Pathophysiologic, Inflammatory and Haemostatic Responses to Various Endotoxaemic Patterns

An Experimental Study in the Pig

MIKLŐS LIPCSEY
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Abstract

Septic shock is frequently seen in intensive care units and is associated with significant mortality. Endotoxin – a major mediator of the pathophysiological responses – is released during lysis of Gram-negative bacteria. These responses can be mimicked in the endotoxaemic pig.

This thesis focuses on the following topics: the inflammatory and pathophysiological responses to various endotoxin doses and infusion patterns; covariations between endotoxin induced inflammatory and pathophysiological responses; whether the biological effects of endotoxin can be modulated by clopidogrel and whether tobramycin or cefazidime reduce plasma cytokine levels.

Endotoxin induced linear log-log cytokine and F2-isoprostane responses. Leukocyte and platelet responses, pulmonary compliance, circulatory variables as well as indicators of plasma leakage and hypoperfusion exhibited log-linear responses to the endotoxin dose. Biological responses to endotoxaemia such as inflammation, hypotension, hypoperfusion and organ dysfunction were more expressed when the organism was exposed to endotoxin at a higher rate. These results may facilitate the possibility to choose relevant endotoxin administration, when experiments are set up in order to evaluate certain responses to endotoxaemia.

Correlation studies between cytokines, leukocytes, platelets and the endotoxin dose were in agreement with the well-known ability of endotoxin to induce cytokine expression and to activate both primary haemostasis and leukocytes. Free radical mediated lipid peroxidation and COX-mediated inflammation correlated to cytokine expression and organ dysfunction in endotoxaemic shock.

Endotoxaemic pigs pretreated with clopidogrel, exhibited a trend towards less expressed deterioration of renal function, although blocking of ADP-induced primary haemostasis is not a key mediator of endotoxin induced deterioration of renal function.

Tobramycin did not neutralise the biological effects of endotoxin or the plasma levels of endotoxin, suggesting that these antibiotics do not bind to endotoxin.

Reduction in IL-6 was greater in pigs treated with cefazidime and tobramycin as compared with those given saline, indicating a possible anti-inflammatory effect of both antibiotics.

Keywords: sepsis, animal model, cytokines, isoprostanes, endotoxic shock, pig

Miklós Lipsey, Department of Surgical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden

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urn:nbn:se:uu:diva-7237 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-7237)
To Krisztina
“What is a scientist after all? It is a curious man looking through a keyhole, the keyhole of nature, trying to know what's going on.”

Jacques Yves Cousteau, marine ecologist
List of papers

This work is based on the following papers, which will be referred to in the text by their roman numerals:

I  Inflammatory, coagulatory and circulatory responses to logarithmic increases in the endotoxin dose in the anaesthetised pig.
Miklós Lipcsey, Anders Larsson, Mats B. Eriksson and Jan Sjölin.

II  Effect of the administration rate on the biological responses to a fixed dose of endotoxin in the anaesthetised pig.
Miklós Lipcsey, Anders Larsson, Mats B. Eriksson and Jan Sjölin.
In manuscript.

III  F₂-isoprostane, inflammation, cardiac function and oxygenation in the endotoxaemic pig
Miklós Lipcsey, Ewa Söderberg, Samar Basu, Anders Larsson, Jan Sjölin and Mats B. Eriksson.
Submitted.

IV  Early endotoxin-mediated haemostatic and inflammatory responses in the clopidogrel-treated pig.
Miklós Lipcsey, Anders Larsson, Matts Olovsson, Jan Sjölin and Mats B. Eriksson.

V  Endotoxin neutralization and anti-inflammatory effects of tobramycin and ceftazidime in porcine endotoxin shock.
Gunilla Goscinski, Miklós Lipcsey, Mats Eriksson, Anders Larsson, Eva Tano and Jan Sjölin.
Critical Care 2004; 8:35-41.
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<td>8-iso-PGF$_{2\alpha}$</td>
<td>8-iso-prostaglandin F$_{2\alpha}$</td>
</tr>
<tr>
<td>15-KDH-PGF$_{2\alpha}$</td>
<td>15-keto-dihydro-PGF$_{2\alpha}$</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>aPTT</td>
<td>Activated partial tromboplastin time</td>
</tr>
<tr>
<td>BPI</td>
<td>Bactericidal permeability increasing protein</td>
</tr>
<tr>
<td>C</td>
<td>Complement factor</td>
</tr>
<tr>
<td>C$_{\text{dyn}}$</td>
<td>Dynamic airway compliance</td>
</tr>
<tr>
<td>C$_{\text{stat}}$</td>
<td>Static airway compliance</td>
</tr>
<tr>
<td>CI</td>
<td>Cardiac index</td>
</tr>
<tr>
<td>CL$_{\text{cr}}$</td>
<td>Creatinine clearance</td>
</tr>
<tr>
<td>cm H$_2$O</td>
<td>Centimetre water</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>CI</td>
<td>Cardiac index</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficients of variation</td>
</tr>
<tr>
<td>CVP</td>
<td>Central venous pressure</td>
</tr>
<tr>
<td>DO$_2$</td>
<td>Oxygen delivery</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>F</td>
<td>French</td>
</tr>
<tr>
<td>FiO$_2$</td>
<td>Inspired fraction of oxygen</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilopascal</td>
</tr>
<tr>
<td>LAL assay</td>
<td>Limulus amebocyte lysate assay</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS binding protein</td>
</tr>
<tr>
<td>Log</td>
<td>Logarithm, Logarithmic</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>mL</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mmHg</td>
<td>Millimetre mercury</td>
</tr>
<tr>
<td>mmol</td>
<td>Millimol</td>
</tr>
<tr>
<td>MPAP</td>
<td>Mean pulmonary arterial pressure</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>p</td>
<td>Probability</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Arterial partial pressure of oxygen</td>
</tr>
<tr>
<td>PCWP</td>
<td>Pulmonary capillary wedge pressure</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>P&lt;sub&gt;peak&lt;/sub&gt;</td>
<td>Peak airway pressure</td>
</tr>
<tr>
<td>PvO₂</td>
<td>Venous partial pressure of oxygen</td>
</tr>
<tr>
<td>PVRI</td>
<td>Pulmonary vascular resistance index</td>
</tr>
<tr>
<td>r</td>
<td>Regression coefficient</td>
</tr>
<tr>
<td>SaO₂</td>
<td>Arterial oxygen saturation</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SvO₂</td>
<td>Venous oxygen saturation</td>
</tr>
<tr>
<td>SVRI&lt;sup&gt;TM&lt;/sup&gt;</td>
<td>Systemic vascular resistance index</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TV</td>
<td>Tidal volume</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
</tr>
<tr>
<td>UO</td>
<td>Urinary output</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
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Introduction

The development of the eukaryotic cell and later the multicellular structure has given us several evolutionary advantages compared to the prokaryotic organisms. Nevertheless these advantages have by no means lead to the disappearance of the prokaryotes. These organisms still share the biosphere with us and with most of them we live in peaceful coexistence. With some of the prokaryotes we live in symbiosis and only a minor fraction of them are threats to us by being pathogens.

The vertebrates have developed sophisticated systems to preserve their integrity during the continuous encounters with pathogenic organisms. These protective systems include compartmentisation with barriers, colonisation with non-pathogenic organisms and the adaptive immune system.

It is the interaction between pathogenic microorganisms and the immune system that causes the state of disease. The activation of the immune system by microorganisms or their fragments causes the inflammatory reaction that gives most of the disease symptoms. This activation of the immune system by the microbial disease processes is usually within its capacity and the aim of the inflammatory reaction is to preserve the integrity of the body. However, in septic shock, fulminant microbial activation of the immune system leads to disregulation of the inflammatory reaction that may - while protecting the body - cause extensive tissue damage leading to organ failure and ultimately death.

As our understanding of these inflammatory reactions is inadequate, considerable efforts are made to study these phenomena. Apart from doing bedside studies in patients with septic shock, studies can also be performed in animal models.
Initially, when bacteria penetrate the body and cause an infection it is usually a localised process initially. If the site of the infection is visible the cardinal signs of inflammation – tumor (swelling), rubor (redness), dolor (pain), calor (heat) and functio laesa (dysfunction of the organs involved) – may be present as described by Aulus Cornelius Celsus and Rudolf Virchow [1]. If this bacteria from this local process gain access to the circulation, the inflammation becomes generalised and the signs similar to that of the local inflammatory process may have been seen in the whole body. This clinical condition, when a bacterial infection triggers a generalised inflammatory response with symptoms, is sepsis.

Sepsis is commonly caused by Gram-negative bacteria. One of the best-studied factors triggering generalised inflammation in Gram-negative sepsis is endotoxin. In sepsis research endotoxin is widely used in human and animal models. The papers in this thesis are based on studies of the endotoxin model in the pig.

Sepsis

Concepts of sepsis through history

Treatment of septic fever with herbal medicines was already described in 2735 B.C., by the Chinese Emperor, Sheng Nung [2]. In the ancient greek and roman medicine infection of wounds with the appearance of pus was not only seen as the natural process of healing, but interestingly it was considered a commendable sign (pus laudable).

Bacteria were visualised already in 1683 by Anton van Leeuwenhoek, the first to use the light microscope in biologic research. Nevertheless it took almost two more centuries until the emerging concepts of microbiology came to patient’s benefit when Ignaz Semmelweis in 1848 hypothesised that “putrid”, rotten particles, were the cause of disease and that by antiseptic measures the death rate in puerperal fever could be decreased [3]. In 1879, Louis Pasteur identified the streptococcus bacteria as the cause of puerperal sepsis [1]. Thirteen years later, Richard Pfeiffer discovered that bacteria released a poisonous heat-stable substance that caused symptoms of sepsis and introduced the term endotoxin [4]. Alexandre Besredka was the first to produce antibodies that were capable of suppressing the poisonous effects of endotoxin in 1906 [5].

In the beginning of the 20th century the host was regarded to have a passive role in the pathogenesis of the septic syndrome, while bacterial virulence and bacterial toxins were seen as the dominating factor. It was not until Giuseppe Sanarelli [6] and Gregory Shwartzman [7] published their observa-
tions with repeated endotoxin exposure that the immune state of the host came into focus [8]. In the 1940ies the chemical structure of the endotoxins was characterised and endotoxaemic animal models were introduced in sepsis research. In the 70ies the significance of the complement system was pointed out. In the 80ies the importance of the cytokine network as well as the arachidonic acid metabolites were elucidated, and in the 90ies the role of the haemostatic system in the inflammatory reaction was further highlighted, emphasising the importance of these host factors in the sepsis. It was also realised that the infectious aetiology is just one of the pathways to initiate the systemic inflammatory reaction since e.g. pancreatitis and burns initially exhibit a similar clinical picture.

The definitions and clinical manifestations of sepsis

Although the term sepsis (Greek, from “sēpō” meaning “make rotten”) has been used to describe a condition with fever, chills and altered mental state, no general consensus existed on the terminology of systemic bacterial infection. For many years a variety of terms such as sepsis, septicemia, bacteremia, infection, septic shock, toxic shock were used to describe illnesses associated with infection, or illness that looked like infection.

