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THE ROLE OF THROMBIN IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

presented by

Frederic Jean-Marie Moulin

has been accepted towards fulfillment
of the requirements for

~~Ph.D.~~ degree in ~~Pharmacology~~ & Toxicology

Robert A. Roth

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Major professor

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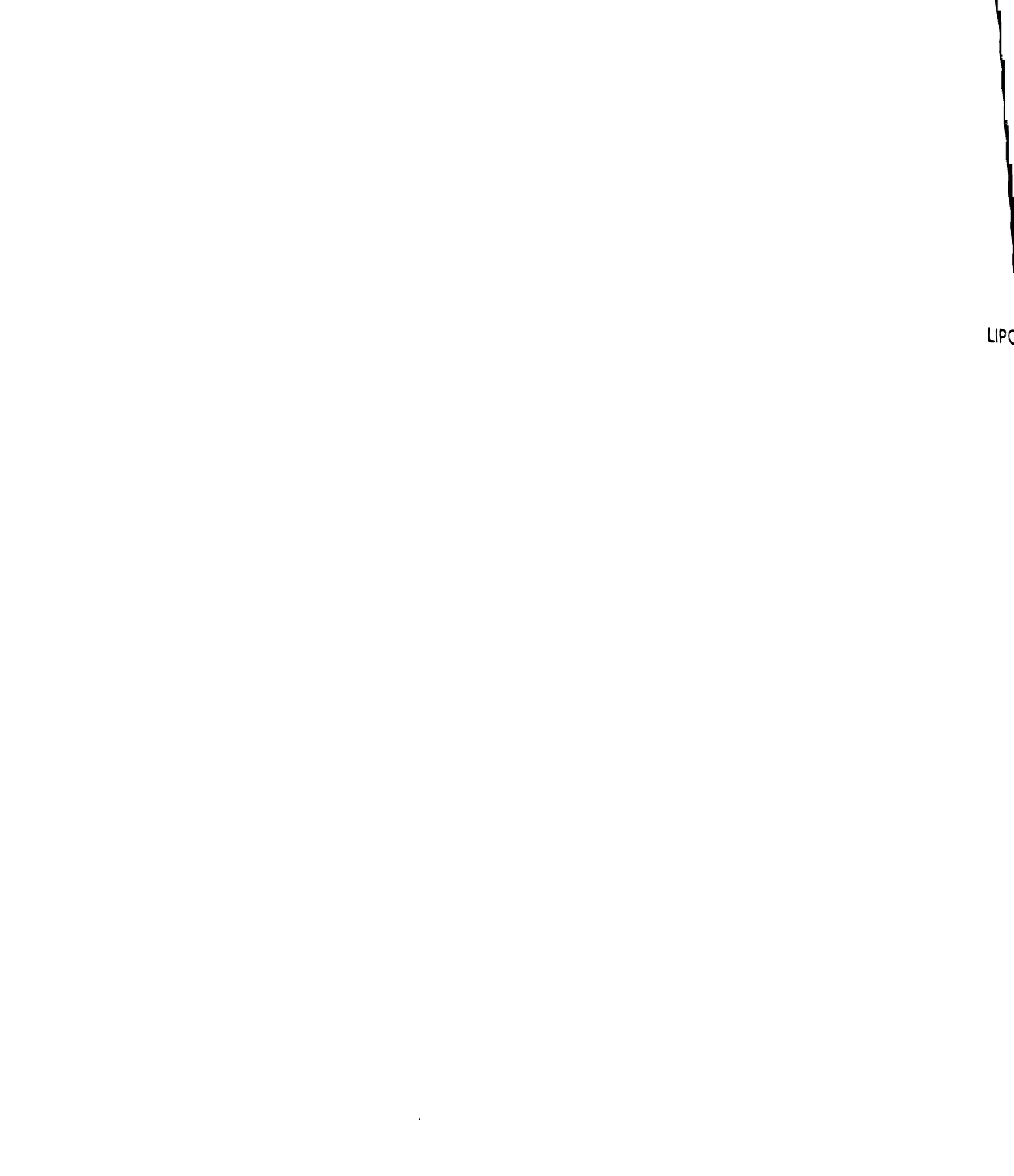
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THE ROLE OF THROMBIN IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

By

Frederic Jean-Marie Moulin

A DISSERTATION

Submitted to
Michigan State University
In partial fulfillment of the requirements
For the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

