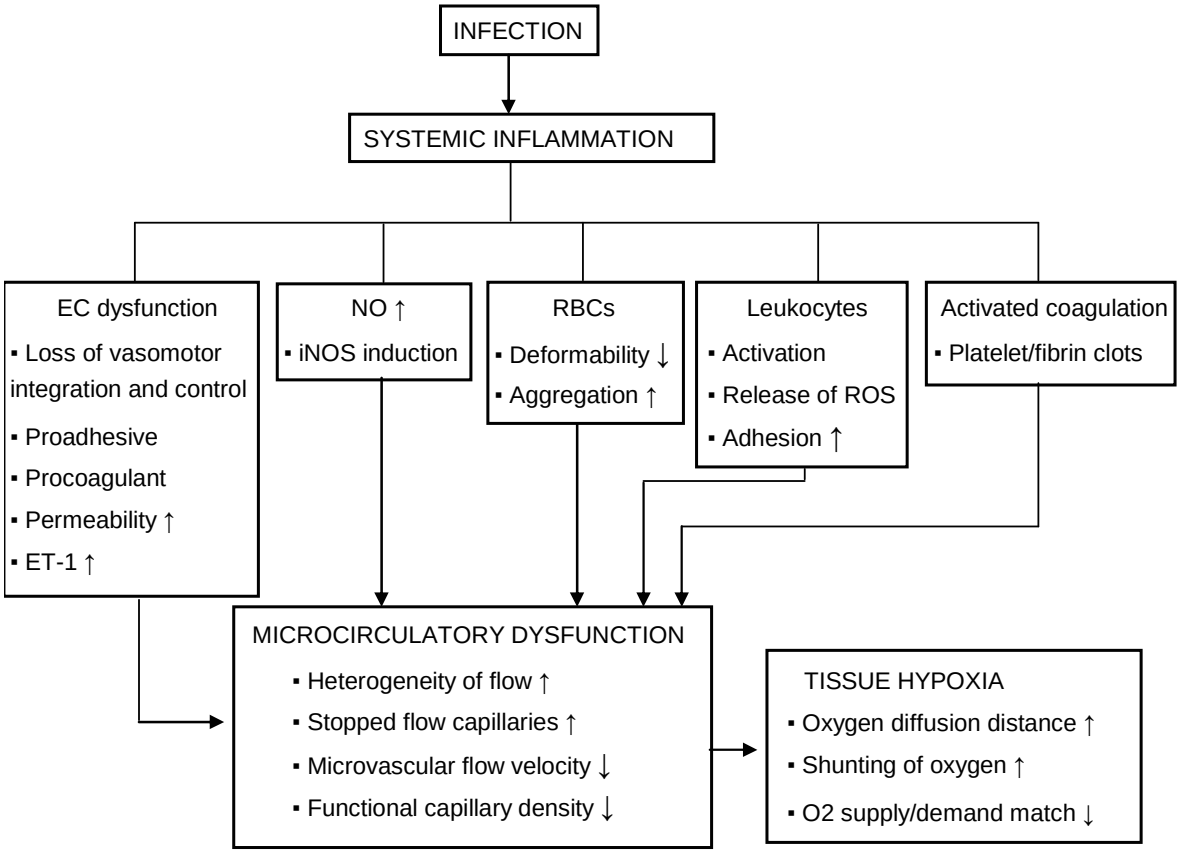

Figure 2. An overview of the microcirculatory alterations in sepsis. EC, endothelial cell; ET-1, endothelin-1; NO, nitric oxide; iNOS, inducible nitric oxide synthase; RBCs, red blood cells; ROS, reactive oxygen species.

The microcirculatory abnormalities seen in severe sepsis and septic shock typically include increased heterogeneity of microvascular flow, an increased number of stopped-flow microvessels, a decreased microvascular flow velocity, and a lowered functional capillary density[40]. The sepsis-induced heterogeneity of flow is important, since tissues tolerate a homogenous decrease in blood flow better than a heterogeneous one[41]. The maldistribution of blood flow at the capillary level results in hypoxic areas in the microcirculation due to an increased diffusion distance for oxygen[41], and in shunting of oxygen from the arterial to the venous compartment. This inability to match oxygen delivery to oxygen demand in the tissue leads to tissue hypoxia, and persistence

of microcirculatory dysfunction has been proposed as the leading cause of organ failure in severe sepsis and septic shock[42].

Sepsis and the gut

The gut has long been presumed to have an important role in the pathogenesis of multiple organ dysfunction in critical illness and septic shock. As early as the 1960s, experimental studies showed that gut bacteria and endotoxins could gain access to the systemic circulation in shock states[43,44], a process later termed *bacterial translocation*. In the 1980s, the idea of septic states originating from commensal gut bacteria penetrating the intestinal wall, with the gut acting as the motor of multiple organ dysfunction, gained clinical attention[45]. The increased intestinal permeability seen in critical illness has been associated with an increased risk of complications, multiple organ dysfunction syndrome, and mortality[46-49]. Still, the clinical relevance of bacterial translocation has remained controversial, mostly due to the failure to consistently find gut-derived bacteria or endotoxins in the blood or mesenteric lymph nodes of critically ill patients[50,51]. Recently, there has been a growing understanding that the role of the gut in sepsis is more complex, and that it is not only limited to the notion of commensal gut bacteria reaching the systemic circulation[52]. The gut has important immunological functions, and recent studies indicate that during conditions associated with gut hypoperfusion, the gut itself can become a source of inflammatory and tissue injurious factors. Magnotti et al. showed that systemic inflammation and distant organ injury can be induced by nonbacterial, proinflammatory factors released from the ischaemic gut into the mesenteric lymphatics[53], a concept termed *the gut-lymph hypothesis*[54].

Current theories on how the gut contributes to the development of systemic inflammation and multiple organ dysfunction syndrome include both translocation of bacteria or toxins to the systemic circulation, and the release of gut-derived, proinflammatory factors into the circulation and the mesenteric lymphatics[52].

Gut microcirculation in sepsis

Gut microcirculatory disturbances are present early in experimental septic shock[36], and include a decreased percentage of perfused villi, a reduction of mean erythrocyte velocity and functional capillary density, and an increased heterogeneity of flow[55]. Due to the combination of the counter-current oxygen exchange, making the tip of the villi prone to hypoxia, and the high metabolic demand in the intestinal mucosa, the mucosa is particularly vulnerable to microcirculatory disturbances. In septic shock, microcirculatory failure has been proposed as the main factor behind gut mucosal acidosis[56]. Moreover, microcirculatory hypoperfusion could contribute to the increased intestinal wall permeability seen in sepsis. A better knowledge of the factors contributing to sepsis-induced gut microcirculatory failure is needed, and therapies restoring gut microcirculatory perfusion could potentially be of major importance in the treatment of sepsis.

Sepsis and the liver

The liver is an important modulator of the systemic inflammatory response in sepsis. Kupffer cells, the largest mass of macrophages in the body, act as scavengers for systemic and gut-derived inflammatory mediators and cytokines[57], and also clear the portal vein blood of bacteria[58]and endotoxins[59]. However, when activated, Kupffer cells also produce proinflammatory mediators, thereby contributing to the development of systemic inflammation[60]. The liver also plays a major role in metabolism and protein synthesis, and in sepsis, the hepatocytes increase synthesis and release of coagulation factors, complement factors, and acute-phase proteins[61].

In patients with septic shock, the development of acute liver failure has been associated with increased mortality. Sepsis-induced liver failure can be divided into an early, primary dysfunction followed by a later, secondary dysfunction[61]. The primary dysfunction is usually related to shock and hypoperfusion, and hepatic function is often severely compromised. The secondary dysfunction is more insidious. Although most of the hepatic functions remain intact, this secondary dysfunction can cause spillover of bacteria, endotoxin, and inflammatory mediators, possibly promoting systemic inflammation.

Microvascular dysfunction and ischaemia are considered to be main factors behind the hepatic dysfunction seen in sepsis[62]. Also, the hepatic arterial buffer is impaired in endotoxaemia, making the liver more vulnerable to hypoperfusion[63]. Other factors contributing to liver damage in sepsis is mitochondrial dysfunction, an increase in hepatocyte apoptosis, and tissue damage mediated by activated neutrophils.

Initial treatment of severe sepsis

In recent years, the concept of early recognition and aggressive goal-directed treatment of severe sepsis has been widely accepted. In 2001, Rivers et al. demonstrated an improved survival in patients with severe sepsis or septic shock treated with early goal-directed therapy (EGDT) compared to patients treated with standard therapy[64]. A simplified version of the EGDT protocol used by Rivers et al. is presented in Figure 3. In 2002, the European Society of Intensive Care Medicine, the International Sepsis Forum, and the Society of Critical Care Medicine launched the Surviving Sepsis Campaign (SSC). The SSC is an international campaign to improve outcome in severe sepsis and septic shock through the implementation of evidenced-based guidelines and treatment protocols.

The implementation of EGDT seems to improve the prognosis of septic shock patients[65], but even though the haemodynamic goals of the treatment protocols are met, tissue hypoperfusion can still persist, eventually leading to organ failure and death[31]. Interestingly, Sakr et al. found that sublingual microcirculatory alterations was a better prognostic indicator than global haemodynamic variables in septic patients[3]. Moreover, recent findings indicate that early increases in microcirculatory blood flow during

EGDT are associated with reduced organ failure in septic patients without substantial differences in global haemodynamics[33]. Based on the accumulating data indicating the importance of microcirculatory failure in sepsis, the direct monitoring of microcirculatory perfusion and the incorporation of therapies targeting the microcirculation into EGDT protocols has been proposed as a new strategy in the treatment of severe sepsis.