In 1992, the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) Consensus Committee defined “sepsis” as a state of disease with the presence of both an infectious process and a systemic inflammatory response [9]. An infectious process was defined as a pathologic process caused by the invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganisms, and systemic inflammatory response as the presence of in addition to at least two of the following: hypothermia or fever, tachycardia, tachypnoea and leucopenia or leucocytosis representing an acute alteration from baseline in the absence of other known causes. The term “severe sepsis” refers to sepsis complicated by organ dysfunction, hypotension and/or hypoperfusion. The term “septic shock” refers to a state of acute circulatory failure characterised by persistent arterial hypotension despite adequate volume resuscitation.

The sepsis diagnosis criteria were updated in 2001 expanding the list of signs and symptoms with altered mental status, significant oedema or positive fluid balance, hyperglycaemia in the absence of diabetes, normal white blood cell count with >10% immature forms, elevated plasma C-reactive protein, elevated plasma procalcitonin, arterial hypotension, elevated SvO₂, high CI, arterial hypoxemia, acute oliguria, elevated plasma creatinine, coagulopathy, ileus, thrombocytopenia, hyperbilirubinaemia, hyperlactataemia and decreased capillary refill or mottling [10].

It should be understood that none of these are specific for sepsis, but rather a broad spectrum of signs and symptom that may aid to the clinician to consider sepsis, if the presence of these cannot be explained with other
pathologic processes. The sepsis definitions from 1992 made it possible for investigators that with simple entry criteria define patient populations for clinical trials that, at least to some extent, were comparable. In contrast, the revised sepsis definitions from 2001 gave priority bed-side situation describing the septic looking patient.

The epidemiology of sepsis in the intensive care unit
Since the consensus definitions of sepsis were published, epidemiologic research in this field could rely on a terminology that was interpreted similarly worldwide. Several studies have confirmed that sepsis and septic shock are common in intensive care units (ICU). In an American study, where only a fraction (2%) of the patients admitted to tertiary care hospitals had sepsis, a substantial number of the sepsis patients (59%) were admitted to the ICU [11]. In a European study on ICU patients, infection in any form, increased mortality 2.5 times compared to rest of the ICU population [12]. Patients with clinically suspected sepsis with or without microbiologically documented infection are at equally high risk of death [13]. From 1979 to 2000 the incidence of sepsis increased from 164,000 cases to 660,000 per annum in the United States. During the same period the relative mortality fell (from 27.8 to 17.9 percent) but the absolute mortality increased. Although gram-positive bacteria and fungi are becoming more frequent causative agents, gram-negative bacteria are still responsible for a substantial part of the infective agents in sepsis in the USA [14]. Elevated endotoxin levels have been demonstrated in 70-80% of patients with severe sepsis or septic shock and the magnitude of increase in plasma endotoxin levels correlated to lethal outcome [15-17]. It should be remembered that sepsis is the second leading cause of death among patients in non-coronary ICUs [18]. Furthermore, sepsis substantially reduces the quality of life of those who survive [19, 20]. Apart from the impact on the life of an individual patient the economic burden of sepsis on society is substantial, e.g. the care of patients with sepsis costs annually $17 billion in the United States [21].

Sepsis management
Management of the critically ill sepsis patient necessitates rapid diagnosis and management. Early cardio-respiratory resuscitation of the septic patient including oxygen and fluid therapy as well as early adequate antibiotic therapy is crucial. Artificial ventilation is indicated when the free airway is not secured and/or the respiration is insufficient. If circulatory insufficiency persists despite fluid resuscitation, vasopressor and inotropic support should be considered. Low-dose steroid therapy is recommended in vasopressor requiring septic shock. Surgical source control should be performed when the infection focus can be identified and drainage or excision is possible.
Renal replacement therapy should be initiated in case of renal failure. Use of recombinant activated protein C in patients with septic shock and high risk for death is recommended [22].

Interventions in the coagulation system in sepsis
Numerous attempts have been made to modulate the inflammatory reactions during septic shock by antithrombotic treatment [23]. Several points of the coagulation cascade have been subject to experimental and clinical investigations in sepsis studies. Inhibition of factor X, as well as the tissue factor/factor VIIa complex counteracts endotoxin-mediated experimental coagulopathy [24, 25]. Treatment with activated protein C reduces mortality in patients with severe sepsis [26].

Our research group has shown previously that melagatran, a thrombin inhibitor, counteracts the deterioration of the renal function in endotoxaemic pigs. However, melagatran administration caused a marked increase in activated partial thromboplastin time (aPTT) in endotoxaemic pigs [27], which could indicate an increased risk of bleeding complications.

Clopidogrel, a platelet inhibitor
In the activated coagulation system, thrombin both cleaves fibrinogen to fibrin and also acts as a platelet activator. Thrombin binds to PAR1 (protease-activated receptor) on the platelet surface, which leads to secretion of ADP (adenosine diphosphate) from the platelet granules. ADP then activates platelets through binding to the P2Y<sub>12</sub> receptor. Activation of platelets includes a structural confirmation of the GPIIb/IIIa receptor that binds fibrin, hereby linking platelets together and adhering them to the vascular wall. Clopidogrel selectively interferes with thrombin mediated platelet activation via ADP-receptor inhibition [28-32].

In sepsis, activation of platelets leads to increased expression of P-selectin on the platelet membrane [33]. P-selectin enhances the expression of tissue factor on monocytes as well as it mediates platelet adherence to endothel cells [34]. Tissue factor induces activation of the extrinsic coagulation pathway that leads to the activation of thrombin, which in turn activates platelets through the PAR1 receptor [35]. In theory, inhibition of platelet activation by thrombin could prevent one of the feedback loops of coagulation activation and thereby may be of beneficial effect in sepsis [33].
Methodological considerations in sepsis models

The validity of animal models in sepsis research

The vast part of our knowledge of physiology and pathophysiology is a result of studies conducted in animal models, and sepsis research in this respect is no exception. Experimental animal models allow investigations that are not possible in humans because of ethical or practical considerations. However, man is not mice. Although the physiology of humans and animals are similar, research utilising animal models have not always given results that are applicable in humans. Differences between humans and the model species alter the response to pathologic processes and may lead to misleading conclusions about human pathophysiology. For example, sensitivity to endotoxin varies considerably between different species and primates, especially humans are several magnitudes more sensitive to endotoxin than pigs, dogs or rats [36]. Critics of animal models also argue that whereas the most common sepsis patient category in an ICU is an aged person with several systemic diseases, most of the research animals are young and healthy. Also, many models study the animals in early sepsis and mortality with a few hours as the endpoint, while in the clinical setting patient mortality is seldom so close to the first signs of infection [37].

Animal models are a most relevant tool for studying several pathophysiologic phenomena during sepsis. Early inflammatory mediators, for example, have been strongly conserved through evolution. Endotoxin administered to animals or humans induces many features of sepsis such as cytokine release, leukopaenia, fever, cardiovascular changes, nephropathy and coagulopathy. Endotoxin administration to human volunteers has been used in several studies, although it must be remembered that such experiments always carry risk for unforeseen, potentially life-threatening complications [38]. The possibility of performing standardised animal experiments, where extensive monitoring is used, in combination with the opportunity to administer a broad spectrum of dosages of endotoxin, gives us the option to evaluate pathophysiologic events and reactions, ultimately death, with a high degree of reproducibility.

Thus, results from animal models is still a major source of knowledge in sepsis research, but it should be kept in mind that these are just models and that findings in animal experiments are the basis for further investigations in humans.

The animal species

The choice of laboratory animal species is determined by the organ systems to be studied, number of animals needed, local legislation, project budget, housing possibilities, animal size and local traditions with existing models.
In sepsis research considerable knowledge has accumulated on models in different animal species. Despite of being the most abundant laboratory animals, mice and rats are relatively insensitive to endotoxin, causing difficulties when results from such experiments are interpreted and deduced in a human settlement. Rabbits are sensitive to endotoxin but their size still limits the use of intensive care equipment. The sheep’s anatomy in the lower respiratory tract differs considerably from the human, since sheep have collateral ventilation [39]. Lung vascular smooth muscle is a determinant of the pulmonary hypertensive response and contributes to interspecies variability [40]. Although the dog was a sanitary problem a century ago, today the fact that they are popular pets limits their use ethically.

It is not surprising that the closest resemblance to human sepsis response is seen in monkeys, especially primates. Experiments in baboons have contributed to unveil important immunological mechanisms, related to the development of organ dysfunction [41]. Despite of the fact that monkeys are biologically ideal, these experiments tend to be less and less used. Many of these animals are endangered, the legislation restricts or forbids their use and even non-endangered species are costly.

Porcine endotoxin shock models have been widely employed in experimental sepsis research. Although the circulation of the pig has been shown to be most similar to that of humans among non primates [36, 42] the initial response to endotoxin is a hypodynamic state, with low cardiac output, contrary to the human response that is hyperdynamic with high cardiac output. The sensitivity of pig is much greater than that for rats and mice, but is still at least a magnitude less than for humans. The juvenile pig is large enough for instrumentation and the usage of human medical equipment. Also, the blood volume of the juvenile pigs allows extensive blood sampling. Considering these issues the experiments in this thesis are carried out in the pig model.

Sepsis models

Several ways of inducing sepsis or endotoxaemia have been described. These include parenteral administration of endotoxin or living bacteria as well as surgical perforation of the cecum.

As LPS can be relatively easily stored, quantified and administered in a standardised dose it provides a simple and reproducible model. This is an advantage when studying effects of substances with specified modes of action [43]. In particular endotoxin models are of interest when drugs or other treatments with a proposed anti-endotoxin effect are being investigated [44-46].

However while endotoxin elicits a hyperdynamic cardiovascular response in humans the pattern of response is characteristically biphasic in large mammals such as the pig. In the pig the cardiac output decreases with a nadir
at about three hours after the start of continuous endotoxin administration and by eight to twelve hours it reaches supranormal values.

The hyperdynamic porcine models are characterised by long observation periods with the first cardiac output measurements after six hours. These long observation periods are cumbersome since long term anaesthesia or sedation may influence the findings whereas preservation of intravenous access in unanaesthetised animals may be problematic. Furthermore, animal welfare regulations, based on ethical considerations, restrict the use of awake-animals in experiments where considerable suffering is expected. This results in observation periods that are usually less than 8 hours in experimental sepsis. In order to reach the state of multiorgan failure relatively high endotoxin doses are used, which may be rare in clinical sepsis. This may produce a condition of acute intoxication rather than a gradually developing sepsis. The other consequence, of these rather short observation periods, is that such models are most suitable for the investigation of the phenomena of early sepsis [43].

Since other bacterial factors than endotoxin may also contribute to the host response animal models using infusion of live gram-negative bacteria [47], usually Escherichia Coli, have occasionally been proposed to be clinically more relevant than endotoxin models [48]. Nevertheless the pathophysiological changes are virtually identical to endotoxin infusion.

A number of peritonitis models have been described in the literature. The most common are caecal ligation and puncture and implantation of faeces. The shortcomings of these models are the variable microbial composition and dose. To overcome these limitations models with implantation of bacteria in the peritoneal cavity was developed. This gives a reproducible condition resembling clinical sepsis. The hallmark of these models is a rapidly developing hypodynamic circulatory similar to the infusion of LPS. Therefore, some groups use fibrin clot embedded bacteria to produce slower onset of sepsis [49].

With comparison of endotoxaemic and peritonitis models in mice, mortality rates were similar although infusion of endotoxin gave higher cytokine levels and a more severe condition, while the peritonitis model resembled the clinical situation better [50].