1999

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ABSTRACT

THE ROLE OF THROMBIN IN LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

By

Frederic Jean-Marie Moulin

Systemic exposure to Gram negative bacterial lipopolysaccharide (LPS) triggers a cascade of events that culminates in injury to tissues, including the liver. The hypothesis tested was that thrombin participates in LPS-induced liver damage by a receptor-mediated mechanism independent of the formation of occlusive fibrin clots. Perfusion of livers isolated from LPS-treated rats with blood containing ancrod resulted in significant release of alanine aminotransferase (ALT) which was not observed when the blood was treated with heparin. Perfusion of similar livers with buffer containing α-thrombin induced release of ALT in amounts comparable to those observed with ancrod-treated blood. These results indicate that thrombin is a critical mediator of LPS-induced liver damage and contributes to liver injury through a mechanism that is independent of clot formation. Thrombin was not cytotoxic to isolated hepatic parenchymal cells. Neither addition of

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LPS to the culture medium nor treatment of rats with LPS prior to hepatocyte isolation influenced this result. In the isolated, buffer-perfused liver, thrombin causes injury in a dose-dependent manner with an EC_{50} of approximately 0.4 nM. This concentration was consistent with a receptor-mediated event. Moreover, thrombin receptor activating peptide reproduced the damaging effect of thrombin in this system. Platelets and neutrophils (PMNs) are two cell types critical to LPS-induced liver damage and with which thrombin may interact. Platelet depletion of LPS-treated rat liver donors did not influence thrombin hepatic toxicity, but PMN depletion abolished this effect. To identify the extrahepatic factors required for LPS-induced liver injury, naive livers were perfused with buffer containing LPS, PMNs and/or thrombin. Livers were damaged maximally by the combination of all three factors. This demonstrates that the only critical, extrahepatic mediators needed for LPS-induced liver damage are thrombin and PMNs. Thrombin did not stimulate or enhance degranulation of rat PMNs, and it was not directly toxic to isolated rat hepatocytes in the presence of PMNs *in vitro* even after LPS exposure. Accordingly, hepatocyte killing by the PMN/thrombin combination appears to require an additional cellular and/or soluble mediator(s). These results suggest that thrombin causes tissue injury during inflammation through activation of a cellular receptor and that its role in the pathogenesis involves interactions with blood PMNs and perhaps nonparenchymal liver cells.

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In memory of m

To my father, D

of support and e

To my wife Eile

and Muriel, and

loving support r

"To doubt everything or to believe everything are two equally convenient solutions; both dispense with the necessity of reflection."

Jules Henri Poincaré

In memory of my mother, Myriam Moulin.

To my father, Dr. Maurice Moulin, Professor of Pharmacology, for his years of support and encouragement. An apple never falls far from the tree.

To my wife Eileen and my daughter Chloe, my brother and sister Serge and Muriel, and also to my whole family, French and American, whose loving support never dwindled.

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Foremost I would like to thank my thesis advisor, mentor and friend Dr. Robert Roth, who weathered unabashed five years of constant talking, the "cylinder incident", a family vacation to France, various computer crashes and many happy hours. His mentoring and friendship extended far beyond the scope of toxicology.

A heartfelt thank also to Dr. Patricia Ganey, who taught me the isolated liver technique, some fine points of English and whose dreaded red pen labored tirelessly over this manuscript. Her support, patience and humor never faded in the mountains of Colorado, the "lac d'Allos" debacle, or during our weekly meetings.

I thank the other members of my committee, Dr. Kenneth Schwartz whose enthusiasm and confidence sustained my efforts and Dr. Gregory Fink who made statistics almost an understandable subject.

I would like to thank here the many people which in one way or another supported this work. First of all, Dr. Julia Pearson and Dr. Brian Copple, who directly participated to some of the studies presented in this thesis.

Dr. Pearson d

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Dr. Pearson did the *in vivo* experiments involving Heparin and Hirudin, and Dr. Copple contributed the MPO assays and the fMLP stimulation of PMNs. Thanks also to the various members of the laboratory, past and present: Drs. Alan Brown, Patrick Lappin and Jim Wagner, my fellow veterinarian and friend Dr. Rosie Sneed, John Burchweitz, Therese Schmidt and the infernal trio: Steve Yee, Shawn Kinser and Jesus Olivero. Their friendship, help, comments and ideas were invaluable.