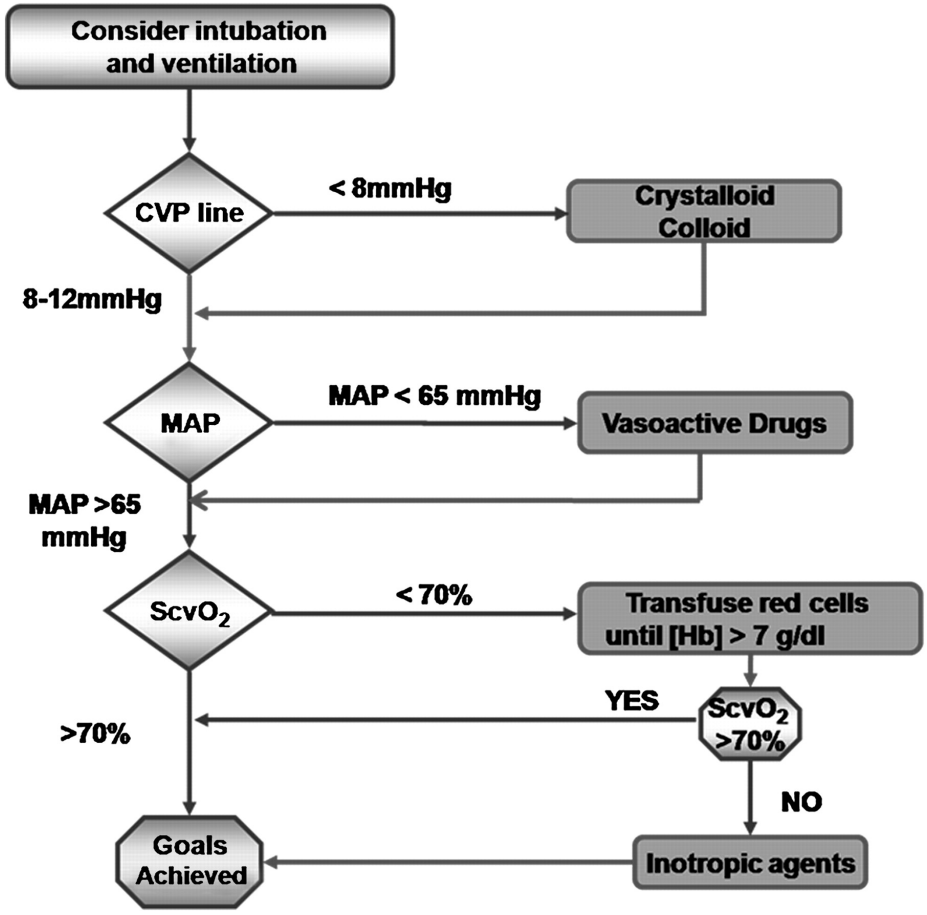

Figure 3. A simplified version of the protocol for early goal directed therapy used by Rivers et al. Reproduced with permission from Daniels et al., Emerg Med J, 2010.

Besides the early administration of correct antibiotics, the SSC resuscitation bundle for the first 6 hours also includes EGDT using intravenous fluids, vasoactive substances and transfusion of red blood cells to reach predefined haemodynamic goals[66]. In septic shock, the systemic hypotension can lead to a loss of the pressure-flow autoregulation in various vascular beds, and organ blood flow can become linearly dependent on perfusion pressure. What level of MAP that should be targeted to preserve organ perfusion has still not been clearly defined, but the SSC guidelines recommend targeting a MAP of 65 mm Hg[66]. Norepinephrine and dopamine are first-line vasopressors to treat persistent hypotension in spite of adequate fluid resuscitation, although concerns have been raised that vasopressors can induce an excessive

vasoconstriction in the microcirculation[67], thereby worsening tissue hypoperfusion rather than improving it. A better understanding of the role of hypotension in the development of septic microcirculatory derangements, and of the microcirculatory effects of perfusion pressure manipulation, could be of significant value for improving septic shock treatment.

Animal models of sepsis

Studies using invasive monitoring to investigate sepsis pathophysiology and preliminary studies investigating unproven pharmacological interventions cannot be performed in humans for ethical reasons, necessitating the use of animal models.
The infusion of endotoxin has been extensively used in animal models to mimic the septic response in patients. Although an endotoxin infusion is not identical to the complex situation of sepsis, endotoxin is an important part of the pathophysiology of gram-negative sepsis, and the administration of small doses of endotoxin to human volunteers induce haemodynamic, metabolic and haematologic changes that are qualitatively similar to those seen in septic patients[68]. Endotoxin is easy to use, and doses are readily measured and controlled. The response to endotoxaemia is also easy to replicate, facilitating comparisons between groups[68,69].

A large number of animal species have been used in sepsis models. Rats and mice are inexpensive to purchase and maintain, and are often used when a large number of animals are needed for the experiment. However, in many ways rodents are physiologically and pharmacologically different from humans, among other things being considerably more resistant to the effects of endotoxin. The size of rodents also limits the possibilities for monitoring and blood sampling[68]. The sensitivity of pigs and sheep to endotoxin is more similar to that of humans, and their size makes extensive surgical preparation and monitoring possible. Pigs are similar to humans in renal, cardiovascular and intestinal physiology and anatomy[70], and have been extensively used in sepsis models. In short-term models of endotoxaemia, pigs normally develop a hypodynamic circulatory response, but this can be converted to a hyperdynamic circulation through heavy fluid loading. The sheep is a docile animal, having the advantage that studies can also be performed in unanaesthetized animals. Moreover, sheep are also prone to developing a hyperdynamic response, similar to that seen in septic patients, when infused with endotoxin[71].

ETHYL PYRUVATE

Besides having a central role in intermediary metabolism, pyruvate also functions as a scavenger of reactive oxygen species. However, the possibility to use pyruvate as a therapeutic agent is limited by its poor solubility in solution. To improve its solubility, Sims et al. used the ethyl ester of pyruvic acid, ethyl pyruvate (EP), in a balanced salt solution called Ringer's ethyl pyruvate solution (REPS)[72]. Treatment with EP was shown to ameliorate gut mucosal injury in a rat model of ischaemia and reperfusion,

and in subsequent studies EP was found to have positive effects on intestinal barrier function[73], renal function[74], and survival[75,76] in small animal models of endotoxaemia and sepsis.

EP is a potent anti-inflammatory agent, decreasing NFκB-activation and the release of proinflammatory cytokines as HMGB-1[77]. It has also been suggested that pyruvate have direct inotropic effects through glycolytic substrate augmentation, and initial studies with EP indicated positive effects on cardiac function[78] and gut microcirculation[79] in small animal models of cardiac ischaemia[78] and mesenteric ischaemia and reperfusion[79]. Given the results from initial small animal models of sepsis, it was of interest to further evaluate the potential role of EP as a therapeutic agent in sepsis, using a large animal model to investigate the effects of EP on systemic haemodynamics and splanchnic perfusion.

THE ENDOTHELIN SYSTEM

Endothelin-1 (ET-1) was originally identified in 1988 as a vasoconstrictive peptide released from the endothelium[80]. Later studies isolated two additional isoforms belonging to the endothelin (ET) family, endothelin-2 and endothelin-3. ET-1 is the predominant form of the endothelins, and it is considered to be the most important isoform in human physiology and pharmacology.

ET-1 synthesis and secretion

The peptide ET-1 is 21 amino acids long, and the most potent vasoconstrictor in the human body so far known. ET-1 is synthesized through enzymatic cleaving of precursor proteins. Initially, the primary peptide pre-pro-ET-1 is cleaved by endopeptidases to big ET-1, a peptide with weaker vasoconstrictive properties. Finally, big ET-1 is cleaved by endothelin-converting enzymes, resulting in active ET-1[81]. The production occurs mainly in endothelial cells, from which ET-1 is continuously released, contributing to the maintenance of vasomotor tone[82]. In the endothelial cell, ET-1 is also stored in intracellular storage granules. Among the factors known to stimulate ET-1 synthesis and release are proinflammatory cytokines, endotoxin, catecholamines, hypoxia, thrombin, angiotensin II, growth factors, and ET-1 itself[83,84]. Factors inhibiting ET-1 production include ANP, prostaglandins, NO, and heparin[84,85]. Eighty per cent of ET-1 is secreted abluminally by the endothelial cells, acting on underlying smooth muscle cells. ET-1 mainly acts as a paracrine and autocrine mediator, and in normal conditions circulating levels of ET-1 are low. However, in some pathological conditions, such as sepsis, plasma levels increase dramatically, and ET-1 may then also have endocrine properties[86].

Besides vascular effects, ET-1 exerts a number of physiological functions in the human body, and the effects of the endothelin system have been extensively reviewed elsewhere[87]. Release of ET-1 has also been associated with the development of sepsis,

vascular disease, heart failure, diabetes, pulmonary hypertension, and several other pathological conditions.

Endothelin receptors

In humans, ET effects are mediated by two subtypes of G protein-coupled endothelin receptors, the endothelin$_A$ (ET_A) and endothelin$_B$ (ET_B) receptors. The ET_A receptor is primarily expressed on vascular smooth muscle and mediates vasoconstriction. The actions of the ET_B receptor are more complex and dependent on the location of the receptor. ET_B receptors located on vascular smooth muscle cells mediate vasoconstriction, but ET_B receptors on endothelial cells induce vasodilation through the release of nitric oxide and prostacyclin[88]. There is also evidence of the existence of a crosstalk between ET_A and ET_B receptors, possibly through receptor heterodimerization[89].

Stimulation of ET_A receptors and vasoconstrictive ET_B receptors activates phospholipase C which in turn activates the second messengers diacylglycerol (DAG) and inositol triphosphate. This results in an increase of cytosolic calcium, inducing smooth muscle contraction and vasoconstriction[90]. ET-1 generally induces a more powerful vasoconstriction in veins than in arteries[91]. Since ET-1 receptors mediate both vasodilation and vasoconstriction, the net effect of ET-1 in a vascular bed depends on the properties of the local receptor population.

The ET_B receptor also functions as a clearance receptor, removing circulating ET-1 from the circulation via endocytosis[92]. The plasma half-life of ET-1 is approximately 1-2 minutes[93]. The pulmonary circulation is the main site for ET-1 removal[94], but the kidney and liver also contributes to the clearance of ET-1.

ET-1 in sepsis

Levels of ET-1 are increased in all forms of shock, but they are particularly high in septic shock. The increased plasma levels of ET-1 also correlate with increased mortality and morbidity in septic patients[27,28]. Besides vascular effects, ET-1 increases the activation and adhesion of leukocytes[95] and induces production of reactive oxygen species[96], thereby contributing to the proinflammatory response in sepsis. In septic shock, ET antagonists have been shown to have beneficial effects on haemodynamics[97], pulmonary hypertension[97], acute lung injury[98], renal failure[99], and hepatosplanchnic hypoperfusion[100]. Most of these data originate from the use of ET receptor antagonists in animal models of septic or endotoxaemic shock.