The clinical situation with a local infection, which is initially within the capacity of the host immune system that gradually progresses into systemic sepsis with fully activated inflammatory reaction, is virtually impossible to model. On the other hand the patients in the intensive care units have often a complex clinical picture that makes studies of isolated phenomena difficult. The simple, easily reproducible, relatively cheap and dose dependent endotoxaemic model offers the possibility of screening effects of drugs or equipment or studying the pathophysiology of sepsis.
Endotoxin

Endotoxins are extensively studied trigger factors of the inflammatory response seen in sepsis. They are potent immunostimulators of eukaryotes - from the small two-winged fruit flies used in genetic research (drosophila melanogaster) - to the homo sapiens [51-59]. The sensitivity to endotoxin is thus a phylogenetically conserved “alarm system” that functions as an activator of the immune system if gram-negative bacteria are present.

While the term endotoxin is occasionally used to refer to any cell-associated bacterial toxin, its use ought to be limited to the lipopolysaccharide complex associated with the outer membrane of Gram-negative bacteria. The terms endotoxin and LPS are used synonymously, although the former is usually used in the context of biological activity while the latter used to describe the macromolecule. LPS are a group of compounds with varying biological activity. Not all LPS have endotoxin activity and not all endotoxins have LPS structure [60, 61]. For example, bacterial mutants deficient of endotoxin have been shown to elicit only a partial response in comparison with that caused by wild type bacteria [62].

Lipopolysaccharides are complex molecules with molecular weight in the 10 kDa range. They are stable at 100 °C for over 30 minutes due to a molecular structure with covalent bounds. Two main regions can build up the amphiphilic LPS molecule: the lipophilic lipid A region and a hydrophilic polysaccharide region [63]. Biological activity, or endotoxicity, is associated with the lipid component (Lipid A) and immunogenicity is associated with the polysaccharide components. The polysaccharide region can be further divided into the O antigen and a core region, where the latter connects the former with the lipid A region.

A major antigenic determinant of the Gram-negative cell wall resides in the O antigen and constitutes the chemical basis of the bacterial serological classification. The hydrophilic O antigen polysaccharide consists of up to 50 repeating oligosaccharide subunits made up of 2-8 different monosaccharides. Specificity of this site is due to an extremely high structural and chemical variation of the O antigen.

With minor variations, the core region is common to all members of a single bacterial genus. It is attached to one of the two N-acetylg glucosamines of the lipid A region.

Lipid A contains the membrane-anchoring region of LPS and consists of a phosphorylated N-acetylg glucosamine (NAG) dimer with 6 or 7 saturated fatty acids attached. Although the structure of the lipid A region is relatively conserved between bacterial species the variations in this part of the LPS determines its endotoxicity.

The LPS layer of the outer membrane of Gram-negative bacteria is an effective permeability barrier against external stress factors. It is permeable...
only to low molecular weight, hydrophilic molecules, which makes it a barrier to e.g. lysozyme, bile acids and many antimicrobial agents.

LPS is liberated in small amounts spontaneously during bacterial growth [64-66]. Larger amounts of endotoxin are released with bacterial lysis irrespective of its mechanism. These may be autolysis, external lysis mediated by complement or antibiotics and lysozyme phagocytic digestion of bacterial cells.

Antibiotic induced endotoxin release varies with the type of antibiotics. For instance, the cephalosporin induced endotoxin release can be reduced if tobramycin is added. To explain this phenomenon low endotoxin liberation per bacteria or a non-specific binding between aminoglycosides and endotoxin has been suggested. The finding that tobramycin does not exhibit endotoxin-neutralising effects in the LAL assay contradicts the latter explanation [67]. Nevertheless, since endotoxin interacts with several biologic binding sites in the mammalian organism the possibility of an anti-endotoxin effect can not be excluded in vitro.

Endotoxin-host interactions

The mammal organisms have several proteins and cells that, like watchmen, are constantly susceptible for activation by danger signals, such as endotoxin, to initiate an immune reaction against gram negative “intruders”.

Many of the proteins activated are serine proteases, e.g. the Hageman factor and C3, which are parts of cascades such as the coagulation and complement system, respectively [68-71].

The cells in the first line of endotoxin activation are monocytes, macrophages and neutrophil granulocytes, phagocytic cells of the innate or natural immune system [72-76]. A key immunological feature of these cells is the constitutive expression of the CD 14 antigen (CD 14) and Toll-like receptor 4 (TLR4) on their membrane [77]. Activation of the CD14 and TLR4 leads to the release of cytokines, biologically active lipids and free radicals.

Monocyte-mediated activation of the innate immunity

Free LPS forms aggregates due to its amphiphilic nature and spontaneous diffusion of LPS monomers from these aggregates to CD14 occurs at a very slow rate [78]. LPS binding protein in plasma (LBP) [79], a 65 kDa liver derived protein, however, dramatically accelerates binding of LPS monomers from aggregates to CD14, thereby enhancing the sensitivity of cells to LPS [78, 80-83]. Even cells with no expression of CD14; such as endothel, fibroblasts, and smooth muscle cells; are activated by endotoxin probably through the soluble form of CD14 antigen.

The intracellular events following CD14 activation includes a G-protein and second messenger kinase system that activates transcription factors such
as activator protein-1 and cyclic AMP-responsive element. Binding to the TLR4 initiates intracellular second messenger signal systems, involving mitogen-activated protein kinases that through series of phosphorylations lead to the activation of nuclear factor-kB (NF-kB), a protein modulating transcription of DNA. These transcription factors then initiate the synthesis of a myriad of proinflammatory mediators [84].

The most studied cytokines released from monocytes are TNF-α, IL-1, IL-6 and Il-8. These trigger the systemic inflammatory response with lymphocyte and platelet activation, initiation of arachidonic acid metabolite synthesis, hepatic secretion of acute phase proteins, resetting of the thermostat of the body resulting in fever, the activation of hypothalamus-pituitary-adrenal axis with the release of stress hormones, increasing of the oxygen transport to the tissues, causing peripheral vasodilatation, increasing endothelial permeability and initiation of lymphoid differentiation in the bone marrow [85].

The complement system
The complement system is a cascade system of serum proteins that mediate non-adaptive immune response. Similarly to the coagulation system, proteins are cleaved by other complement proteins in serial fashion, leading to activated fragments. Endotoxin activates complement factor 3 (C), thereby triggering the alternative pathway. A number of the complement fragments generated during activation play a major role in host defence.

The smaller fragments of C3 and C5, C3a and C5a are called anaphylatoxins, trigger cells of inflammatory reactions. C3a activates mast cells that release histamine from granulae and leading to vasodilatation and increased vascular permeability that is seen in local inflammation [86]. The other anaphylatoxin, C5a, binds to neutrophils, monocytes, and macrophages leading to their activation [87-89]. C5a facilitates chemotaxis and the recruitment of neutrophils and induces the production and release of reactive oxygen species and lysosomal enzymes [87]. In monocytes and macrophages C5a stimulates secretion of proinflammatory cytokines, such as TNF-α [90–92].

The larger protein fragments derived from C3 and C5, C3b and C5b, are involved in opsonisation, phagocytosis and immunomodulation. C5b binds to complement factors 6-9 to form the membrane attack complex (MAC) [93, 94]. The formation of MAC correlates to the plasma endotoxin levels [41]. Among other inflammatory actions, the membrane attack complex stimulates arachidonic acid metabolism resulting in the release of prostaglandin E₂ from macrophages, leukotriene B₄ from neutrophils, thromboxane B₂ from human platelets, as well as the release of prostanoids, interleukin-1, and reactive oxygen species from human monocytes [95-97].
Products of arachidonic acid metabolism

The metabolites of eicosanoids play a central role in inflammatory reactions. The cyclooxygenase enzymes metabolise the eicosanoid arachidonic acid (AA) into prostaglandin (PG) H\textsubscript{2} which can be further converted into the three main products of these pathways: thromboxane (TX) A\textsubscript{2}, PGE\textsubscript{2} and PGI\textsubscript{2}.

As TXA\textsubscript{2} and PGI\textsubscript{2} have short half-lives, laboratory measurement of their stable metabolites, TXB\textsubscript{2} and 6-keto-prostaglandin F\textsubscript{2\alpha}, respectively, is more convenient. Assessing the overall activity of the cyclooxygenase enzymes is possible by measurement of the PGF\textsubscript{2\alpha} metabolite, 15-keto-dihydro-PGF\textsubscript{2\alpha}. Prostaglandin-like compounds, isoprostanes such as of 8-iso-PGF\textsubscript{2\alpha}, are synthesised in vivo non-enzymatically due to lipid peroxidation and can be used as indicators of oxidative injury in sepsis [98].

The arachidonic acid metabolites are part of pathophysiological changes of the inflammatory response in sepsis and endotoxaemia too. Studies have shown that circulatory and respiratory changes in severe endotoxaemia and early sepsis are mediated by these compounds [98, 99]. In porcine endotoxaemia metabolites of cyclooxygenase increase in myocardial microdialysate and their levels correlate inversely to myocardial function [100]. Both PGI\textsubscript{2} and the TXA\textsubscript{2} metabolite, TXB\textsubscript{2}, increase in humans in early Gram-negative septic shock and their plasma concentrations correlate to the severity of organ failure [99].

TXA\textsubscript{2} is synthesised in platelets, and in sepsis may contribute to organ failure through a number of mechanisms. Platelet aggregation promoting the formation of microvascular thrombosis and smooth muscle contraction causing vasoconstriction are two such mechanisms. Aggregation of neutrophils and adhesion of leukocytes and neutrophils to endothelium is also mediated by TXA\textsubscript{2} [101]. Although a study of clinical sepsis showed ten-fold higher TXB\textsubscript{2} levels in non-survivors compared to survivors [102], the exact role of TXA\textsubscript{2} in clinical sepsis remains to be elucidated since non-selective inhibition of TXA\textsubscript{2} synthesis does improve the outcome [103].

In contrast to TXA\textsubscript{2} prostaglandins, PGE\textsubscript{2} and PGI\textsubscript{2}, counteract the proinflammatory and procoagulatory processes. PGE\textsubscript{2} is synthesised by macrophages as well as by the endothel and contributes to the vasodilatation in sepsis. As PGE\textsubscript{2} has been shown to attenuate the cytokine response of macrophages and lymphocytes its net effect is probably antiinflammatory [104]. PGI\textsubscript{2}, produced mainly by the endothel, dilates vessels and inhibits of platelet aggregation [105].

Although effects elicited by the prostaglandins, PGE\textsubscript{2} and PGI\textsubscript{2}, could theoretically be beneficial in sepsis through e.g. increased oxygen delivery, administration of PGI\textsubscript{2} and PGE\textsubscript{1} have not yielded convincing evidences for clinical use [106, 107].
The coagulation in endotoxaemia

The coagulation system is activated by endotoxin and inflammatory mediators. The final common pathway of coagulation is the activation of factors X and thrombin and the cleavage of fibrinogen into fibrin by thrombin that forms a clot.

Factor X can be activated by both the intrinsic and the extrinsic pathways. Endotoxin has a direct activating effect on the factor XII that activates both the intrinsic pathway, the bradykinin and kallikrein systems. In contrast to previous theories, however, studies of endotoxaemia indicate that the intrinsic pathway is not involved in the activation of coagulation by sepsis [108]. The role of factor XII activation is probably more important in bradykinin and kallikrein systems as blocking factor XII activation, in sepsis models, counteract hypotension that the bradykinin and kallikrein systems contribute to [109].