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KEY TO ABBREVIATIONS

AALAS	american association of laboratory animal science
ALT	alanine aminotransferase
AP	activator protein
APS	anti-platelet serum
ARDS	adult respiratory distress syndrome
AST	aspartate aminotransferase
BSA	bovine serum albumin
CAPK	ceramide-activated protein kinase
DAG	diacylglycerol
DIC	disseminated intravascular coagulation
ELAM	endothelial-leukocyte adhesion molecule
EU	endotoxin unit
fMLP	N-formyl-met-leu-phe peptide
G-CSF	granulocyte colony stimulating factor
$GdCl_3$	gadolinium chloride
GM-CSF	granulocyte-monocyte colony stimulating factor
HC	hepatocyte
HDL	high density lipoprotein
ICAM	intercellular adhesion molecule
IL	interleukin
ip	intra-peritoneal
iv	intra-venous
KDa	kilodalton
KDO	2-keto-3-deoxy-D-mann-octulosonic acid
LAL	*limulus* amebocyte lysate
LBP	lipopolysaccharide binding protein

ABBREVIATIONS

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IκB	inhibitory element kappa-B
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
mCD14	membrane-bound CD14 receptor;
M-CSF	macrophage colony stimulating factor
MODS	multiple organ dysfunction syndrome
MPO	myeloperoxidase
NFκB	nuclear factor kappa-B
NO	nitric oxide
NOS	nitric oxide synthase
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PAR	protease-activated receptor
PBS	phosphate-buffered saline solution
PLA_2	phospholipase A_2
PLC	phospholipase C
PMN	polymorphonuclear phagocyte
sc	subcutaneous
sCD14	soluble CD14 receptor
SEM	standard error of the mean
SIRS	systemic inflammatory response syndrome
Smase	sphingomyelinase
TF	tissue factor
TNF-α	tumor necrosis factor-alpha
TRAP	thrombin receptor activating peptide
VCAM	vascular adhesion molecule

CHAPTER 1

GENERAL INTRODUCTION

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Sepsis and its consequences

Historical context

Shock is a phenomenon that was already well recognized by military surgeons at the beginning of the 19th century. It was however, much less well understood and characterized than wounding. In a surgical text published in 1859 (Fallon, 1997), only three pages out of over a 1000 were devoted to the description of shock. Despite the brevity of the description, many of the observations were quite accurate and reflect much of what we know today about the systemic inflammatory response and its potential consequences, e.g. the multiple organ failure syndrome. At that time, shock was considered to be a constitutional effect of the injury, which could lead to disturbances of the normal functions of the circulatory, respiratory and nervous system. The perturbations were noted to last for a variable period of time, depending on the "nervous susceptibility" of the victim. It was also well recognized that this susceptibility could result in a progression beyond the normal limits of the initial injury and lead to fever as well as tissue damage in locations remote from the original site of the wound. Based on autopsy data obtained from patients that died of shock late after the initial injury, the authors noted alterations in structure with extensive local damage usually of an inflammatory nature. Read in the context of our present understanding of shock and its evolution, this appears to be one of the first references describing the progression from

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The advent in the 1960s of modern, potent antibiotics directed against gram-positive bacteria revealed a new threat to the survival of critically ill surgical patients (Shenep *et al.*, 1985; Hurley, 1995). Attention turned to gram-negative bacterial infection and its hemodynamic manifestations. The subsequent advances in cardiovascular and respiratory supportive therapy unmasked the physiologic disorders associated with gram-negative sepsis and led to the description of a progressive and sequential failure of multiple organ systems as the final common pathway of death from bacterial infection (Baue and Chaudry, 1980; Baue, 1990).

By the end of the 1970s, however, the multiple organ failure syndrome was becoming distinctly separated from septicemia, and physicians started to describe a group of patients who manifested the clinical symptoms and outcome of sepsis and yet had persistent negative blood culture and did not demonstrate a convincing focus of infection (Penner, 1998). It was not clear whether the physiological perturbations that occurred in those patients were the same as the changes observed in patients with Gram-negative bacteremia (Bone, 1996), and the lack of precise criteria for the terms "infection", "sepsis", "sepsis syndrome" and "septic shock" made it difficult to assess the severity of the infectious