Intestinal effects

In models of endotoxaemic shock, mixed endothelin receptor antagonism with bosentan effectively restores portal vein flow and improves intestinal mucosal acidosis[100], demonstrating positive effects on hepatosplanchnic circulation.

ET-1 has been implicated as one of the possible mediators of the microcirculatory failure seen in the gut in sepsis. In 1993, Wilson et al. demonstrated that an infusion of anti-endothelin antibodies improves intestinal microcirculation in a rat model of bacteraemia[39]. In a model of porcine septic shock, Krejci et al. found that the mixed endothelin receptor antagonist bosentan improved microcirculation in the gastric and colonic mucosa, but a significant difference in microcirculatory flow in the jejunal mucosa and muscularis could not be demonstrated[101].

Selective ET_A receptor antagonism has previously been demonstrated to improve intestinal microvascular blood flow in a rat model of endotoxaemia[102]. This finding indicates that the ET_A receptor could be the main receptor responsible for the ET-1-induced vasoconstriction in the intestine, but the knowledge of how the different ET receptors influence intestinal microcirculation in sepsis is still incomplete.

There are several mechanisms by which ET-1 could affect the intestinal microcirculation. Intravenous infusion of ET-1 decreases intestinal mucosal functional capillary density and red blood cell velocity[103], and also causes increased leukocyte activation and aggregation in the intestinal microcirculation, thereby increasing microvascular thrombosis[104]. ET-1 is a powerful venous vasoconstrictor, and increases portal vein pressure in endotoxaemia[105]. An increase in portal vein pressure has been shown to divert blood away from the intestinal mucosa towards the muscularis[10], possibly contributing to mucosal hypoperfusion in sepsis. Moreover, ET-1 can contribute to the development of tissue oedema through an increase in endothelial permeability[106], reduced lymphatic flow[107], and increased venous pressure.

Hepatic effects

Hepatic sinusoids contract in response to stimulation of ET_A and ET_B receptors, but ET_B receptors also induce vasodilation through activation of the endothelial NO synthase system. Studies in rat models have indicated that ET-1 is an important factor contributing to hepatic microcirculatory failure in sepsis and endotoxaemia. ET-1 has been shown to increase portal venous resistance and portal vein pressure through elevation of both sinusoidal and presinusoidal resistance in the portal circulation[108]. Furthermore, the portal circulation was found to have an increased vascular responsiveness to ET-1 in rat sepsis[109]. In line with this, the mixed endothelin receptor antagonist tezosentan has been found to decrease liver injury in small animal models of endotoxaemia[110,111] and sepsis[111]. Previous studies investigating the role of the different ET receptors in mediating hepatic microcirculatory effects in intact animals in sepsis are very sparse, but the selective ET_A receptor antagonist BQ-485 was found to have detrimental effects on liver microcirculation in a rat model of endotoxaemia[112]. Moreover, studies investigating effects of selective ET_A receptor antagonism on liver injury in endotoxaemia have yielded conflicting results[113,114].

In contrast to the positive hepatic effects seen with endothelin receptor antagonism in rat models, Krejci et al could not demonstrate a significant improvement of hepatic microcirculatory perfusion using bosentan in a porcine model of fecal peritonitis[101]. Also, tezosentan failed to improve hepatic microcirculation and liver injury in a murine model of systemic inflammation[115].

In conclusion, the role of ET-1 and the different ET receptors in the development of hepatic microcirculatory failure in sepsis and endotoxaemia is still under debate, and data from large animal models are still scarce.

NOREPINEPHRINE

In fluid resuscitated patients, norepinephrine infusion is a first-line treatment for septic hypotension. Norepinephrine increases mean arterial pressure and systemic perfusion pressure through α_1 receptor-mediated vasoconstriction in arterioles, leading to an increase in systemic vascular resistance. Norepinephrine also has positive inotropic and chronotropic effects through its effects on cardiac β_1 and β_2 receptors, and increases venous return through constriction of venous capacitance vessels, thereby increasing cardiac preload. The cardiac effects of norepinephrine usually lead to an increased cardiac output in septic patients[116], also contributing to the increase in MAP. Norepinephrine does not seem to impair splanchnic blood flow in septic shock patients[117,118], but the existing data are limited. The effects of norepinephrine on the intestinal microcirculation have been investigated in various animal models of sepsis, with different studies finding improved[119], worsened[67,120], or unaltered[121,122] microcirculatory perfusion after norepinephrine administration.

AIMS

The overall aims of the thesis were

- To establish an experimental animal model, using endotoxin infusion to mimic the septic response, suited for studies investigating splanchnic microcirculatory changes.

- To investigate changes in the splanchnic microcirculation in response to experimental endotoxaemia and to the following interventions aimed at improving the microcirculation:
    1. Ringer's ethyl pyruvate solution
    2. Mixed endothelin and selective endothelin$_A$ receptor antagonists
    3. Increasing perfusion pressure with norepinephrine.

- To investigate the relationship between splanchnic microcirculatory changes and changes in systemic haemodynamics and regional blood flow in different animal models of endotoxaemia.

METHODOLOGICAL CONSIDERATIONS

ANIMALS

In papers I-III, crossbred (Landrace/Yorkshire/Hampshire) female pigs weighing 28-37 kg were used. Porcine and human splanchnic organs are anatomically and physiologically similar[69], and the size of the pig allows for the necessary instrumentation and blood samples. The ET system is also similar between pigs and humans[123].

In paper IV, ewes weighing 47±6 kg were used. Long-term (≥24 hours) sheep models of endotoxaemic shock provide a suitable animal model reproducing many of the pathophysiologic features found in septic patients, including a hyperdynamic syndrome and multiple organ dysfunction[124].

All experimental protocols were approved by the ethics committee for experiments in animals, Stockholm, Sweden, and all studies 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).

ANAESTHESIA

The porcine experiments

In the porcine experiments (papers I-III) the animals were premedicated with ketamine 10 $mg \cdot kg^{-1}$, midazolam 0.5 $mg \cdot kg^{-1}$, and atropine 0.05 $mg \cdot kg^{-1}$ intramuscularly. After cannulation of the marginal ear vein, anaesthesia was induced with propofol (40-80 mg) and the animals were orally intubated. Anaesthesia was maintained with a combination of sevoflurane (2.6% end-tidal concentration during surgical procedures, followed by 1.0% end-tidal concentration throughout the experiment) and an infusion of fentanyl 10 $mg \cdot kg^{-1} \cdot h^{-1}$ and midazolam 0.15 $mg \cdot kg^{-1} \cdot h^{-1}$. Additional doses of fentanyl and midazolam were given if needed. Muscle paralysis was achieved by an infusion of pancuronium bromide 0.5 $mg \cdot kg^{-1} \cdot h^{-1}$. Before the administration of muscle relaxants, anaesthetic depth was ensured through pain stimulation of the fore hoof.

The animals were mechanically ventilated; the settings of the ventilator were adjusted to reach an arterial PCO_2 value of 4.7–5.3 kPa at baseline, and were then kept constant throughout the experiment. The body temperature was maintained at 37-39ºC by heating pads and blankets. After the surgical preparation, the animals were allowed 60 minutes of rest before the endotoxin infusion was started.

All animals received a continuous infusion of saline with glucose 25 $mg \cdot ml^{-1}$ at a rate of 20 $ml \cdot kg^{-1} \cdot h^{-1}$ during surgical preparation. In paper I, fluid therapy was modified according to the experimental protocol after 60 minutes of endotoxaemia. In paper II, the infusion was continued throughout the experiment. In paper III, an infusion of hydroxyethyl starch 130/0.4 (Voluven 60 $mg \cdot ml^{-1}$, Fresenius Kabi AB, Uppsala,

Sweden) at a rate of 10 $ml \cdot kg^{-1} \cdot h^{-1}$ was also given during surgical preparation. After the surgical preparation, the animals in paper III received 5 $ml \cdot kg^{-1} \cdot h^{-1}$ of saline with glucose 25 $mg \cdot ml^{-1}$ and 15 $ml \cdot kg^{-1} \cdot h^{-1}$ of Ringer's acetate (RA) throughout the experiment.

The sheep experiments

In the sheep experiments anaesthesia was induced with intravenous sodium thiopental 10 $mg \cdot kg^{-1}$ after cannulation of the external jugular vein. The animals were orally intubated and mechanically ventilated. A combination of isoflurane (2.0% end-tidal concentration during surgical procedures followed by 1.2-1.6% end-tidal concentration throughout the experiment) and an intravenous infusion of midazolam 0.1 $mg \cdot kg^{-1} \cdot h^{-1}$ was used to maintain anaesthesia during the experiments. No muscle relaxants were administered. The settings of the ventilator were continually adjusted during the experiments aiming at an arterial PCO_2 value of 4.8-5.7 kPa. After the surgical preparation, the animals were allowed 120 minutes of rest before the endotoxin infusion was started. All animals received a continuous infusion of RA 3 $ml \cdot kg^{-1} \cdot h^{-1}$, and bolus doses of hydroxyethyl starch 130/0.4 (Voluven 60 $mg \cdot ml^{-1}$, Fresenius Kabi AB, Uppsala, Sweden) were administered according to the experimental protocol.

The surgical preparation and catheterization of the animals are described in detail in the separate papers of this thesis. An overview of the monitoring equipment used in papers I-IV is presented in table 2.

Table 2.	**Paper I (Pig)**	**Paper II (Pig)**	**Paper III (Pig)**	**Paper IV (Sheep)**
Pulmonary artery catheter	X	X	X	X
Arterial line	X	X	X	X
PV flowprobe	X	X	X	
SMA flowprobe			X	X
RA flowprobe	X			
Mesenteric vein catheter			X	
Air tonometry ileum		X	X	X
LD ileal mucosa	X	X	X	X
LD ileal muscularis	X		X	X
LD liver	X	X	X	
LD colon mucosa		X		
LD renal cortex	X			
SDF ileal mucosa				X

An overview of the monitoring equipment used in paper I-IV. PV, portal vein; SMAF, superior mesenteric artery; RA, renal artery; LD, laser Doppler; SDF, sidestream dark field.

ENDOTOXIN

Escherichia coli lipopolysaccharide (LPS; serotype 0111:B4, Sigma-Aldrich Sweden AB, Stockholm, Sweden) was used in papers I-IV to induce a response similar to that seen in patients with severe sepsis. LPS, also called endotoxin, is a component of the gram-negative bacterial cell wall, and consists of a polysaccharide side chain, a core oligosaccharide, and a lipid component called lipid A. In the body, LPS interacts with CD14 and activates the toll-like receptor 4, subsequently initiating the transcription of proinflammatory genes and inducing pathophysiological changes similar to those seen in human gram-negative sepsis.