The activation of the coagulation in endotoxaemia is hence mediated by the extrinsic pathway where tissue factor induced activation of factor VII and the subsequent activation leads to thrombin activation and fibrin clot formation [110, 111]. How endotoxaemia initiates tissue factor expression is not fully clarified. One source may be expression of tissue factor on monocytes in response to proinflammatory cytokines [112]. Studies suggest that the key cytokine that activates the extrinsic pathway is IL-6 [113, 114]. The thrombin activation takes place on platelet membranes, and the thrombin formed initially in turn enhances platelet adhesion as well as platelet and coagulation factor activation [28, 115].

With the activated coagulation a procoagulative state emerges that is further enhanced by the lack of function in both the inhibitor systems and the fibrinolysis.

In fulminant sepsis or endotoxaemia, the generalised activation of the coagulation rapidly consumes antithrombin III, the main inhibitor of factor X and thrombin. Also a marked reduction in the function of the protein C system, inhibitors of factors V and VIII, is present. The impairment in the activity of the protein C system is caused by a combination of decreased protein C and S synthesis, a decrease in the free plasma level of the protein C cofactor, protein S, due to complement binding and a decrease in the endothelial thrombomodulin activity mediated by cytokines [116-118].

The fibrinolytic activity is inhibited at the time of maximal activation of coagulation in experimental endotoxaemia, due to persistent increase in the plasma level of plasminogen activator inhibitor 1, the main inhibitor of the fibrinolytic system [108, 119]. In addition to data from studies in sepsis models, clinical studies have also shown that inhibition of fibrinolysis is mediated by plasminogen-activator inhibitor 1. The fibrinolytic activity present in response to the formation of fibrin is insufficient to oppose systemic
deposition of fibrin [120]. This suppression of fibrinolysis in sepsis is likely to be mediated by TNF-α [121].

Decreasing platelet counts is a feature of endotoxaemia; however, the mechanisms for this phenomenon are not clear. Both to decreased platelet production and increased consumption due to coagulation activation may contribute to thrombocytopenia [35].

The lack of anticoagulant fibrinolytic capacity with concurrent activation of coagulation, eventually leads to overwhelming fibrin formation and consumption of clotting factors and inhibitors as well. Massive intravascular fibrin formation leads to microvascular thrombosis that may induce widespread organ damage [122]. Ultimately, consumption of clotting factors and decreasing platelet counts may lead to hemorrhage.

LAL assay
In 1964 Levin and Bang described that endotoxin caused coagulation of Limulus polyphemus’, or American horseshoe crab’s, blood [123]. In the blood cells of this crab, the amebocytes, granules contain serine protease zymogens that are released in the presence of endotoxin. Extracellularly the activated proteases initiate a cascade reaction that eventually leads to clot formation [124-127]. These observations lead to the development of the limulus amebocyte lysate (LAL) assay for endotoxin or lipid A detection. In brief, lipid A region induces autocatalysis and activation of factor C that cleaves and activates factor B. Activated factor B than cleaves the proclotting enzyme into the active clotting enzyme. The latter cleaves coagulogen into coagulin, which forms a polymeric clot [123]. This system shows similarities with the coagulation factors of the mammals [124].

The amplification in this cascade accounts for the sensitivity of the Limulus coagulation system to endotoxin concentrations as low as picogram per millilitre. The LAL assay has very high specificity for endotoxin [128]. However, endotoxin detection in blood with the LAL assay is associated with at least two additional difficulties compared with other biological fluids: the presence of ill-defined endotoxin inhibitors and the endotoxin levels that are at the detection limit of the assay.

In order to develop the LAL assay into a quantitative method and improve other shortcomings, several techniques have been described since the original gel-clot LAL assay [129-131].

Porcine dose-response experiments
Although the effects of endotoxin in laboratory animals were characterised already in the fifties [8], and endotoxaemia became a popular model for sepsis studies [132-134], only few investigations have described the role of the endotoxin dose in experimental animal models. Dose-response studies are
now accessible for a number of species [135, 136]. Also, the endotoxin dose in porcine endotoxaemia has been investigated in a few cases. Two of these did not use fixed incremental escalation of the dose and conclusions regarding endotoxin dose-response are therefore difficult [137, 138]. In one study by Hand et al., the effect of increasing doses of continuous endotoxin (Difco 055:B5) infusion in the pig was studied and the effect on circulatory, respiratory and metabolic changes were monitored along with mortality rate [139]. To our knowledge, the endotoxaemic dose-response of inflammatory cytokines and isoprostanes has previously not been established.
Aims

The aims of this thesis were to investigate the following issues in the endotoxaemic pig model:

- the pathophysiological responses to increasing endotoxin doses and defining the endotoxin dose-response relationship to pathophysiological responses.
- whether the mode and administration rate of endotoxin is a factor of importance for the biological response.
- the pathophysiological covariations and relationships between inflammatory, coagulatory and haemodynamic responses.
- whether 8-iso-prostaglandin F$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ correlate to the endotoxin dose, inflammatory mediators, pathophysiological events.
- whether clopidogrel treatment has an effect on renal function, the inflammatory response or the coagulation endotoxaemic shock.
- whether the biological effects of endotoxin can be neutralised by addition of tobramycin.
- whether tobramycin or ceftazidime could reduce cytokine concentrations at the end of the observation period.
Materials and Methods

Subjects
The piglets were handled according to the guidelines of the Swedish National Board for Laboratory Animals and the European Convention on Animal Care. The experiment was approved by the Animal Ethics Committee of Uppsala University, Sweden. The following inclusion criteria were applied: no apparent pre-existing diseases, PaO$_2$ of > 10kPa (75 mmHg) and mean pulmonary arterial pressure (MPAP) of < 2.7 kPa (20 mmHg) at baseline, which was 20 min after the completed preparatory procedure (see below). None of the 48 animals in the experiments were excluded. The animals in all of the studies were 12–14 weeks old and were without any evidence of illness.

Paper I
The study included 20 domestic breed piglets of both sexes weighing between 21 and 33 kg (mean 26 kg).

Paper II
The study included 18 domestic breed piglets of both sexes weighing between 22 and 31 kg (mean 27 kg).

Paper III
The study included 23 domestic breed piglets of both sexes weighing between 21 and 34 kg, (mean 26).

Paper IV
The study included 15 domestic breed piglets of both sexes weighing between 21 and 33 kg (mean 28 kg).

Paper V
The study included 13 domestic breed piglets of both sexes from different litters, weighing between 20 and 26 kg (mean 24 kg).
Anaesthesia and fluid administration

General anaesthesia was induced by intramuscular injection of a mixture of 6 mg x kg\(^{-1}\) tilétilam-zolazepam (Zoletil forteTM, Virbac Laboratories, Carros, France), 2.2 mg x kg\(^{-1}\) xylazin (Rompun VetTM, Bayer, Leverkusen, Germany) and 0.04 mg x kg\(^{-1}\) atropine (AtropinTM, NM Pharma, Stockholm, Sweden). Anaesthesia was maintained with sodium pentobarbital (8 mg x kg\(^{-1}\) x h\(^{-1}\); PentobarbitalnatriumTM, Apoteket, Umeå, Sweden), pancuronium bromide (0.26 mg x kg\(^{-1}\) x h\(^{-1}\); PavulonTM, Organon, Oss, The Netherlands) and morphine (0.48 mg x kg\(^{-1}\) x h\(^{-1}\), MorphineTM, Pharmacia, Uppsala, Sweden) dissolved in 2.5% glucose solution given as a continuous infusion. Sodium chloride infusion was administered, resulting in a total fluid administration rate of 30 mL x kg\(^{-1}\) x h\(^{-1}\).

Preparatory procedure

A bolus dose of 20 mg morphine was given intravenously (i.v.) before the tracheotomy procedure, which was done in an effort to secure a free airway during the experiment. The animals were artificially ventilated throughout the experimental procedure (Servo 900CTM, Siemens-Elema, Stockholm, Sweden). During surgical stimulation (i.e. catheter insertion), 30% oxygen in N\(_2\)O was given, after which the gas mixture was set to FiO\(_2\): 0.3 in medical air for the rest of the experiment. The ventilation after preparation was adjusted to yield a PaCO\(_2\) between 5.0 and 5.5 kPa. The respiratory rate was 25 min\(^{-1}\) and the inspiratory-expiratory ratio was 1:3. Respirator settings were then kept constant throughout the experiment. Atelectasis was prevented by placing the piglets into prone position as soon as the preparation was completed, as well as by maintaining 5 cm H\(_2\)O positive end-expiratory pressure.

A cervical artery was catheterised for pressure monitoring and blood sampling. A central venous line and a 7 F Swan-Ganz catheter, equipped with a thermistor, were inserted through the internal jugular vein into the superior caval vein and into the pulmonary artery, respectively. A minor vesicotomy was performed and a urinary catheter was introduced into the bladder. A heating pad (Operatherm 200WTM, KanMed, Bromma, Sweden) was used to keep the animals at a constant body temperature.

Protocol

As the preparation was completed and 20-min stabilisation time, baseline values were registered and baseline blood samples were taken. If the mean arterial pressure decreased to the same level as the mean pulmonary pressure during the first hour of the experiment, a single dose of 0.2 mg adrenalin was given i.v.. Physiologic variables were registered and blood samples were taken at every hour after baseline for 6 hours, after which all surviving pig-
lets were killed by potassium chloride injection i.v. Randomisation to groups in all studies was performed by the sealed envelope method employing the ‘randomisation-in-block’ principle.

Paper I
The piglets were randomly allocated to seven groups. Endotoxin (Escherichia Coli: 0111:B4; Sigma Chemical, St. Louis, MO, USA) obtained from a single batch was given as an infusion to six groups in doses of 0.063 (n=3), 0.25 (n=3), 1.0 (n=3), 4.0 (n=3), 8.0 (n=3) and 16 μg x kg⁻¹ x h⁻¹ (n=2). A non-endotoxin group constituted the control group (n=3).

Paper II
The piglets were randomly allocated to two groups. As depicted in Figure 1, one group received endotoxin infusion (Escherichia Coli: 0111:B4; Sigma Chemical, St. Louis, MO, USA) in doses of 4 μg x kg⁻¹ x h⁻¹, 0.5 μg x kg⁻¹ x h⁻¹, and 0.063 μg x kg⁻¹ x h⁻¹ during the first, second and third hour of the experiment, respectively (Fig.1). Another group received endotoxin infusion in the reverse order, i.e. in doses of 0.063 μg x kg⁻¹ x h⁻¹, 0.5 μg x kg⁻¹ x h⁻¹, and 4 μg x kg⁻¹ x h⁻¹ during the first, second and third hour of the experiment, respectively. After the first three hours, endotoxin infusion was terminated in both groups and the piglets were observed for another three hours.

Figure 1. The scheme of the experimental model, showing the difference in the endotoxin administration to the two groups in Paper II.

Paper III
The piglets were randomly allocated to seven groups. Endotoxin (Escherichia Coli: 0111:B4; Sigma Chemical, St. Louis, MO, USA) obtained from a single batch was given as an infusion to six groups in doses of 0.063 (n=3), 0.25 (n=3), 1.0 (n=3), 4.0 (n=6), 8.0 (n=3) and 16 μg x kg⁻¹ x h⁻¹ (n=2). A non-endotoxin group constituted the control group (n=3).
**Paper IV**

The piglets were randomly allocated to one of three groups. One group of 6 pigs received an injection of clopidogrel i.v. (10 mg x kg⁻¹; Plavix™ a gift from Sanofi-Synthelabo, Paris, France), which 30 min later was followed by a 6 hours continuous i.v. endotoxin infusion (4 μg x kg⁻¹ x h⁻¹; Escherichia Coli: 0111:B4; Sigma Chemical, St. Louis, MO, USA). Another group of 6 pigs received an i.v. injection of saline (corresponding to the volume of clopidogrel), 30 min later followed by a continuous infusion of i.v. endotoxin infusion at 4 μg x kg⁻¹ x h⁻¹. Three pigs received i.v. clopidogrel at 10 mg x kg⁻¹, 30 min later followed by a saline infusion corresponding to the volume of the endotoxin infusion. Multiple biopsies were taken from a standardised location in the right kidney of each pig immediately after their sacrifice.