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process (Odeh, 1996), the prevalence in the population (Proulx *et al.*, 1996; Barriere and Lowry, 1995; Brun-Buisson *et al.*, 1995; Pittet *et al.*, 1995) and the mechanism of injury as well as the efficacy of therapeutic attempts directed against it (Bone *et al.*, 1998). Only recently has our understanding of the molecular and cellular events that occur during sepsis started to shed light on the complex cascade of events underlying the mechanism of multi-organ failure (Johnston, 1993; Wenzel *et al.*, 1996; Hayashi *et al.*, 1995), and the need for more exact definitions has become clear. In response to this need, a consensus conference was held in 1997 by the American College of Chest Physicians and the Society of Critical Care Medicine (Muckart and Bhagwanjee, 1997). Out of this conference emerged the following definitions:

Definitions

Systemic Inflammatory Response Syndrome (SIRS)

The "systemic inflammatory response syndrome" is the recommended term to describe the inflammatory process independently of the cause (Bone *et al.*, 1992a). It can be seen often following a wide variety of insults and includes, but is not limited to, more than one of the following clinical manifestations:

1/ A body temperature greater than 38°C or less than 36°C.

2/ A heart rate greater than 90 beats per minute.

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3/ Presence of tachypnea, manifested by a respiratory rate greater than 20 breaths per minute or hyperventilation, indicated by a $PaCO_2$ of less than 32 mm Hg.

4/ An alteration in the white blood cell count, such as a count greater than 12,000/ml or less than 4,000/ml, or the presence of more than 10% immature neutrophils ("bands").

These physiologic changes should represent an acute alteration of standard physiological baseline in the absence of otherwise known causes of such abnormalities like chemotherapy (Nystrom, 1998; Rangel-Frausto *et al.*, 1995; Bone, 1992b).

Bacteremia and Infection

"Bacteremia" is defined as the presence of viable bacteria in the blood. "Infection" describes the inflammatory response to the presence of microorganisms or the invasion of normally sterile host .

Sepsis

When SIRS is the result of a confirmed infectious process, it is termed "sepsis". In essence, sepsis represents the systemic inflammatory response to the presence of a live foreign pathogenic organism in the patient (Knaus *et al.*, 1992).

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"Septicemia" was defined in the past as the presence of microorganisms or their toxins in the blood; however, this term has been used in a wide variety of ways, leading to confusion and difficulties in the interpretation of data. It was recommended that this term be eliminated from usage (Odeh, 1996). Sepsis and its sequelae represent a continuum of clinical and pathological responses from the host. Some clinically recognizable stages along this continuum have been characterized.

Severe sepsis

"Severe sepsis" is defined as sepsis associated with organ dysfunction, hypoperfusion abnormalities like lactic acidosis, or sepsis-induced hypotension (i.e. systolic blood pressure of less than 90 mm Hg or reduced by at least 40 mm Hg from baseline in the absence of other causes for hypotension).

Septic shock

Septic shock is a subset category of severe sepsis manifested by sepsis-induced hypotension persisting despite adequate fluid administration and the presence of hypoperfusion abnormalities or organ dysfunction. Patients receiving inotropic or vasopressor agents may no longer be hypotensive by the time the manifestations of hypoperfusion arise and yet would still be considered in septic shock.

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Multiple organ failure

The terms "progressive or sequential organ failure", "multiple organ failure" and "multiple organ systems failure" were introduced to describe the evolving clinical syndrome characterized by the development of abnormalities of organ functions in critically ill patients not clearly related to wounds or direct organ damage (Fry, 1995; Graham and Brass, 1994; Ruokonen *et al.*, 1991). The pathology that these terms describe is clearly increasing in prevalence as a result not only of improvements in life-support technologies, but also their application to an increasing high-risk patient population (Proulx *et al.*, 1996; Saffle *et al.*, 1993; Zimmerman *et al.*, 1996). Since criteria for defining abnormalities of specific organ function have been widely dissimilar from one study to another, the use of "organ failure" allowed retrospective evaluation of older studies. However, this static definition excludes the possibility of a dynamic continuum of physiologic changes in organ functions as is normally observed clinically in septic patients (Baue, 1993; DeCamp and Demling, 1988). For that reason, a new terminology was added.

Multiple Organ Dysfunction Syndrome (MODS)

The detection of altered organ functions in the acutely ill patient constitutes a syndrome that was termed "multiple organ dysfunction syndrome." Dysfunction is defined as the state at which an organ is not

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capable of maintaining homeostasis. MODS can be either primary or secondary.

Primary MODS is the result of a well defined insult in which organ dysfunction occurs early and is clearly the result of the insult itself. An example of primary MODS is organ dysfunction as the direct result of multiple trauma following a crash. In this case, the participation of abnormal and excessive host inflammatory response in both the onset and progression of the injury is not evident.