In papers I-III, the LPS infusion was started at a rate of 0.31 $\mu g \cdot kg^{-1} \cdot h^{-1}$ and increased stepwise until reaching 2.5 $\mu g \cdot kg^{-1} \cdot h^{-1}$ after 30 minutes. In paper IV, the LPS infusion was started at a rate of 0.3 $\mu g \cdot kg^{-1} \cdot h^{-1}$ and subsequently adjusted according to the experimental protocol.

PULMONARY ARTERY CATHETER

In papers I-IV, a balloon-tipped pulmonary artery catheter (7.5 F Swan-Ganz; Edwards Lifesciences, Irvine, CA) was connected to a Vigilance Monitor system (Edwards Lifesciences), and used for continuous measurements of cardiac output (thermodilution technique), central venous pressure (CVP), mean pulmonary artery pressure (MPAP), mixed venous oxygen saturation (SvO_2), and body temperature. The catheter was inserted through the right jugular vein, and positioned in the pulmonary artery by pressure guidance.

ULTRASONIC FLOW PROBES

Ultrasonic flow probes (Transonic Systems Inc., Ithaca, NY) were used to measure portal vein flow (papers I-III), renal artery flow (paper I) and superior mesenteric artery flow (papers III-IV). The flow probe has two ultrasonic transducers positioned on one side of the vessel and an acoustic reflector positioned on the opposite side of the vessel. One of the transducers is positioned upstream and one downstream from the reflector. The two transducers pass ultrasonic signals back and forth, alternately intersecting the flowing liquid in upstream and downstream directions, and the difference between the upstream and downstream integrated transit times is used to calculate a measure of volume flow in ml/min.

MICROCIRCULATORY MEASUREMENTS

Laser Doppler flowmetry

Laser Doppler flowmetry (LD) was used to measure microcirculatory perfusion in the ileal mucosa (papers I-IV), ileal muscularis (papers 1, III-IV), liver (papers I-III), colonic mucosa (paper II), and renal cortex (paper I).

LD is an established method for the real-time measurement of microcirculatory perfusion in various tissues. The method is based on the measurement of the small frequency shift, the Doppler shift, which arises when a near-infrared laser light is scattered by moving red blood cells in the tissue. Monochromatic light is carried to the tissue by a fibre optic probe containing one emitting and one receiving fibre. In the tissue, the light is scattered and partly absorbed. When the light hits moving blood cells in the tissue there will be a change in wavelength, while the wavelength of light hitting static tissue structures is unchanged. The backscattered light is picked up by the receiving fibre, and the magnitude and frequency of the changes in wavelength are analysed to calculate the number and velocity of red blood cells in the tissue[125].

The tissue volume assessed by LD is hemisphere-shaped, but the exact sampling volume and measuring depth cannot be calculated. Depending on the properties of individual tissues, as, for instance, the structure and density of capillary beds, tissues will absorb and scatter light in different ways, and this influences measuring depth. The measuring depth also depends on the properties of the LD probe. An increase of the fibre diameter, the distance between the emitting and the receiving fibres, or the wavelength of the light will increase penetration depth[126]. The probes used in this thesis were standard probes (Perimed AB, Järfälla, Sweden), with a fibre separation of 0.25 mm, a fibre diameter of 0.1 mm, and a wavelength of 780 nm.

Since the measuring volume is unknown, it is not possible to obtain absolute values of blood flow expressed as $ml \cdot min^{-1} \cdot g\ tissue^{-1}$. Instead, values are obtained as arbitrary perfusion units (PU), reflecting the product of velocity and concentration of blood cells in the tissue sample, and the quantity measured with laser Doppler flowmetry is generally referred to as perfusion[125]. Results are presented as changes relative to baseline in percentage. Before the start of the experiments, the laser Doppler probes were calibrated according to the manufacturer's instructions, at optical zero using a plastic disc and at 250 PU using motility standard latex solution. LD measurements were obtained and analysed using Perisoft for Windows data acquisition software (Perimed AB).

Sidestream dark field imaging

Sidestream dark field imaging (SDF) was used in paper IV to evaluate the microcirculation in the ileal mucosa. SDF is a recently introduced technique allowing the direct visualization of the microcirculation. The SDF consists of a light guide surrounded by a ring of stroboscopic light-emitting diodes (LEDs). The device utilizes a new method of reflectance avoidance. The LEDs at the tip of the device are optically isolated from the inner image-conducting core, thereby completely avoiding tissue surface reflections and permitting visualization of subsurface structures. The emitted light has a wavelength of 530 nm, a wavelength of light that is absorbed by haemoglobin in red blood cells, making red blood cells visible as dark globules. The

technique provides clear images of blood vessels in the microcirculation containing red blood cells, without the need for contrast dyes.

In paper IV, an ileostomy was constructed, and SDF videos of the ileal mucosal microcirculation were obtained at baseline, after 24 hours of endotoxaemia, and after one hour of norepinephrine treatment. The SDF videos were obtained and analysed in accordance with previously published guidelines[127]. Due to the heterogeneity in microvascular flow seen in sepsis, steady SDF images of 10-20 seconds' duration were acquired at three to five sites in the ileum at each time point. The videos were stabilized using the AVA 3.0 software (MicroVision Medical, Amsterdam, The Netherlands) and analysed blindly and in random order.

Images were divided into four quadrants, and the microvascular flow index (MFI) was determined. MFI is a semiquantitative score of the types of microvascular flow predominantly seen in each quadrant. Type of flow in each quadrant is determined using an ordinal scale; 0, no flow; 1, intermittent flow; 2, sluggish flow; 3, normal flow. The MFI value was calculated as the average score of all quadrants at each time point. The heterogeneity index was calculated at each time point as the highest flow velocity minus the lowest flow velocity divided by the mean MFI across all mucosal sites at that time point, as previously described by Trzeciak et al[128].

In all videos, the number of villi was counted and all villi were semiquantitatively classified as perfused, heterogeneously perfused or unperfused. In every villus, areas containing vessels with continuous flow (MFI 2 and 3) were classified as perfused, and areas containing vessels without continuous flow (MFI 0 and 1) were classified as unperfused. The percentage of perfused villi was calculated at each time point as number of perfused villi per total number of villi visible. Figure 4 shows an image of the ileal villi microcirculation obtained from the SDFvideos.

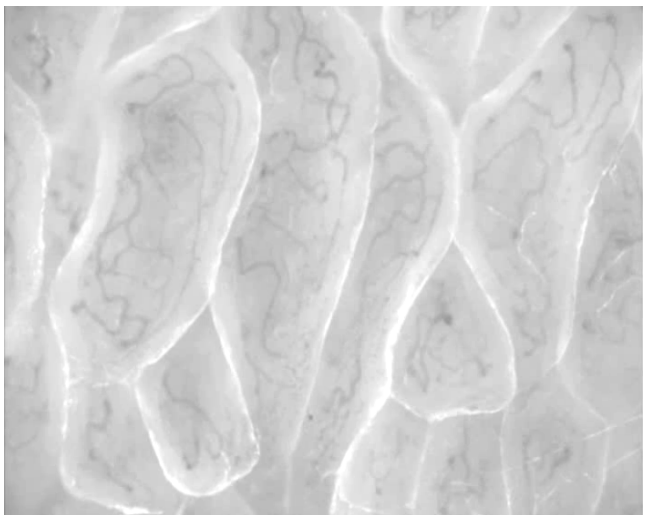

Figure 4. A still image of the ileal mucosal microcirculation obtained from the videos taken with the SDF technique.

TONOMETRY

In papers II-IV a 14 F tonometry catheter (GE Healthcare Information Technologies AB, Stockholm, Sweden) for air tonometry was inserted into the lumen of the ileum through a small incision in the ileal wall. The catheter was connected to an S/5 Tonometry Module (GE Healthcare Information Technologies AB) and used to measure mucosal partial pressure of carbon dioxide every 10 minutes. The mucosal-arterial PCO_2-gap ($PCO_{2muc-art}$) was calculated as the difference between ileal mucosal PCO_2 and arterial PCO_2.

MITOCHONDRIAL ENZYMES

In paper IV, tissue samples from the ileal wall were homogenized in a KCl-based buffer using a Potter-Elvhjem homogenizer. Activity of citrate synthase, mitochondrial complex I and complex IV was analysed as described in detail elsewhere[129].

INTERVENTIONS

Ringer's ethyl pyruvate solution

The resuscitation fluid Ringer's ethyl pyruvate solution (REPS) was used in paper I. EP was bought from Sigma-Aldrich Sweden AB (Stockholm, Sweden), and used to prepare REPS (NaCl 130 $mmol \cdot L^{-1}$, KCl 4 $mmol \cdot L^{-1}$, CaCl 2.7 $mmol \cdot L^{-1}$, and EP 28 $mmol \cdot L^{-1}$) with help from the hospital pharmacy. After 60 minutes of endotoxaemia, a bolus dose of 40 $mg \cdot kg^{-1}$ EP (corresponding to 12.3 $ml \cdot kg^{-1}$ of REPS) was given over 10 minutes. After the bolus dose was administered, a continuous infusion of EP 40 $mg \cdot kg^{-1} \cdot h^{-1}$ (12.3 $ml \cdot kg^{-1} \cdot h^{-1}$ of REPS) was started and continued throughout the experiment.

Tezosentan

The dual ET receptor antagonist tezosentan was used in papers II and III. Tezosentan is approximately 30-fold more potent on ET_A receptors than on ET_B receptors[130]. It is a very specific, competitive antagonist without agonistic effects. Tezosentan also has been found to have weak inhibitory effects on H1 central, 5-HT_2A, and vasopressin V1 receptors[130], but these effects are likely to be of little importance compared to the potent effects on the ET receptors. Tezosentan is relatively short acting, and designed for intravenous use. There are no known active metabolites. In humans, the distribution half-life of tezosentan is 0.1 hours and the elimination half-life 3 hours[131].

In papers II and III tezosentan was intravenously administered after 120 minutes of endotoxaemia. A bolus dose of 1 $mg \cdot kg^{-1}$ was given over 10 minutes and followed by a continuous infusion of 1 $mg \cdot kg^{-1} \cdot h^{-1}$ throughout the experiment.