**Paper V**

All animals were subjected to endotoxin infusion (Escherichia coli O111:B4; Sigma Chemical, St. Louis, MO, USA) with an initial infusion rate of 4 μg x kg⁻¹ x h⁻¹. After 30 min the infusion rate was reduced to 1 μg x kg⁻¹ x h⁻¹, which was continued during the rest of the experiment.

The animals were randomised using to receive tobramycin, ceftazidime, or saline solution. Tobramycin was given as an intravenous infusion of 140 mg in 100 ml saline solution for 20 min, starting 10 min before the initiation of endotoxin infusion. Ceftazidime was injected as an intravenous bolus dose of 1 g in 10 ml saline 5 min before the start of endotoxin infusion, followed by 90 ml saline for a total infusion time of 10 min. In the control group, an infusion of 100 ml saline was initiated 5 min before the start of endotoxin administration and was given for 10 min. The antibiotics were obtained as reference substances with known potencies. Ceftazidime was purchased from Glaxo Wellcome AB (Gothenburg, Sweden) and tobramycin from Eli Lilly Sweden AB (Stockholm, Sweden). The doses were calculated to achieve similar concentrations to those with commonly recommended maximal doses for treatment of clinical sepsis.

**Measurements**

Mean arterial pressure (MAP) and mean pulmonary arterial pressure (MPAP) were monitored continuously while pulmonary capillary wedge pressure (PCWP) was measured hourly. The central line was used for measurement of the central venous pressure (CVP). Cardiac output (CO) was assessed by the thermodilution method using the thermistor in the Swan-Ganz catheter. The average value of at least three serial CO measurements was registered. Heart rate (HR) was continuously monitored by means of
ECG. Proximal airway pressure values (P_{peak}) and tidal volume (TV) were recorded from ventilator readings.

Urine output (UO) was registered and urine samples were taken hourly. Blood samples were taken at baseline and at every hour. Arterial and mixed venous blood gases (PaO_{2}, PvO_{2}), oxygen saturation (SaO_{2}, SvO_{2}) and base excess (BE) were analysed (ABL^{TM} 5 and Hemoximeter^{TM}, Radiometer, Brønhøj, Denmark). Blood cells were analysed on a CELL-DYN 4000^{TM} (Abbott Scandinavia AB, Kista, Sweden) for haemoglobin (Hb), leukocytes and platelets.

Laboratory analysis

**IL-6 and TNF-α**

Commercial sandwich ELISA was used for the detection of interleukin-6 (IL-6; Quantikine^{TM} porcine IL-6, P6000, R&D Systems, Minneapolis, MN, USA) and tumour necrosis factor alpha (TNF-α; KSC3012, Biosource International, Nivelles, Belgium) in all studies, except in Paper II where TNF-α was analysed with (Porcine TNF-α KSC3011, Biosource International, Nivelles, Belgium). The lower detection limit was 10 ng x L^{-1} for both IL-6 and TNF-α. The assays had intra-assay coefficients of variation (CV) of < 5% and total CVs of < 10% [140, 141].

**Radioimmunoassay of 8-iso-PGF_{2α} (Paper III)**

Unextracted heparinised plasma samples were analysed for 8-iso-PGF_{2α} as an index of oxidative injury by radioimmunoassay [142]. The cross-reactivity of the 8-iso-PGF_{2α} antibody with 15-keto-13,14-dihydro-8-iso-PGF_{2α}, 8-iso-PGF_{2π}, PGF_{2α}, 15-keto-PGF_{2α}, 15-keto-13,14-dihydro-PGF_{2α}, TXB_{2}, 11β-PGF_{2α}, 9β-PGF_{3α} and 8-iso-PGF_{3α}, respectively was 1.7, 9.8, 1.1, 0.01, 0.01, 0.1, 0.03, 1.8 and 0.6 %. The detection limit of the assay was about 23 pmol x L^{-1}.

**Radioimmunoassay of 15-keto-dihydro-PGF_{2α} (Paper III)**

Unextracted heparinised plasma samples were analysed for 15-keto-dihydro PGF_{2α} as an index of inflammatory response by radioimmunoassay [143]. The cross-reactivity of the antibody with PGF_{2α}, 15-keto-PGF_{2α}, PGE_{2}, 15-keto-13,14-dihydro-PGE_{2}, 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 11β-PGF_{2α}, 9β-PGF_{2α}, TXB_{2} and 8-iso-PGF_{2α} was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001, 0.01%, respectively. The detection limit was about 45 pmol x L^{-1}.

**Analysis of platelet microparticles by Flow Cytometry (Paper IV)**

5 µL platelet rich plasma was added to 100 µL HEPES-buffer and 10 µL FITC labelled chicken anti-whole platelet antibody (Immunsystem AB,
Sweden). The samples were analysed utilising an Epics Profile XL-MCL cytometer (Coulter Electronics, Hialeah, FL). The instrument gives percentage of antibody positive events, mean fluorescence intensity, complexity, and the mean cell size of the cell population within the field [144, 145].

**Immunohistochemistry (Paper IV)**
Renal biopsies were cut and prepared for microscopical evaluation and marked with antibodies directed against fibrin and fibrinogen bound to porcine platelets [146]. The slides were stained and the fibrin/fibrinogen content was determined in the coded biopsies. The method for preparing immunohistochemical slides has previously been described [147].

**Determination of endotoxin (Paper V)**
Endotoxin-free heparinised tubes (Endo Tube; Chromogenix AB, Mölndal, Sweden) were used for the endotoxin samples. After centrifugation the supernatants were transferred to endotoxin-free tubes and subsequently kept at -70°C until analysis.

Analysis of endotoxin was performed in duplicate with the limulus amoebocyte lysate assay (Coatest™ Plasma Chromo-LAL; Charles River Endosafe, Charleston, SC, USA) [148].

**Calculations of physiologic variables**
Left and right ventricular stroke work indices (LVSWI and RVSWI, respectively), systemic and pulmonary resistance vascular indices (SVRI and PVRI, respectively), cardiac index (CI), delivery of oxygen (DO2), oxygen consumption (VO2), oxygen extraction as well as dynamic and static pulmonary compliance (Cdyn, Cstat) were derived from their conventional formulae [149, 150]. Creatinine clearance was calculated using serum and urine creatinine values as well as hourly diuresis (CLcr, mL x min⁻¹; CLcr=UO x urine [creatinine] x plasma [creatinine]⁻¹).

**Calculations and statistics**

**Paper I**
To reduce inter-animal variation, leukocyte count, platelet count, MPAP, MAP, CI, DO2, SvO2, haemoglobin concentration, airway compliance and serum creatinine were expressed as relative changes to the value obtained at baseline just before the administration of endotoxin. Changes in these parameters approximated to normal distribution, as did base excess values. The cytokine concentrations were log-normally distributed and therefore these values were logarithmically transformed. The optimal time point for determination of the dose-response of a variable was defined as the time point at
which the variable reached its peak or nadir value. The dose-response was based on analysis of the dose-induced changes in the parameter investigated during the time from start of the endotoxin infusion to the optimal time point. In parameters not exhibiting a peak or nadir value changes during the whole period of 6 hours was taken into the dose-response calculations. A repeated measures ANOVA was performed to test changes over time and differences between doses. If the general dose-effect was not opposed by changes in the opposite direction at more than one following dose step and the line passed within the 95% confidence intervals of the different dose-responses, a regression line was calculated. For parameters for which a significant dose-response could be demonstrated, a correlation analysis between the value at the optimal time point and the endotoxin dose was performed and Pearson's correlation coefficient calculated.

To study covariation between haemodynamic, inflammatory and coagulatory responses, TNF-α, IL-6 and leukocytes were chosen as inflammatory variables, platelets as a coagulatory parameter and haemoglobin as an indicator of hemoconcentration due to capillary leakage. The values of all observed parameters over time in all animals were correlated to the peak or nadir concentrations of TNF-α, IL-6, leukocytes, platelets and haemoglobin. Pearson's correlation coefficient was calculated for each time point and, for comparison, the correlation to the logarithmically expressed endotoxin dose was similarly calculated.

A p-value of <0.05 was considered significant. All values were expressed as mean ± SE, unless otherwise stated. The software STATISTICA (Stat. Soft. Inc. Tulsa, OK, USA) was used.

**Paper II**

To reduce inter-animal variation leukocyte count, platelet count, core temperature, MPAP, MAP, CI, DO₂, SvO₂, haemoglobin concentration, pH, static pulmonary compliance and hourly diuresis were expressed as relative changes to the value obtained at baseline just before the administration of endotoxin. Changes in these parameters approximated to normal distribution, as did BE values. The cytokine concentrations were log-normally distributed and therefore these values were logarithmically transformed. Repeated-measures ANOVAs were performed to test changes over time. To describe group differences during the experiment that were caused by accelerating and decelerating endotoxin infusions, data were assessed for differences between the groups at 0-6 hours. If the mean values for a physiologic variable in the groups maintained the same intergroup relationship at 0-6 hours, i.e. their graphs did not cross more than once, and then were <1 standard error (SE) from the mean value of the other variable and yet no significant difference was demonstrated at 0-6 hours, the variable was assessed for a significant difference from 0 to the time point when the difference ceased. A p-value of <0.05 was considered significant. All values were expressed as
mean ± SE unless otherwise stated. The software STATISTICA™ (Stat. Soft. Inc. Tulsa, OK, USA) was used.

**Paper III**

In order to reduce inter-animal variation all variables were assessed as relative changes to the value obtained at baseline just before administration of endotoxin. The distributions of all variables were tested for normality using the Kolmogorov-Smirnov test with D>0.12 considered as non-normal distribution. Variables with non-normal distribution were logarithmically transformed since their distribution was log-normal. Differences over time were then assessed for 8-iso-PGF$_{2\alpha}$, 15-keto-dihydro-PGF$_{2\alpha}$, LVSWI and PaO$_2$ comparing endotoxaemic and non-endotoxaemic pigs by ANOVA for repeated measures, as was the effect of the endotoxin dose on 8-iso-PGF$_{2\alpha}$, 15-keto-dihydro-PGF$_{2\alpha}$ levels. If the general dose-effect was not opposed by changes in the opposite direction at more than one following dose step and the line passed within the 95% confidence intervals of the different dose-responses, a regression line was calculated.

P<0.05 was considered a statistically significant result. Correlations between variables were estimated by Pearson’s correlation coefficient (R). Correlations were considered of interest if coefficients at one time point were >0.5 for the endotoxin dose and at no less than three adjoining time points were >0.5 for 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$.

**Paper IV**

Statistical differences between the groups were calculated by an ANOVA, where each treatment group, regarded as explaining variable, was compared to its corresponding area under the curve (AUC), and used as the variable of response. Least square means were calculated and used for paired comparisons. Correlations were calculated by Spearman’s rank test. Values are given as mean ± SD. p < 0.05 was considered significant.