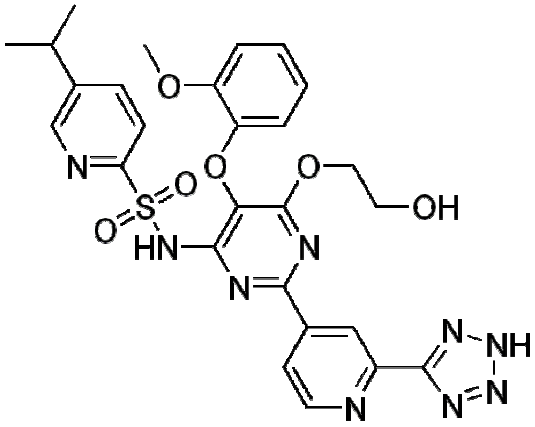

Figure 5. The molecular formula of tezosentan.

TBC3711

The selective ET_A receptor antagonist TBC3711 was used in paper III. TBC3711 has an affinity ratio between the ET_A receptor and the ET_B receptor of 441000:1[132]. The elimination half-life for TBC3711 is 6.6 hours in humans[132]. In paper III, TBC3711 was administered intravenously as a bolus dose of 2 $mg \cdot kg^{-1}$ given over 15 minutes.

Norepinephrine

Norepinephrine is an agonist of α_1, β_1 and β_2 adrenergic receptors, and a first-line vasopressor in the treatment of septic shock hypotension according to the SSC guidelines[66]. In paper IV, norepinephrine was infused intravenously after 24 hours of endotoxaemia to restore mean arterial pressure to baseline levels. The mean intravenous dose of norepinephrine was 318 ± 45 $ng \cdot kg^{-1} \cdot min^{-1}$.

STATISTICS

Paper I

Changes in parameters over time were analysed according to a two-way repeated measures analysis of variance (ANOVA), the repeating variable being time. In case of a significant main effect, differences between groups at each time point were analysed using Bonferroni-corrected pairwise comparisons.

Paper II

Main effects were analysed using a two-way ANOVA with time as a repeating variable before intervention (T0-T120) and after intervention (T120-T300). The effects of tezosentan were determined by analysis of the time–treatment interaction between T120 and T300.

Paper III

Main effects were analysed using a two-way ANOVA with time as a repeating variable before intervention (T0-T120) and after intervention (T120-T300). If the overall F-ratio

was significant, pairwise planned comparisons of the changes over time were performed. P-values were adjusted according to Bonferroni.

Paper IV

In the endotoxin group, changes over time between T0 and T24 were analysed with one-way ANOVA, Student's paired t-test or Wilcoxon matched pair test, and interpreted as effects of endotoxemia. Effects of norepinephrine were evaluated with Student's paired t-test or, in case of non-normal distribution, Wilcoxon matched pair tests using time-points T24 and T25.

RESULTS

PAPER I

Systemic and pulmonary effects

Endotoxaemia induced a hypodynamic response with decreasing CI and increasing systemic vascular resistance index (SVRI) and mean pulmonary artery pressure (MPAP), whilst MAP was maintained at baseline levels. CI was significantly lower in the REPS group at T60 before treatment. Mixed venous oxygen saturation (SvO_2), systemic oxygen delivery index (DO_2I), and systemic oxygen consumption index (VO_2I) remained unchanged for the first 60 minutes of endotoxaemia. Arterial pH levels decreased, but base excess (BE), arterial lactate levels and anion gap remained unchanged. Haematocrit increased as a sign of haemoconcentration.

Resuscitation with either REPS or RA after 60 minutes of endotoxaemia induced a temporary increase in CI, but then progressive deterioration ensued again. SVRI temporarily decreased after the initial fluid bolus, but increased again to reach levels above baseline at T300. MPAP remained elevated, but MAP deteriorated throughout the experiment. SvO_2 and DO_2I decreased, but VO_2I increased over time. There were no significant differences between the REPS and the RA group in the effect on central haemodynamic and pulmonary parameters. Arterial pH decreased throughout the experiment due to a combination of metabolic and respiratory acidosis. BE decreased, and was significantly lower in the REPS group compared to the RA group at later time points. A significantly higher plasma chloride concentration in the REPS group explained the lower BE to some extent, but there was also a lower anion gap in the REPS group, indicating an additional source of the metabolic acidosis. Haematocrit remained elevated without any intergroup differences.

Splanchnic perfusion

Endotoxaemia appeared to decrease portal vein flow indexed to body weight (PVFI) and microcirculatory perfusion in the ileal mucosa and muscularis during the first 60 minutes, but this was only statistically significant for the ileal mucosa. Hepatic microcirculation was initially unchanged by endotoxaemia. No intergroup differences were found for the above-mentioned parameters before treatment at T60, but renal artery flow indexed to bodyweight was lower in the REPS group at T60, presumably as a reflection of the difference seen in CI. There were no differences in renal microcirculation or diuresis before the start of treatment.

Treatment with REPS or RA after 60 minutes of endotoxaemia did not induce any differences between the groups in regional blood flow, microcirculatory perfusion or urine output. PVFI and renal artery flow index decreased compared to baseline levels, although this did not reach significant levels for renal artery flow index. Microvascular

perfusion decreased over time at all sites, without intergroup differences. In the ileum, microvascular perfusion seemed to be preserved in the mucosa compared to the muscularis, indicating a functional autoregulation of mucosal microvascular perfusion.

PAPERS II-III

Systemic and pulmonary effects

Effects of endotoxaemia

Endotoxin administration resulted in a hypodynamic response in papers II-III. The decrease in CI was due to a reduction of stroke volume index, since heart rate concomitantly increased. In paper II MAP decreased compared to baseline levels, but in paper III MAP was maintained. SvO_2 and DO_2I decreased over time (paper II), but VO_2I increased in response to endotoxin (paper II). Pulmonary capillary wedge pressure (PCWP) increased over time (paper II).

Endotoxaemia induced pulmonary hypertension, and PO_2 and PCO_2 deteriorated in parallel (papers II-III). Metabolic and respiratory acidosis developed over time (papers II-III), with increased arterial lactate levels and a decrease in BE (papers II-III). Haemoglobin levels increased (papers II-III) indicating haemoconcentration. Arterial levels of ET-1-like immunoreactivity increased in response to endotoxin (papers II-III).

Effects of ET receptor antagonism

Tezosentan improved CI and stroke volume index compared to controls (papers II-III) and TBC3711 (paper III), but no differences in heart rate were found. Tezosentan also preserved SvO_2 compared to controls (papers II-III), but compared to TBC3711 this effect did not reach statistical significance ($p=0.07$; paper III). The deterioration in DO_2I seen in the control group was prevented by tezosentan (paper II), but there was no significant difference in VO_2I (paper II).

The effect of tezosentan on MAP compared to controls differed between papers II and III. In paper II there was no difference, but in paper III both tezosentan and TBC3711 lowered MAP compared to controls. In the control group, PCWP increased compared to the tezosentan group (paper II). The pulmonary hypertension seen in controls was ameliorated by tezosentan (papers II-III) and TCBC3711 (paper III), resulting in an improvement of PO_2 and PCO_2 in treatment groups.

Tezosentan improved pH compared to controls (papers II-III), but only in paper II did tezosentan significantly improve BE and arterial lactate. No differences in BE or arterial lactate levels could be found between TBC3711 and controls, but TBC3711 improved pH due to a decrease in PCO_2 (paper III). In paper II, tezosentan did not affect haemoglobin levels, but in paper III both tezosentan and TBC3711 decreased haemoglobin levels compared to controls. As expected when blocking the ET_B

receptor, tezosentan increased arterial levels of ET-1-like immunoreactivity compared to TBC3711 and controls.

Splanchnic perfusion

Effects of endotoxaemia

Total splanchnic blood flow measured as PVFI (papers II-III) and splanchnic oxygen delivery index (paper II) were reduced in response to endotoxin administration. An initial increase in the superior mesenteric artery flow index (SMAFI) was followed by a progressive decline in flow. Microcirculatory perfusion in the ileal mucosa (papers II-II), ileal muscularis (paper III), colonic mucosa (paper II) and liver (papers II-III) all decreased over time in a similar pattern. In parallel with this, the mucosal-arterial PCO_2-gap (papers II-III) and mesenteric vein lactate levels (paper III) increased. Endotoxaemia also raised mesenteric vein levels of ET-1-like immunoreactivity (paper III).

Effects of ET receptor antagonism

PVFI was significantly improved with tezosentan compared to controls (papers II-III) and TBC3711 (paper III); this also resulted in an improved splanchnic oxygen delivery index (paper II). However, no differences in SMAFI were found between the groups (paper III).

Microcirculatory perfusion in the ileal mucosa (papers II-III) and colon mucosa (paper II) were significantly improved with tezosentan compared to controls (papers II-III) and TBC3711 (paper III). In parallel with this, the increase in $PCO_{2muc\text{-}art}$ seen in controls (papers II-III) and the TBC3711 group (paper III) was attenuated. In the muscular layer of the ileum, the microcirculatory perfusion did not differ between groups (paper III). TBC3711 failed to improve ileal microcirculation or $PCO_{2muc\text{-}art}$ compared to controls. Tezosentan also had beneficial effects on mesenteric vein lactate levels compared to controls (paper III), but compared to TBC3711 this effect failed to reach statistical significance ($p=0.05$; paper III).

The hepatic microcirculation did not significantly improve after administration of tezosentan (papers II-III) or TBC3711 (paper III). Also, no significant difference in hepatic microcirculatory perfusion could be found when comparing all of the animals ($n=14$) from the two controls groups and tezosentan groups in papers II and III ($p=0.43$; Figure 6).

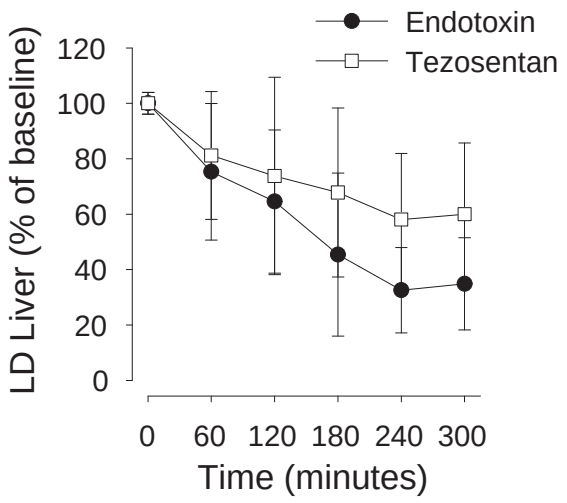

Figure 6. Hepatic microcirculatory perfusion values in control animals and tezosentan animals from paper II and III. N=14 in both groups. P=0.43 for time–treatment interaction between T120 and T300 using two-way ANOVA.

PAPER IV

Effects of endotoxaemia

In paper IV, a state of hyperdynamic shock with an increased CI and systemic hypotension had developed after 24 hours of endotoxaemia. Although SvO_2 increased, metabolic acidosis with a decrease in base excess and increased arterial lactate levels ensued. PCWP increased and urine output was preserved, indicating that fluid resuscitation was adequate. SaO_2 and PCO_2 did not significantly change during the first 24 hours.

SMAFI increased, but in spite of this ileal microcirculation deteriorated. Microvascular perfusion measured with LD decreased to a similar extent in the mucosa and in the muscular layer. The SDF derived parameters showed a similar pattern, with a decrease in MFI and percentage of perfused villi and an increase in heterogeneity index. In parallel with these microcirculatory changes, $PCO_{2muc\text{-}art}$ increased.

Endotoxaemia significantly decreased citrate synthase (CS) and complex I activity, but complex IV activity remained unchanged. To correct for mitochondrial density, the ratio of complex I and complex IV to CS were calculated (complex I/CS and complex IV/CS respectively). No significant differences were found for complex I/CS (p=0.09) or complex IV/CS (p=0.21).

Effects of increasing perfusion pressure with norepinephrine

After 24 hours of endotoxaemia, a norepinephrine infusion was used to restore mean arterial pressure to baseline levels. CI and SvO_2 increased further, but SMAFI remained unchanged. Norepinephrine did not induce any significant differences in PCWP, acid-base parameters, pulmonary gas exchange parameters or urine output.

Neither the intestinal microcirculation nor $PCO_{2muc\text{-}art}$ was significantly affected by norepinephrine treatment. On the mitochondrial level, norepinephrine increased

complex I activity and complex I/CS in 5 out of 6 animals, but this did not reach statistical significance (p=0.12 and 0.18, respectively). No statistical differences were found in the activity of CS, complex IV or complex IV/CS.

Sham animals

In paper IV, 5 sham animals were used to rule out any non-endotoxaemic effects over time. Due to technical difficulties, SDF measurements and ileal wall biopsies were only obtained from three animals in the sham group. Sham animals did not exhibit any major changes in central haemodynamics, SMAFI, acid-base parameters, urine output, arterial lactate or body temperature over time. Microcirculatory parameters and $PCO_{2muc\text{-}art}$ also remained stable over time. In sham animals, CS was reduced to a similar extent as in the endotoxin animals after 24 hours, possibly due to the preoperative fasting period or to the fact that the animals were unable to ruminate.

DISCUSSION

THE ENDOTOXIN MODEL

The experiments in this thesis were performed using endotoxin infusion in two animal models: a shorter (5 hours) porcine model (papers I-III) and a longer (25 hours) sheep model (paper IV). Although many different models of sepsis have been used, there is no single ideal preclinical model, but rather a large number of complementary models with different strengths and weaknesses[133]. Endotoxin is considered to be an important part of the pathophysiology of gram-negative sepsis, and the administration of small doses of endotoxin to human volunteers induces haemodynamic, metabolic and haematologic changes that are qualitatively similar to those seen in septic patients[68]. Endotoxin is easy to use, dosing can be controlled, and the response is reproducible. However, results from animal models of endotoxaemia are not directly applicable to the complex clinical situation of bacterial sepsis in humans, and this should be kept in mind when interpreting the results.

In shorter models of acute porcine endotoxaemia, the haemodynamic response is normally hypodynamic, with a decrease in CI and increase in systemic vascular resistance. This was also the case in the porcine experiments (papers I-III), where a hypodynamic cardiovascular response was elicited by endotoxin administration. The hypodynamic situation is different from the hyperdynamic response usually seen in septic shock patients, and this limitation of the model has led to concerns about the clinical relevance. Another limitation of the endotoxin model is that the cytokine response is more rapid and quantitatively larger than in models of cecal ligation and puncture, representing a focus of infection model[134]. However, several authors have concluded that endotoxin infusion can be a reasonable model of human sepsis, provided that the model chosen adequately replicates those features of the clinical syndrome that are the focus of the experiment[68,69]. With this in mind, studies using shorter models of endotoxaemia with a predominantly hypodynamic response[56,135] can replicate many of the microcirculatory changes seen in septic patients, supporting the relevance of these models in this context. Still, since shorter, hypodynamic endotoxin models do not fully mimic the complexity of the human septic syndrome, findings should primarily be interpreted from a mechanistic point of view, and results cannot be extrapolated into the clinical and therapeutic setting.

In porcine models of endotoxaemia, aggressive fluid resuscitation using up to 70 $ml \cdot kg^{-1} \cdot h^{-1}$ has been used[136] to avoid a hypodynamic response and normalize or increase CI. However, resuscitation with large amounts of fluid can also induce pulmonary oedema, and the management of fluid therapy in pigs[137] is a delicate balance. Fluid resuscitation with 20 $ml \cdot kg^{-1} \cdot h^{-1}$ of saline with glucose 25 $mg \cdot ml^{-1}$ elicited a hypodynamic response in paper II. In paper III, the total amount of fluid given was kept constant at 20 $ml \cdot kg^{-1} \cdot h^{-1}$, but to achieve better intravascular resuscitation

saline with glucose was to a large extent replaced by Ringer's acetate (15 $ml \cdot kg^{-1} \cdot h^{-1}$). As a result, animals in the control group maintained MAP at baseline levels, but CI still decreased. Possibly, a more aggressive fluid resuscitation could have induced a hyperdynamic response to endotoxaemia, but in our pilot experiments using larger amount of fluids several of the animals developed pulmonary oedema.

In paper IV, we aimed to create a model of endotoxaemic shock that would as far as possible mimic the clinical situation of hyperdynamic septic shock in patients. Sheep models using low-dose endotoxin infusion are known to replicate the human cardiopulmonary changes seen in septic shock, with increased CI and systemic hypotension[71]. Furthermore, the clinical course of sepsis in patients is often prolonged with multiple organ failure developing over days. In paper IV, the study period was extended to 25 hours, thereby allowing for a slowly progressing development of septic organ failure more clinically relevant than the shorter model of acute endotoxaemic shock.

The sheep is also a very calm animal, and establishing a sheep model has the advantage that studies can also be performed in unanaesthetized animals, thereby avoiding any cardiovascular or inflammatory effects of the anaesthetics used. However, the use of SDF monitoring in paper IV necessitated the use of an anesthetized model in order to avoid suffering of the animals and to obtain steady pictures.

The animals used in this thesis were subjected to extensive surgical preparation, and the results should be interpreted in this context. However, in order to achieve a comprehensive monitoring of systemic haemodynamics and splanchnic perfusion, the use of invasive monitoring equipment is necessary considering the currently available techniques.

MONITORING OF MICROCIRCULATORY PERFUSION

Laser Doppler flowmetry

LD is an established method of measuring microcirculatory perfusion in the intestinal mucosa, and over the years it has been used in numerous studies in humans[138] as well as in experimental animal models[139]. Previous studies have shown that LD values correlate strongly to whole organ blood flow in the intestine[140] as well as in the liver[141] in normal conditions. Moreover, microcirculatory perfusion measured in the intestinal mucosa with LD correlate strongly to simultaneous mucosal blood flow measurements using the microsphere technique or hydrogen gas clearance[142].

Laser Doppler flowmetry allows for the real-time, continuous, quantitative measurement of microvascular perfusion, but the inability to determine measurement depth and volume remains a limitation of the technique. Using Monte Carlo simulations of light propagation in tissue, Fredriksson et al. recently estimated the measuring depth of probes similar to the ones used in this thesis to be 0.4 mm in the liver, 0.55 mm in

skeletal muscle, and 0.53 mm in skin[126]. Considering the optical properties of the intestinal wall, it is reasonable to assume that the intestinal measuring depth is also in this range. Ileal villi height in pigs similar in size to those used in this thesis is 0.5-0.6 mm[143,144] and crypth depth approximately 0.45 mm[143], and available data indicate similar conditions in the ileal mucosa of sheep[145]. This indicates that the laser Doppler signal from the mucosal probes was mainly derived from the villous part of the mucosa.

A prominent feature of the microcirculatory changes seen in sepsis and endotoxaemia is spatial hetereogeneity within the tissue. A limitation of laser Doppler flowmetry in this setting is that it measures perfusion in the entire measurement volume, and hence it cannot be used to evaluate changes of microcirculatory perfusion heterogeneity. The fact that values are arbitrary and should be regarded as the product of red blood cell velocity and tissue haematocrit, rather than linear blood flow per se, can complicate comparisons with whole organ blood flow. The measurement depth and volume of laser Doppler flowmetry are affected by instrumental factors as well as tissue factors. If tissue properties change over time, the absorption or scattering of light in the tissue can be altered, possibly affecting measurement volume and laser Doppler perfusion values[126].

Measurements are sensitive to probe migration, pressure from the probe itself, and motion artefacts[125]. In the experiments performed in this thesis, motion artefacts did not occur, presumably due to the combined effects of anaesthetics and endotoxaemia. Probe migration was avoided by attaching the probe to a small holder gently sutured to the surface of the organ with great care being taken to avoid pressure artefacts.

Sidestream dark field imaging

SDF is a recently developed videomicroscopy technique that has been used to directly visualize the microcirculation of the intestinal mucosa in patients as well as in experimental animal models. The device is small and handheld, and no contrast dye is needed to visualize the microcirculation. The optical field of view when using a 5× objective is approximately 0.94 mm × 0.75 mm, and the on-screen magnification is ×380[127].

SDF makes it possible to directly evaluate changes in the microvascular network, including capillary density and heterogeneity of perfusion. The technique is sensitive to pressure and motion artefacts, and contrast and focus must be adjusted to obtain images of adequate quality. The vascular wall cannot be visualized with SDF, meaning that only vessels containing red blood cells can be detected[146]. The technique does not allow for continuous evaluation of the microcirculation for extended periods of time. Software for computer-assisted analysis of microvascular parameters can be used when evaluating the videos, but manual intervention is still needed for vessel identification and blood flow measurements.