**Paper V**

The TNF-α response at 1 hour and the IL-6 response at 2 hours were compared in the primary analyses of the immediate initial cytokine response. Because there was a variation in the immediate cytokine response, the reduction in concentrations of TNF-α and IL-6 from 1 hour and 2 hours, respectively, to 6 hours, as well as the concentrations at 6 hours, were considered late cytokine responses. In order to detect a difference in the initial log concentration of cytokines of at least 20% with an α error of 0.05, a β error of 0.2, a power of 0.8, and a calculated interindividual variation of 14%, eight evaluable animals were needed in each treatment group. In this exploratory trial an interim analysis was planned when there were at least four animals in each group that had survived for 6 hours. The distribution of the logarithm of
initial cytokine response approximates the Normal distribution in this model, and was therefore analysed using an unpaired t-test. Other differences between treatment groups were calculated using the non-parametric Mann–Whitney U-test. The results are expressed as mean ± standard error or as median ± range. P < 0.05 was considered statistically significant.
Results

All animals fulfilled the inclusion criteria. The piglets were comparable on all baseline data. All animals in the endotoxaemic groups responded to the endotoxin infusion with marked inflammatory, circulatory and respiratory derangements as displayed in Figure 2.

A total of 69 pigs were included in the experiments. In total 9 pigs died during the experiments all receiving endotoxin and 9 pigs were given adrenaline during the first hour of the experiment as a rescue medication. None of the animals that died received adrenaline. All of the pigs receiving adrenaline survived throughout the experiment and their results were well within the range of the other animals.

Figure 2. Relative changes in TNF-α, IL-6, 8-iso-PGF2α, MAP and PaO2 in endotoxaemic pigs. The changes in TNF-α and IL-6 were calculated from their logarithmic values.
Inflammatory variables and endotoxin levels

TNF-α and IL-6 increased from baseline values in all endotoxaemic pigs peaking at one hour and two to four hours respectively, while remaining at baseline levels in non-endotoxaemic pigs (Figure 3). 15-keto-dihydro-PGF$_{2α}$ and 8-iso-PGF$_{2α}$ increased from baseline in all endotoxaemic pigs with highest levels at one and three hours respectively, with a slight decrease from baseline in non-endotoxaemic animals (Figure 3). The increase in these cytokines and 8-iso-PGF$_{2α}$ was dependent on the endotoxin dose in a log-log fashion (Figure 4).

Figure 3. Relative changes in inflammatory variables and cells over time. All endotoxemic animals are grouped together, irrespective of the endotoxin dose. TNF-α and IL-6 are presented as absolute values. Non-endotoxemic group, Endotoxemic groups (**, *** (values are mean ± SEM). Asterisks denote significance tested with one-way ANOVA (* p<0.05; ** p<0.01; *** p<0.001).
Figure 4. Endotoxin dose-response. Relative changes in inflammatory variables and cells from the start of the experiment to the optimal time point of measurement at logarithmic increases in the endotoxin dose. TNF-α and IL-6 are given as absolute values. If the dose effect was significant and not opposed by changes in the opposite direction at more than one following dose level and the line passed within the 95% confidence intervals of the different dose responses, a regression line is drawn (values are mean + SEM).

In animals receiving a given endotoxin dose in a decelerating infusion pattern, TNF-α levels were higher, as compared with animals receiving accelerating endotoxin infusion of the same dose (Figure 8). No such difference in response to the endotoxin infusion rate was detected in the IL-6 concentration. At 6 hours the IL-6 concentration was significantly lower in endotoxemic pigs receiving ceftazidime than in pigs receiving saline instead (Figure 5). The reduction in IL-6 seen both in the animals treated with cef-
tazidime and in those treated with tobramycin was significantly greater than that in the animals receiving saline.

Decreasing leukocyte counts were observed in all endotoxaemic pigs with a nadir at the first few hours of the experiments (Figure 3). Leukocyte counts were virtually unchanged over time in non-endotoxemic pigs. The leukocyte levels during the experiment were dose dependent in a log-linear fashion (Figure 4) and were significantly lower in animals with endotoxin infusion in accelerating administration during the first five hours and first two hours, respectively, as compared to animals receiving endotoxin with a decelerating infusion rate.

In Paper V initial plasma endotoxin samples obtained before the start of endotoxin infusion were taken after the preparatory procedure (i.e. vesicotomy and insertion of a central venous catheter through the skin), and therefore endotoxin contamination cannot be excluded. Despite this, plasma endotoxin was low (1.61±0.47 endotoxin units/ml) in eight of the animals. However, in five animals – three in the tobramycin group and one from each of the other two groups – there were considerably higher levels of endotoxin (15.5±2.9 EU/ml). In these animals there were no concomitant increases in TNF-α or IL-6 concentrations or signs of infection, and all of them responded physiologically to the endotoxin infusion with a doubling of the MPAP. In three animals – two in the tobramycin group and one in the saline group – there was even a decrease in endotoxin concentration after one hour of endotoxin infusion. Neither tobramycin nor ceftazidime had an endotoxin neutralising effect in comparison with that conferred by saline. The endotoxin levels remained stable after one hour into the experiment.

Figure 5. Plasma IL-6 concentrations 2-6 hours after start of endotoxin infusion in ceftazidime, tobramycin or saline treated endotoxaemic pigs.
Figure 6. Relative changes in physiologic variables over time. All endotoxemic animals are grouped together, irrespective of the endotoxin dose. BE is presented as the absolute difference from baseline. Non-endotoxemic group, ** Endotoxemic groups * (values are mean ± SEM). Asterisks denote significance tested with one-way ANOVA (* p<0.05; ** p<0.01; *** p<0.001).

Coagulatory variables

While a gradually decreased in platelets was during the experiments in non-endotoxemic pigs, platelet levels declined faster and earlier in endotoxemic pigs with a nadir at three hours (Figure 3). Decrease in platelets was dependent on the endotoxin dose in a log-linear manner (Figure 4). Platelets decreased slightly more in animals receiving endotoxin in decelerating infusion pattern during the first two hours after baseline as compared to animals
with the same endotoxin dose given with accelerating infusion rate (*Figure 8*).

APTT and PK-INR increased in the endotoxaemic pigs by the end of the experiment while being unchanged in the non-endotoxaemic controls. The coagulation was not significantly affected by clopidogrel.

*Figure 7.* Endotoxin dose-response. Relative changes in physiologic variables from the start of the experiment to the optimal time point of measurement at logarithmical increases in the endotoxin dose. BE is presented as the absolute difference from baseline. If the dose effect was significant and not opposed by changes in the opposite direction at more than one following dose level and the line passed within the 95% confidence intervals of the different dose responses, a regression line is drawn (values are mean + SEM).
Circulatory variables

MPAP was doubled in all endotoxaemic pigs within one hour from baseline levels and remained increased throughout the experiment (Figure 6). No significant changes were observed in non endotoxaemic pigs. Dose-response to endotoxin could not be established for MPAP (Figure 7).

MAP and LVSWI decreased in endotoxaemic animals with a nadir at the second or the third hour of the experiment (Figure 6). A moderate increase was observed in both variables among the non-endotoxaemic pigs. MAP decreased with increasing endotoxin doses in a log-linear fashion (Figure 7). Both MAP and LVSWI were significantly lower in animals with endotoxin infusion in a decelerating pattern during the first two hours after baseline as compared to animals receiving the same endotoxin dose in an accelerating pattern (Figure 8).

Oxygen delivery and utilisation

CI, DO2 and oxygen exhibited a biphasic course that decreased initially with nadir values at the third hour of the experiments (Figure 6). In the non-endotoxaemic pigs these variables decreased modestly during the experiments. Neither the endotoxin dose nor the endotoxin administration rate affected CI and DO2 (Figure 7, Figure 8).

PaO2, pH and base excess decreased in endotoxaemic pigs during the experiments while haemoglobin levels increased (Figure 6). Log-linear dose-response to endotoxin could be established for base excess and haemoglobin (Figure 7). BE and pH were lower to the third hour of the experiment if a given dose of endotoxin was administered in decelerating doses compared to accelerating doses (Figure 8).
Figure 8. Relative changes in certain inflammatory and physiologic variables over time. TNF-α and IL-6 are presented as absolute values. Group receiving endotoxin in decelerating infusion pattern ——, group receiving endotoxin in accelerating infusion pattern ——— (values are mean ± SE).

Organ function

Alternations static and dynamic pulmonary compliance were concomitant. Both measures of pulmonary compliances decreased in endotoxaemic pigs, while being practically unchanged in non-endotoxaemic piglets (Figure 6). Dynamic pulmonary compliance exhibited log-linear dose-response to endotoxin (Figure 7). The changes in static pulmonary compliance developed faster and were more marked in animals that received an endotoxin dose in
decelerating pattern as compared to animals that received the same endotoxin dose in an accelerating pattern of administration (Figure 8).

Diuresis and CL_{cr} decreased in endotoxaemic pigs by the end of the experiment, while both renal function variables were preserved in the non-endotoxaemic pig. Although clopidogrel-injected endotoxaemic pigs had higher mean CL_{cr} values than pigs that received placebo this was not statistically significant (Figure 9).

![Figure 9. Changes in creatinine clearance in saline-endotoxin, saline-clopidogrel and endotoxin-clopidogrel groups (mean ± SEM).](image)

**Correlations to the endotoxin dose and cytokine, leukocyte, platelet and haemoglobin responses**

Initial TNF-α concentration and IL-6, platelet and leukocyte values after the first hour correlated closely to the maximal response value of each other and the endotoxin dose.

The haemodynamic variables MAP, CI, DO_2 and SvO_2 correlated more to peak cytokine responses than to the endotoxin dose. The correlation to the platelet response, which was almost as close as that of the cytokines, was generally somewhat higher than that of the leukocytes. During the last hours, the haemodynamic variables correlated increasingly negatively to the maximum haemoglobin response.
Changes in haemoglobin over time correlated more to the endotoxin dose than to the other responses. Base excess and dynamic airway compliance correlated after 3 hours roughly as good with the cytokine, leukocyte response and platelets as with the endotoxin dose. As with the haemodynamic parameters, there was a negative correlation to the maximum haemoglobin response for both base excess and dynamic airway compliance.

**Correlations to the endotoxin dose, 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$**

8-iso-PGF$_{2\alpha}$ correlated strongest to TNF-α although both endotoxin dose and 15-keto-dihydro-PGF$_{2\alpha}$ showed strong correlations early in the experiment. IL-6 and leukocytes correlated stronger to the endotoxin dose than both to 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$. Early 15-keto-dihydro-PGF$_{2\alpha}$ values correlated to 8-iso-PGF$_{2\alpha}$ throughout the experiment.

MPAP correlated mainly to 15-keto-dihydro-PGF$_{2\alpha}$. MAP and LVSWI correlated strongest to 8-iso-PGF$_{2\alpha}$. MAP did not correlate to 15-keto-dihydro-PGF$_{2\alpha}$ at all. Late Troponin-I values showed close correlations to 15-keto-dihydro-PGF$_{2\alpha}$. MPAP and Troponin-I did not exhibit correlations to the endotoxin dose. CI did not correlate to any of the tested variables.

PaO$_2$, oxygen extraction and pH correlated closely to 8-iso-PGF$_{2\alpha}$ and to a lesser extent also to 15-keto-dihydro-PGF$_{2\alpha}$, but did not correlate to the endotoxin dose. Haemoglobin correlated more to 8-iso-PGF$_{2\alpha}$ than to 15-keto-dihydro-PGF$_{2\alpha}$ and the endotoxin dose in the beginning of the experiment, whereas it correlated only to 15-keto-dihydro-PGF$_{2\alpha}$ at the end of the experiment.