The SDF-derived variables used to evaluate the microcirculation in paper IV were in line with previously published guidelines[127]. The microvascular flow index was used to semiquantitatively evaluate the main type of capillary blood flow in each quadrant. Using this variable, four categories of flow are identified using a scale ranging from 0 to 3; 0 represents no flow, 1 represents intermittent flow, 2 represents sluggish flow, and 3 represents normal flow. The MFI score is relatively easy to measure, but the score is ordinal, and this complicates interpretation of the results. Moreover, the scale used does not take into consideration flow velocities in the supranormal range. Based on the MFI values, the heterogeneity index was calculated, as previously described. The percentage of perfused villi was calculated as a surrogate parameter for functional capillary density. The fact that the SDF-derived variables used are semiquantitative is a limitation of the analysis. However, the possibility to visualize individual vessels and determine heterogeneity of flow is an advantage, compared to laser Doppler flowmetry. When used in paper IV, the SDF device mainly visualized vessels in the ileal villi, and hence, the SDF-derived parameters should be regarded as representing the microcirculation in the villous part of the mucosa.

Tonometry

In papers II-IV, an air tonometry catheter was placed in the lumen of the ileum and used to measure gut mucosal PCO_2. The air in a semi-permeable silicon balloon is allowed to equilibrate with the mucosal PCO_2 for 10 minutes, after which the air is automatically aspirated and the PCO_2 level is analysed. Monitoring of gut mucosal PCO_2 with tonometry is based on the principle that tissue PCO_2 levels increase in conditions with inadequate tissue perfusion[147]. The tissue level of PCO_2 is influenced by the arterial PCO_2, and the mucosal-arterial PCO_2-gap is usually calculated. When arterial CO_2 content is stable, the two main factors influencing PCO_2 in the mucosa are perfusion and the metabolic production of PCO_2[147], but an impaired perfusion seems to be the main determinant of tissue CO_2 accumulation[148]. Several studies have shown a close relationship between PCO_2 levels and mucosal microcirculation[101,149,150]. Still, the PCO_2-gap should not be interpreted as a direct measure of microcirculatory blood flow, but rather as a reflection of the adequacy of microvascular perfusion in relation to oxygen demand.

The linearity between mucosal CO_2 content and PCO_2 is dependent on stable conditions, and stability is hard to achieve when studying the progression of endotoxaemia and shock. Blood oxygen saturation affects the relationship between blood CO_2 content and PCO_2, a phenomenon called the Haldane effect. This means that alterations of arterial oxygen saturation or tissue oxygen extraction can affect the mucosal-arterial PCO_2-gap independent of changes in mucosal CO_2 content, and this is a potential source of error[151].

EFFECTS OF ETHYL PYRUVATE

The anti-inflammatory and reactive oxygen species scavenging properties of EP was documented in several pre-clinical animal models[77]. Theoretically, EP could also have positive inotropic effects through metabolic support resulting from the administration of pyruvate. Early studies suggested direct positive cardiac effects of EP[78], and EP also improved intestinal microcirculation in a mouse model of mesenteric ischaemia/reperfusion[79]. Since the data on the effects of EP in sepsis and endotoxaemia were mainly derived from small animal models at the time, we wanted to evaluate the effects of resuscitation with REPS on haemodynamics and metabolic parameters in a large animal model of acute endotoxaemia (paper I).

Resuscitation with a bolus dose of either REPS or RA after 60 minutes of endotoxaemia temporarily improved CI compared to baseline values, but this was soon followed by a progressive deterioration of CI, and at the end of the experiment all animals were again severely hypodynamic. The change in systemic haemodynamic parameters from intervention to the end of the experiment did not differ between the REPS group and the RA group, indicating that EP did not have direct positive inotropic effects in this model. Microcirculatory perfusion in the liver, kidney, and ileal mucosa and muscularis deteriorated throughout the experiment without any intergroup differences.

In contrast to this, EP improved central haemodynamics in a 24-hour model of porcine endotoxaemic shock[152], and significantly prolonged the time to arterial hypotension in a 30-hour model of ovine peritonitis[153]. The results from paper I indicate that positive cardiovascular effects seen in studies of longer duration could have been secondary to the anti-inflammatory effects of EP rather than due to direct positive cardiac effects. However, it is possible that hypovolaemia developing during later time points in paper I could have concealed a positive inotropic effect of EP in our model.

One explanation for the lack of effect seen with EP is the relatively short timeframe of the study. EP is a potent inhibitor of the late-acting proinflammatory cytokine HMGB-1[77], previously shown to be a mediator of LPS-induced lethality. Positive central haemodynamic and microcirculatory effects of inhibiting HMGB-1 are unlikely to appear in the 5-hour study period used in paper I.

Animals in the REPS group showed significantly lower BE and a higher anion gap at later time points. This could in part be explained by the higher chloride content in REPS compared to RA, but the higher anion gap suggests that the lower BE was not explained only by differences in chloride content. Pyruvic acid is a strong acid ($pK_a \approx 3$), and this is a possible explanation for the difference in anion gap. Also, even though no differences in perfusion parameters were found, a release of tissue acids secondary to inadequate perfusion in the REPS group cannot be ruled out.

There are several limitations to paper I. The relatively short model and the difference in chloride content between REPS and RA have been addressed above. Also, the study did not evaluate possible positive effects of EP on inflammatory cytokines or tissue damage. Considering CI and renal blood flow, the REPS group had significantly lower values compared to the RA group before treatment, after 60 minutes of endotoxaemia. In this model, the initial peak in pulmonary hypertension occurred between 50 and 70 minutes after the start of endotoxaemia, but in two pigs in the RA group the initial increase in MPAP started to revert before intervention after 60 minutes. The difference in CI and renal blood flow seen after 60 minutes of endotoxaemia was most likely due to this individual variability in the time point for reversal of the initial peak in MPAP. This is also supported by the fact that the degree of change of systemic haemodynamic variables from 60 to 300 minutes was identical between the groups. Still, the fact that a significant intergroup difference in a central parameter was found before treatment makes data interpretation more complicated.

Interestingly, although EP has been proven beneficial in several preclinical animal models of endotoxaemia or sepsis, there have also been reports of negative effects of EP administration. In a mouse model of endotoxaemia testing several different doses of EP, EP administration decreased early (3 hours) NF-κB and cytokine levels, but at a later (9 hours) time point the levels were increased compared to controls[154]. When all groups receiving EP were combined, EP also significantly increased the hazard ratio of death[154]. Activation of NF-κB pathway is an important part of the dysregulated inflammatory response that is characteristic of sepsis. However, the effects of NF-κB are complex, including pro-inflammatory, anti-inflammatory and anti-apoptotic properties, and concerns have been raised that a therapeutic strategy based on the inhibition of NF-κB could, in some cases, have unintended negative effects[155,156].

A phase II trial of the effects of EP in high-risk patients undergoing cardiopulmonary bypass was recently performed. The trial was terminated by the sponsor after 102 patients due to problems with the drug container closure system. Data from the trial showed that the administration of EP did not appear to confer any benefit to high-risk cardiac surgical patients undergoing cardiopulmonary bypass[157]. So far, no data on the effects of EP on patients with sepsis have been presented.

EFFECTS OF ENDOTHELIN RECEPTOR ANTAGONISTS

ET-1 has been implicated as one of the possible mediators of the gut microcirculatory failure in sepsis. Tissue levels of ET-1 are increased in the intestinal mucosa in endotoxaemia[102], and antagonizing ET-1 has proven beneficial to the intestinal microcirculation in rat models of bacteraemia[39] and endotoxaemia[102].

Mixed endothelin receptor antagonism with tezosentan was effective in improving microcirculatory perfusion in the ileal (papers II-III) and colonic (paper II) mucosa. Although this improvement probably to some extent was due to improved systemic

haemodynamics, we did not find any statistically significant differences in SMAFI in paper III, indicating that tezosentan also had direct positive effects on the microcirculation in the ileal mucosa.

In a porcine model of peritonitis, Krejci et al found that the mixed ET receptor antagonist bosentan improved microvascular perfusion in the jejunal mucosa compared to baseline, but could not demonstrate a statistically significant improvement compared to controls[101]. Of the ET receptors, the ET_A receptor has been suggested to be the most important receptor mediating vasoconstriction, while the ET_B receptor also mediates vasodilation and clearance of circulating ET-1. Tezosentan has more pronounced effects on ET_A than on ET_B receptors compared to bosentan, 30:1 compared to 20:1, possibly rendering tezosentan more beneficial to the intestinal microcirculation. Differences in experimental models, fluid loading and drug dosing could also explain the better effect seen with tezosentan in this model.

Tezosentan also improved $PCO_{2muc\text{-}art}$ (papers II-III), indicating a better balance between oxygen supply and demand in the intestinal mucosa. This is in line with previous studies using bosentan in similar models of porcine endotoxaemia[100,105].

The improvement of mucosal microcirculation did not occur at the expense of perfusion in the muscular layer, since perfusion in the muscular layer did not differ between controls and the tezosentan group (paper III). The fact that tezosentan did not affect perfusion in the muscular layer is in accordance with the results of Krejci et al[101]. In endotoxaemia and sepsis, mucosal microcirculatory perfusion is initially better preserved than muscular perfusion through a redistribution of microvascular flow[158]. Our findings indicate that ET-1 is not of major importance to this redistribution of flow in the intestinal wall.

The physiological response to endothelin receptor activation is hard to predict and varies depending on the receptor population in different tissues. Theoretically, selective ET_A receptor antagonism has advantages in preserving ET_B receptor-mediated vasodilation and clearance of ET-1, and in a previous study selective ET_A receptor antagonism improved microcirculation in the ileal submucosa in a rat model of endotoxaemia[102]. However, selective ET_A receptor antagonism with TBC3711 failed to improve microcirculation in the ileum and mucosal PCO_2 levels, indicating that also the ET_B receptor can mediate intestinal microcirculatory dysfunction in this model of endotoxaemia. Still, the interaction between the endothelin receptors is very complex, and it has been proposed that a cross-talk exists between the ET_A and the ET_B receptors, making interpretation of the role of the different ET receptor subtypes complicated. Ozaki et al showed that activation of the ET_A receptor induced changes in the ET_B receptor through ET_A receptor-mediated intracellular signalling, thereby altering the affinity of the ET_B receptor to ligands[89]. In line with this, Just et al found that the effects of the ET_B receptor on renal blood flow differed depending on the degree of ET_A receptor activation[159]. When both receptors were stimulated

simultaneously, the ET_B receptor induced vasodilation, but when the ET_B receptor was selectively activated without concomitant ET_A receptor stimulation the response seemed to be predominantly vasoconstriction. The existence of a cross-talk between the ET receptors was not investigated in this thesis, but the results show that mixed ET receptor antagonism is necessary to improve microcirculation in the ileal mucosa in porcine endotoxaemia.