Early static pulmonary compliance correlated strongly to 8-iso-PGF$_{2\alpha}$ while late in the experiment correlations to 15-keto-dihydro-PGF$_{2\alpha}$ were more expressed. Static pulmonary compliance correlated to the endotoxin dose too.

**Light microscopy**

Immunohistochemical evaluation of intrarenal fibrin depositions revealed that there were numerous fibrin depositions both in the glomeruli and also in the renal parenchyma of both clopidogrel and saline-injected endotoxemic pigs. There was no significant difference in the number of stains indicating presence of fibrin between the two groups of animals. These intrarenal depositions of fibrin did not correlate with CL$_{cr}$ ($r$=-0.2) or with urinary production at 6 hours ($r$=-0.3).
Discussion

In this endotoxaemic porcine model a broad early pathophysiological events of endotoxaemia can be studied. The model results in a hypodynamic circulation and it has been argued that it is less relevant in human sepsis research. Conventional human sepsis is characterised by a hyperdynamic phase. However, if sepsis-mediated myocardial depression, prevents CO to increase 50-100% above normal the hyperdynamic compensated phase may rapidly be converted to hypodynamic uncompensated septic shock [151].

As outlined above, the model of porcine endotoxic shock used in this thesis has, among other advantages, the benefit of simplicity, reproducibility and limited observation periods. Thus, this model is of considerable interest when interventions in the early pro-inflammatory response, especially when anti-endotoxin effects are being evaluated.

The inflammatory response to endotoxin is an early and reproducible phenomenon. Activation of white blood cells, cytokine and prostaglandin release are proximal events of endotoxaemia. In these experiments, TNF-α and 15-keto-dihydro-PGF$_{2\alpha}$ peaked at one hour, leukocytes reached nadir at two hours, IL-6 and 8-iso-PGF$_{2\alpha}$ peaked at three hours. These findings may seem to oppose to results of two other studies of porcine endotoxaemia, which describe peak 8-iso-PGF$_{2\alpha}$ and 15-keto-dihydro-PGF$_{2\alpha}$ concentrations at a half an hour [152] and TNF-α below detection levels at a half an hour peaking at one hour [153]. The data from these two studies could, apart from suggesting that the formation of these eicosanoids is not caused by expression of these cytokines, also have implication on assessment of the optimal time point of assessment of response in F$_2$-isoprostanes. As cytokine and F$_2$-isoprostane levels were only assessed at one hour in the current experiment an F$_2$-isoprostane surge at a half an hour after baseline may have been passed without being expressed in the blood samples taken at one hour. One consequence of assessing at submaximal response maybe inappropriate setting of the optimal time point of dose-response assessment, which could be one explanation why no dose-response could be demonstrated for 15-keto-dihydro-PGF$_{2\alpha}$. The responses of TNF-α, IL-6 and 8-iso-PGF$_{2\alpha}$, in the current experiments were principally of log–log character, whereas the leukocyte and platelet responses were log–linear. The log–linear response in MAP, CI and SvO$_2$ was not as evident as for the inflammatory and coagulatory parameters but there were significant dose-responses; for DO$_2$, however, no dose-effect could be demonstrated. The increased deviation in these re-
responses might be due to the variability of several factors, including preceding pathophysiological mediators and volume load. During the experiment, the same, rather generous, amount of fluid was given but there might have been some discrepancies in the hydration status and circulation at the beginning as demonstrated by deviations in the baseline circulatory parameters.

Haemoglobin is a macromolecule that is confined to the red blood cells and thus to the vascular system; because fluid resuscitation was constant, changes in haemoglobin levels were used as an indicator of endothelial leakage. The haemoglobin concentration in this study demonstrated a more evident log–linear response than CI and DO₂. This was also true for deterioration in base excess, reflecting hypoperfusion. The dose-response of the latter is surprising considering that DO₂ did not demonstrate any dose-response. Cellular hypoxia and anaerobic metabolism are also affected by the plasma leakage, which might have rendered base excess more dose-dependent. Of the two organ dysfunction parameters, only the dynamic pulmonary compliance, indicating lung dysfunction, demonstrated a log–linear, dose-dependent response.

Thus, if a drug or equipment with a proposed anti-endotoxin mode of action were studied, these findings would suggest that the IL-6 response, with its clear dose-dependency, is selected as the primary effect variable. If not, the full response, that also takes the values before the optimal time point into calculation, is chosen as such an effect variable; the value at the time of optimal assessment will do very well as the correlation coefficient at this time point correlated strongly to the endotoxin dose. TNF-α, 8-iso-PGF₂α, leukocyte and platelet responses represent alternatives with dose-dependency and correlation values of similar magnitude or just slightly lower. If these parameters are chosen, the best discriminatory power will be obtained when the endotoxin dose selected is 1 μg x kg⁻¹ x h⁻¹ or less, at which levels the cytokine response with a greater certainty and less variation is linear and with a slope of the regression line that is steeper than at higher doses. The optimal time point for assessment seems to be at two hours or, or if 8-iso-PGF₂α or platelet response is included in a more composite end-point, at three hours. If an observation time of less than two hours is selected, only the TNF-α response seems to be reliable. If changes in circulatory parameters constitute the primary end-point, the recommendation of dose is weaker, but even here doses of 1 μg x kg⁻¹ x h⁻¹ appear to offer an advantage over higher doses. However, if using haemoglobin concentration, base excess or dynamic pulmonary compliance to study an endotoxin effect on plasma leakage, hypoperfusion or lung dysfunction, higher doses might preferably be used.

Even if regression lines could be drawn for the inflammatory cytokine and leukocyte responses, these tended to level off at higher doses. Together with the fact that the slope coefficients of the log–log regression lines were lower than one indicates a higher responsiveness to endotoxin at lower lev-
els. This, in turn, implies that a given endotoxin dose that might have been released from growing or antibiotic-exposed bacteria has a larger biological effect at lower than at higher pre-existing endotoxin levels. Thus, besides such factors as differences in genetic predisposition and in the time interval between the present and previous endotoxin exposure [8, 154], the endotoxin level at the time of exposure may also affect the induced cytokine response. Variation in all these factors may offer explanations to the difficulties in demonstrating significant clinical trial effects when antibiotics, with different propensities to release endotoxin, are being compared or anti-endotoxin strategies evaluated.

When comparing whether the rate of increase in endotoxin concentration affects the biological response, besides the endotoxin dose, a two groups of animals were given identical cumulative doses of endotoxin with different administration patterns. In one group the highest endotoxin dose was given during the first hour and over the next three hours the administration rate was tapered in a decelerating fashion and the other group endotoxin administration rate was escalated once an hour during a three hour period.

The duration of the endotoxin infusion in the present study was set to three hours, which is in analogy with the results in the dose-response experiments, demonstrating that all responses, except that of haemoglobin, reached their peaks by the third hour. In the dose-response experiments all three chosen levels of endotoxin dosing have been demonstrated to result in significant responses in an interval of the dose-response curve in which the slope is optimally steep. The post-infusion follow-up period was set to three hours.

If the dose of endotoxin was the sole determinant of the biologic response, a greater response would be expected in the group with the decelerating endotoxin administration rate during the first three hours of the experiment, which then would be followed by an intersection of the response curves during the subsequent three hours of the experiment when identical total endotoxin doses have been given. Such a response was only observed for IL-6. For the rest of the endotoxin dose-dependent variables, significantly greater biologic responses were recorded in the group with the decelerating endotoxin administration rate, either initially or during the whole experiment and in none of them was an opposite response observed during the last part of the experiment. No significant dose-responses have been demonstrated in MPAP, CI and DO2 in this porcine endotoxin model. The response in MPAP is early and not cytokine-driven and for CI and the CI-dependent DO2, other confounding factors, such as preexperimental hydration status or stress level of the laboratory animals may play important roles. Accordingly, in this experiment no differences between the groups were noted in these variables.

The results in these experiments indicate that the biological response is increased if the organism is exposed to a fixed amount of endotoxin more
quickly. This was not only found in the inflammatory variables, TNF-α, leukocytes and core temperature, but also, and even more evidently, in the more delayed parameters, hypotension, BE, pulmonary dysfunction and pH. The half-life of endotoxin has been reported to vary from 15 min to a few hours [155-157]. Taking this fact into account, the concentration of endotoxin should be higher after three hours in group with the accelerating endotoxin administration rate than after one hour in the group with the decelerating endotoxin administration rate. Despite this, the response is more pronounced in the group with the decelerating endotoxin administration rate, suggesting that the response is not only dependent on the concentration but also on the rate by which this concentration is achieved.

The explanation to these findings is not clear. Taking the half-life into consideration, it may be argued that the area under the endotoxin concentration curve was greater in the group with the decelerating endotoxin administration rate and that the differences observed could at least partially be explained by this event. Even if this explanation cannot totally be excluded, the magnitude of such an effect would be minimal, because IL-6, which is strongly correlated to the biological activity of endotoxin, did not differ between the groups. A more probable explanation is that a slow increase in endotoxin administration may lead to tolerance. The development of endotoxin tolerance has been demonstrated in several studies [154, 158] and can easily be observed in experimental models with continuous infusions of endotoxin [159]. Another possible mechanism may be that intracellular signalling is greater when the ligand binds to the receptor with a higher rate. An example of such a biological response pattern is the haemodynamic response to stimulation with arginine vasopressin [160].

The TNF-α reaction in this study was, in similarity to the biological response, dependent on the endotoxin administration rate, whereas the IL-6 response was not. Data from this porcine model thus support the notion that IL-6, in contrast to TNF-α, is no key mediator in the important reactions leading to inflammation, hypotension, hypoperfusion and organ dysfunction. Consistent with results in this thesis, several studies using different animal models have demonstrated that infusion of TNF-α results in the same signs and symptoms as endotoxin [161]. Regarding IL-6, data are more limited. In the mouse it has been found that although being a marker for the outcome in septic shock, IL-6 contributes only marginally in the pathogenesis leading to circulatory collapse and death [162]. Yet, data in this thesis indicate that the IL-6 response is a good indirect parameter of the endotoxin load, irrespective of the administration rate. This finding is particularly noteworthy given that the quantitative measurement of plasma endotoxin with the chromogenic modification of the limulus amebocyte assay has been shown to be notoriously difficult [163].

The finding that biological responses are dependent on both the dose and the administration rate of endotoxin is, of course, important when setting up
experimental endotoxaemic models in which gradual increases to a certain dose will be associated with a lower response than if the final dose is being set directly. The clinical relevance of the present findings, however, is not clear. Since endotoxin infusion can cause sepsis-related symptoms in healthy persons [164, 165], it is reasonable to believe that these mechanisms may operate also in man and thus that not only the high plasma endotoxin concentration [166], but also the rate by which this concentration is achieved may be relevant to the severity and mortality, for instance in meningococcal disease. The results may also implicate that a rapid increase in the free endotoxin release in the early course of Gram-negative sepsis as a resultant of bacterial growth or antibiotics [167] will lead to a more apparent response than would a more gradual absorption from the gut because of organ dysfunction at a later stage of the disease. Such a mechanism would add to the list of factors known to confound the correlation between endotoxin levels and the clinical picture and outcome and influence the maximally achievable effects of potential anti-endotoxin strategies [168]. These speculations, however, need further investigation.