Previous findings from rat models have indicated that ET-1 is an important mediator of hepatic microcirculatory dysfunction in endotoxaemia[108,160], but mixed endothelin receptor antagonism failed to improve hepatic microcirculation in porcine peritonitis[101] and in a model of systemic inflammation in rats[115]. Although tezosentan improved portal vein flow, no statistically significant improvement in hepatic microcirculatory perfusion could be demonstrated in paper II or III. TBC3711 failed to improve either portal vein flow or hepatic microcirculation compared to controls (paper III).

The increase seen in portal vein flow with tezosentan indicated an increased blood flow to the liver, but since we did not measure hepatic artery flow, total hepatic blood flow could not be calculated. However, the hepatic arterial buffer system is impaired in endotoxaemia[63], and hence it is not likely that the lower portal vein flow in controls was fully compensated for by a higher hepatic artery flow. There are several possible explanations for the lack of improvement of hepatic microcirculatory perfusion in spite of the apparent higher hepatic blood flow in the tezosentan group. Microcirculatory perfusion was measured at the surface of the liver, and possibly perfusion could have been redistributed to deeper layers in the tezosentan group. Moreover, it has been suggested that laser Doppler flowmetry readings are more sensitive to changes in hepatic artery flow than portal vein flow[161], and if true, this could, to some extent, explain our results. The use of the laser Doppler technique did not allow us to evaluate changes in microcirculatory heterogeneity, and ET receptor antagonism could have improved heterogeneity within the microcirculation without affecting the measure of perfusion obtained with LD. The relatively small number of animals studied must also be taken into account, and possibly differences could have been found if the number of animals were increased. However, when combining controls and tezosentan-treated animals from papers II and II, thereby increasing the number of animals in each group to 14, there was still no significant difference in hepatic microcirculatory perfusion ($p=0.43$) to be found, further supporting the conclusion that ET-1 is not a main determinant of hepatic microcirculatory perfusion in porcine endotoxaemia.

EFFECTS OF CHANGES IN REGIONAL BLOOD FLOW AND PERFUSION PRESSURE

Current protocols for the treatment of septic shock are to a large extent based on achieving predetermined goals for central haemodynamic parameters[66]. However, microcirculatory alterations can persist even though haemodynamic goals of treatment protocols are met, and in septic patients with comparable systemic haemodynamics

persistent microcirculatory alterations have been correlated to patient outcome[3,33]. Data from recent studies indicate that microcirculatory changes seem to be rather dissociated from central haemodynamic parameters, but the microcirculation will obviously be severely affected below certain levels of flow and perfusion pressure. However, these levels so far remain to be defined.

In paper IV, endotoxaemia induced intestinal microcirculatory disturbances measured with both laser Doppler flowmetry and SDF in spite of a substantial increase in SMAFI, strengthening the hypothesis that gut microcirculatory dysfunction can appear independent of changes in systemic and regional blood flow, and demonstrating that increasing whole organ blood flow is not necessarily an effective therapeutic approach for the microcirculation. The data from papers II and III further support the notion that the association between regional blood flow and intestinal microcirculatory changes is rather weak. Moreover, the increase seen in arterial-mucosal PCO_2-gap indicates that an imbalance between oxygen supply and demand in the mucosa developed in parallel with the increase in SMAFI.

In line with the findings in paper IV, two previous studies found severe microcirculatory derangements in the ileal mucosa in spite of a maintained superior mesenteric artery flow, using a 12-hour porcine model of cholangitis[162] and a short-term model of ovine endotoxaemia[56], respectively. Furthermore, severe decreases in ileal percentage of perfused villi have been found in parallel with maintained[163] and increased[164] portal vein flow in 24-hour models of porcine endotoxaemic shock. Taken together, these results indicate that regional blood flow changes are not the main factor behind the microcirculatory failure seen in the ileal mucosa in sepsis and endotoxaemia.

In paper IV, microcirculatory perfusion in the muscular layer and the mucosal layer was reduced to a similar extent by endotoxaemia, indicating that the increase in intestinal blood flow was not directed to either the mucosa or the muscularis of the ileum. A possible explanation for this discrepancy is that perfusion was directed towards the submucosa or the deeper layers of the mucosa or muscularis, layers presumably not penetrated by the laser Doppler probes, and not visualized using SDF. Also, changes in microvascular perfusion can be heterogeneous between different parts of the intestines[36], and regional blood flow could have been directed to another part of the intestine. The reports of the existence of an arteriovenous anastomotic network in the submucosa[165], possibly contributing to the shunting of flow, are not consistent, and the existence of any extensive anastomotic network in the submucosa remains questionable.

Moreover, comparing changes in regional blood flow measured with ultrasonic flow probes and microvascular perfusion measured with laser Doppler probes is somewhat complicated, since the measures of perfusion provided differ between the two devices. While the ultrasonic flow probes provide a measure of linear flow, expressed as ml/min, the laser Doppler perfusion readings are influenced by both the velocity of red

blood cells and the tissue haematocrit. The decrease in systemic haematocrit seen after 24 hours of endotoxaemia probably contributed to the discrepancy between changes in regional blood flow and microvascular perfusion in the mucosa and muscularis. The magnitude of the influence of the change in haematocrit is difficult to determine, since microvascular haematocrit differs from systemic haematocrit due to the Fåhreus effect[166] and the plasma skimming phenomenon[13]. Haemodilution per se can also redistribute flow between different capillary networks within a tissue[167]. The possibility of variations in the volume of tissue monitored by laser Doppler flowmetry must also be considered. Changes in the optical properties of the tissue over time, for instance due to increased tissue oedema, could have influenced penetration depth and thereby measurement volume[126].

In paper IV, microcirculatory perfusion was reduced after 24 hours of endotoxaemia in parallel with a decrease in MAP from 90 mm Hg to 60 mm Hg, indicating that the reduction in perfusion pressure could be a factor contributing to microcirculatory dysfunction. In order to evaluate the effects of elevating perfusion pressure with norepinephrine, MAP was restored using a continuous norepinephrine infusion.

The intestinal microcirculatory effects of norepinephrine have been investigated in various animal models, with different studies indicating beneficial[119], detrimental[67,120], or no effects[121,122], but these studies have mainly been performed in short-term models of sepsis or endotoxaemia. However, adrenergic receptor function is altered over time[168,169], necessitating the use of studies of longer duration in order to better mimic the situation in septic shock patients.

Restoring MAP with norepinephrine did not significantly affect either lased Doppler-derived perfusion or SDF-derived variables of intestinal microcirculatory blood flow. Neither did the mucosal-arterial PCO_2-gap change significantly, indicating that norepinephrine treatment did not alter the balance between oxygen supply and demand in the ileal mucosa.

Since restoring MAP with norepinephrine did not affect SMAFI, the microcirculatory effect of increasing perfusion pressure could be evaluated without changes in regional flow complicating the picture. The findings in paper IV indicate that elevating perfusion pressure is not an effective treatment for intestinal microcirculatory dysfunction in endotoxaemic shock, at least not after several hours of hypotension. This is in line with two recently published investigations where increasing perfusion pressure with norepinephrine failed to significantly improve sublingual microcirculation in septic shock patients[170,171]. However, we cannot rule out that the microcirculation could have improved if perfusion pressure was increased earlier in the course of endotoxaemia, but over time the microcirculation was rendered unresponsive to changes in perfusion pressure.

In sepsis, both microcirculatory failure and mitochondrial dysfunction are thought to play a role in the progression to multiple organ failure and death[29]. After 24 hours of endotoxaemia, mucosal-arterial PCO_2-gap was significantly increased in parallel with

microcirculatory dysfunction and a significant decrease in mitochondrial complex I enzyme activity. Microcirculatory perfusion disturbances have previously been indicated as an important factor contributing to increases in mucosal PCO_2 levels in sepsis[149,150], but the role of mitochondrial dysfunction in this context is still unclear. However, given the findings in paper IV, it is reasonable to assume that both microcirculatory and mitochondrial alterations contributed to the mucosal acidosis induced by endotoxaemia.

Although no significant difference in mitochondrial enzyme activity was found after the initiation of norepinephrine treatment, complex I and complex I/CS activity increased in five out of six animals from which biopsies were obtained. Reguirea et al. have previously demonstrated that norepinephrine improved liver mitochondrial complex I activity in a porcine model of endotoxaemia[172], a finding that supports the possibility of a beneficial effect of norepinephrine on intestinal mitochondrial function. Given the results presented here and those of Reguirea et al., further studies investigating the effects of norepinephrine on mitochondrial function in sepsis and endotoxaemia are warranted.

CONCLUSIONS

- In endotoxaemic shock, gut microcirculatory disturbances develop in spite of an increase in superior mesenteric artery flow, demonstrating the loose connection between changes in regional blood flow and intestinal microcirculatory perfusion in endotoxaemia.

- Elevating perfusion pressure does not seem to be an effective treatment for gut microcirculatory derangements in ovine endotoxaemic shock.

- The finding that gut microcirculatory perfusion has a weak correlation to systemic and regional indices of flow and pressure in endotoxaemia strengthens the hypothesis that monitoring and therapies directed at the microcirculation could be of value in sepsis.

- The endothelin system is an important mediator of microcirculatory dysfunction in the ileal mucosa in endotoxaemia, and mixed endothelin receptor antagonism is needed to counteract these endothelin-induced microcirculatory disturbances.

- The endothelin system does not seem to be an important factor contributing to hepatic microcirculatory failure in endotoxaemia.

- In porcine endotoxaemic shock, resuscitation with Ringer's ethyl pyruvate solution does not have initial beneficial effects on haemodynamics, splanchnic perfusion, or acid-base status compared to Ringer's acetate.

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