The correlation analysis demonstrated a close relationship between the endotoxin dose and the cytokine, leukocyte and platelet responses, which is in agreement with the well-known ability of endotoxin to activate monocytes and macrophages, resulting in cytokine liberation with coagulation and leukocyte activation following [84]. Circulatory variables showed consistently closer correlations to the cytokine responses than to the endotoxin dose. This finding may be explained by that the endotoxin effect on the circulation is mainly indirect and to a substantial extent mediated through cytokines with subsequent leukocyte and cascade system activation. Coagulation activation, as manifested by the decrease in platelet count, showed a somewhat closer correlation to the circulatory variables than did the leukocyte activation. It is noteworthy that the endotoxin dose and the inflammatory parameters did not show a positive correlation to MPAP, which was quickly increased in the endotoxin-exposed animals. Thus, other mechanisms and mediators than TNF-α and IL-6 must be responsible. In fact, data exist indicating that arachidonic acid metabolites and endothelin are involved in the increases in PVRI and MPAP [169-171].

The increase in haemoglobin, indicating plasma leakage, demonstrated surprisingly low correlations to the cytokine and leukocyte responses, while correlation coefficients were higher to F₂-isoprostanes. The higher correlation to the F₂-isoprostanes suggests that oxidative injury and COX-metabolites may play important roles in the development of the capillary leakage. The gradual increase in the correlation coefficients between peak haemoglobin and circulatory variables, base excess and dynamic pulmonary compliance over time is probably caused by the consequences of plasma leakage, a process leading to hypovolaemia and extravasation in the lung.
Troponin I increased during the second part of the experiment and elevated Troponin-I levels correlated to early elevations of 8-iso-PGF$_{2\alpha}$, and even closer to 15-keto-dihydro-PGF$_{2\alpha}$, but not to the endotoxin dose or to reductions in cardiac performance. These findings indicate that myocyte membrane damage and lysis is linked to non-enzymatic lipid peroxidation and COX-activity but is not of major importance for the endotoxin-mediated cardiac dysfunction observed in this study. Correlations between COX-activity and pulmonary hypertension confirm previous findings as described above [169-171].

The endotoxaemia-induced reductions in PaO$_2$ correlated to elevations in both F$_2$-isoprostanes, as did static pulmonary compliance. No relationship was seen between PaO$_2$ and the endotoxin dose, oxygen extraction and pH indicating that these variables are affected by endotoxin through several intermediate steps. It is feasible to assume that endotoxin induced oxidative processes cause lung injury and capillary leakage (assessed by haemoglobin levels) leading to reductions in arterial oxygenation. This is in agreement with previous findings, showing that propofol, serving as a radical-scavenger was able to reduce endotoxin-induced deterioration of PaO$_2$ [153]. Also, administration of propofol significantly increased the plasma levels of α-tocopherol and β-tocopherol [153]. These two endogenous antioxidants contain a phenolic OH-group, similar to the one in propofol. The beneficial effect of propofol in that experiment may also be attributed to the fact that propofol suppresses the activity of cyclooxygenases [172] and reduces COX-catalysed PGF$_{2\alpha}$ and its metabolites [173]. Prostaglandins are involved in various inflammatory events in the mammalian body and to a large extent metabolised in the lungs [174, 175], which are the target organs in the systemic inflammatory response syndrome. Since oxygen extraction in the tissues is dependent on arterial oxygen content, including PaO$_2$, and disturbances in tissue oxygenation may lead to decrease in pH, it is a natural inference from these facts that both variables correlated to COX activity and 8-iso-PGF$_{2\alpha}$.

The thrombin induced platelet activation inhibitor, clopidogrel, did not significantly prevent the endotoxin induced deterioration in renal function or reduce the number of renal fibrin depositions in these experiments. Fibrin is deposited in the kidneys in endotoxemic shock [176]. Since fibrin link activated platelets via their GPIIB/IIIA receptors, and platelet activation is one of the key factors in initiating the activation of the coagulation system, inhibition of platelet activation could lead to an offset in fibrin formation. Hypothetically, clopidogrel may also counteract activation of the GPIIB/IIIA receptors and their subsequent binding of fibrinogen, acting as a ligand, to the neutrophil CD11b/CD18 receptors. Such a mechanism is exerted by GPIIB/IIIA blockers and preserves microvascular function in experimental sepsis [177, 178]. Measurement of renal vascular resistance was not one of the aims in this study, therefore we cannot determine whether modulation of
ADP receptors on the renal vascular wall counteract endotoxemic vasoconstriction, and thereby contribute to the trend of clopidogrel on CLcr during porcine endotoxemia. There were no differences between the two groups of endotoxemic pigs regarding MAP, MPAP or SaO2, suggesting that systemic physiological responses are not of major importance for the action by which clopidogrel might oppose endotoxin-mediated decrease in CLcr.

In the experiments where neutralisation of endotoxin was studied (Paper V), a high-dose model was chosen in order to allow a relatively short observation time and to avoid low precision of measurements as a result of endotoxin levels that were too low. The initial TNF-α and IL-6 concentrations were chosen as primary end-points because endotoxin has been shown to induce a quick and reproducible response both in vitro and in animal models [179], and at one and two hours it is unlikely that any downstream effects will affect the result to any great extent. However, it must be pointed out that there may be a risk that this model will fail to exclude a possible anti-endotoxin effect if endotoxin–aminoglycoside binding is subject to saturation kinetics and if a lower endotoxin dose is used. In order to minimise such a risk, the tobramycin dose chosen was at the upper end of the range used clinically.

There was no tendency toward a reduction in the immediate cytokine response. This indicates that there is no in vivo neutralisation of endotoxin-induced cytokine production, and consequently that there is no interaction with binding to LBP or the CD14 receptor. Furthermore, there was no neutralisation of the circulating endotoxin, which is in agreement with in vitro results [67]. In fact, there was a tendency for an increased endotoxin concentration in animals treated with tobramycin, which might be consistent with reduced binding to the CD14 receptor. However, the corresponding increase in IL-6 concentration and the unchanged TNF-α values strongly indicate that this was not the case. Although there is a need to confirm this result in a low-dose endotoxin model, results in this thesis indicate that there is no neutralisation of endotoxin by tobramycin. As a consequence, the advantage of using an aminoglycoside for treatment of severe sepsis and septic shock may mainly be due to its broad antibacterial spectrum, to its rapid bactericidal effect, and possibly also to low endotoxin release per killed bacterium and inhibition of penicillin binding protein-3 induced endotoxin release [180].

The inflammatory damage to endothelial cells and other tissue cells mediated by oxygen radicals induces cytokine production [181, 182]. In several in vitro experiments it has been demonstrated that ceftazidime and some of the other β-lactam antibiotics inactivate hypochlorous acid [183] and, in the case of ceftazidime, singlet oxygen [184]. In clinically relevant concentrations, ceftazidime has also been shown to protect endothelial cells partly from the oxidative stress of activated neutrophils in vitro [181]. The documentation for aminoglycosides is less extensive but both tobramycin and gentamycin have been shown to protect lung epithelial cells against myeloperoxidase-
dependent oxidant injury by binding to anionic cell surfaces and neutralising hypochlorous acid [185]. These findings led to the hypothesis that the anti-inflammatory effects might affect the cytokine response during the last phase of the experiment. At the end of the experiment, a treatment effect manifested as a significantly greater reduction in IL-6 by ceftazidime and tobramycin, and a lower absolute IL-6 level in the ceftazidime group, as compared with the placebo group was observed. Since there are in vitro data indicating that ceftazidime directly affects neither IL-6 production [186] nor neutrophil function [187], this result might have been caused by its antioxidative properties. For tobramycin, in vitro data indicate that neutrophil function and oxidative metabolism might be influenced, but these results have not been reproduced in vivo [186]. Whether findings in this thesis are due to antioxidative properties of the antibiotics or to other mechanisms cannot be concluded from the data presented, but the findings indicate that anti-inflammatory properties reported in vitro may be demonstrable in animal models as well. With short observation time in these experiments, it was not possible to see whether this reduction was continued or whether it would have any impact on haemodynamic parameters.

Future aspects

Although certain questions were answered by the studies in this thesis several new questions arose. If cephalosporins and aminoglycosides have antioxidative properties assessment of this would be appropriate with the oxidative injury product 8-iso-PGF$_{2\alpha}$. Also, these data and the fact that resistance to aminoglycosides is rare, advocate an increased usage of these drugs in Gram-negative sepsis. However concerns have been raised that aminoglycosides may aggravate the sepsis induced renal insufficiency. This issue has proved to be difficult to evaluate in patients with sepsis partly because of co-existing diseases may complicate the clinical picture. A future study evaluating possible additive effect of aminoglycosides to endotoxin induced deterioration in renal function in this porcine model may add to our knowledge in this area. In fact, such a study has already been initiated by us.

These experiments had an observational period of six hours which allow assessment of proinflammatory responses. Longer experiments could render insights into the antiinflammatory responses that may dominate the clinical picture after the initial proinflammatory period. Many sepsis therapies were promising in animal models, but failed in clinical trials, were specific inhibitors of the proinflammatory activation [188-193]. The administration of agents modifying anti or proinflammatory responses in an inappropriate phase of sepsis may disturb the fine balance between proinflammatory response for immune competence and antiinflammatory response to limit organ dysfunction, and lead to worse outcome by e.g. increased incidence of
opportunistic infections or multi organ failure. Thus, increased understanding of the interplay of pro and antiinflammatory responses, identifying possible points of intervention and development of diagnostic methods for evaluation of the balance between pro and antiinflammatory responses are probably essential in development of future sepsis therapies and some pieces of the enormous puzzle of sepsis may be found in long term experiments of porcine endotoxaemia.
Conclusions

In this pig model, endotoxin induces linear log–log cytokine and F$_2$-isoprostane as well as log–linear leukocyte and platelet responses. Several circulatory parameters, plasma leakage, hypoperfusion and dynamic pulmonary compliance exhibited log-linear response to the endotoxin dose as well.

From dose-effect curves, it may be deduced, that the response to a given endotoxin dose seems to be greater at lower pre-existing endotoxin values. This finding should be considered when setting up experiments where anti-endotoxin effect will be studied.

The biological response, in this pig model, in the form of inflammation, hypotension, hypoperfusion and organ dysfunction is more expressed if the organism is exposed to endotoxin at a higher rate.

Correlation studies between cytokines, leukocytes, platelets and the endotoxin dose were in agreement with the well-known ability of endotoxin to induce cytokine expression concomitant with activation of primary haemostasis and leukocytes.

Free radical mediated lipid peroxidation correlates strongly to deleterious pathophysiologic events, such as enhanced cytokine expression and organ dysfunction in endotoxaemic shock. This oxidative injury is a powerful and potent contributor to the development of multiorgan failure during endotoxaemic shock. COX-mediated inflammation further contributes to this decline.

Pigs pretreated with clopidogrel exhibited a trend towards less expressed endotoxin-mediated deterioration of renal function. Blocking of the ADP-induced primary haemostasis, did not affect haemodynamic, coagulatory or inflammatory responses during porcine endotoxemia.

Neither the biological effects of endotoxin nor the plasma levels of endotoxin were neutralised by ceftazidime or tobramycin.

A significantly greater reduction of IL-6 in pigs treated with ceftazidime and tobramycin as compared with those given saline was observed, indicating a possible anti-inflammatory effect of these antibiotics.
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