Secondary MODS develops not as a direct response to the injury, but as a consequence of a generalized activation of the host inflammatory response in organs remote from the initial site of injury and is identified within the context of SIRS. Since SIRS/sepsis is also a continuous process, MODS can be viewed as the most severe end of the spectrum of illness associated with systemic inflammation. Secondary MODS usually evolves after a period of latency and is the most common complication of severe infections.

Clinical Manifestations

Sepsis and septic shock represent the first cause of death in noncoronary intensive care units (Pittet *et al.*, 1995). It is currently the 13th leading cause of death in the United States (Rangel-Frausto *et al.*, 1995), and sepsis is reported to occur in 400,000 to 500,000 patients each year,

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with an associated mortality of about 35-40% (Lowry, 1994). The magnitude of this problem, both in terms of health care cost for treatment (Baumgartner, 1992) and recovery (Brun-Buisson *et al.*, 1995; Perl *et al.*, 1995) as well as for the projected cost of new therapies (Bone, 1991; Lynn and Cohen, 1995; Suffredini, 1994) has made the understanding of sepsis and its management a prominent public and scientific issue.

One can argue that before the advent of critical care medicine, there was little opportunity for the development of the cascade of events leading to MODS. That is, people with severe trauma and sepsis were likely to die before the onset of MODS (Rignault, 1992; Derby, 1988). Thus, sepsis can be viewed as an indirect result of the advance in intensive care medicine. The natural evolution of the septic response and its sequelae has been well studied physiologically (Siegel *et al.*, 1979), and a four-stage classification system has been defined to summarize the evolution of the disease (Shoemaker *et al.*, 1993).

Stage 1 represents the physiological stress response identified among all patients who have a local infection or any minor surgical procedure. It is a normal adaptive response to a physiologic stress, and it leads to substrate mobilization and increased oxygen delivery to the periphery. There is a modest elevation of cardiac output associated with a modest reduction in systemic vascular resistance. Systemic oxygen consumption is increased and by reflex, the patient experiences tachypnea

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as the result of an increase in the respiratory drive. Mild hypoperfusion of the liver and peripheral vascular beds may lead to mild acidosis, but lactate and pyruvate concentrations are frequently normal. Metabolic responses include a modest elevation in glucagon concentration and acceleration of hepatic gluconeogenesis associated with the mobilization of body protein stores to fuel the reaction. On gross physical examination, the patient still appears normal.

As the patient progresses to stage 2, the physiologic stress response is exaggerated to extreme, and a reasonably careful clinical look at the various organ systems reveals that almost everyone of them is operating at the limit of their physiological reserve. Cardiac output is increased two or three fold in response to a profound loss of peripheral vascular resistance. Metabolic changes include a dramatic increase in glucagon concentration associated with an hypermetabolic state: hepatic gluconeogenesis is dramatically increased. Urinary excretion of nitrogen increases in adjustment to the profound catabolic process taking place fueled by the proteolysis of skeletal muscle to provide the substrate for synthesis of glucose. Despite the hyperdynamic circulation, peripheral utilization of oxygen is inadequate, and the increase in lactate production demonstrates that anaerobic metabolism is taking place. Fever, tachycardia and tachypnea are now apparent. The mean arterial blood pressure may be diminished, but it is still within the physiological range.

The patient demonstrates mental changes that may manifest as extreme lassitude or apprehension. Nausea, vomiting and abdominal pain are often reported.

Stage 3 begins when cardiac performance can no longer meet the demands of the profoundly reduced peripheral vascular resistance. Mean arterial pressure drops outside of the physiological range, and oxygen consumption is reduced. Lactate concentration rises dramatically in response to the low perfusion pressure. Ventilatory gas exchange is problematic and is associated with marked tachypnea. The most commonly reported manifestations are severe hypoxia, azotemia, metabolic acidosis and hyperglycemia, clinical jaundice and the onset of coagulopathy. The patient at this stage is different from a trauma victim with low cardiac output: He is simultaneously febrile and hypotensive. Mental confusion and disorientation are common. Unfortunately, this stage is the point when most commonly treatment begins with intubation and ventilatory support, already far into the cascade of events leading to the final stage of "organ failure".

Stage 4 represents the natural progression of stage 3 at the point when ventricular performance can no longer compensate for the profoundly reduced peripheral vascular resistance, and congestive heart failure adds to the hypotensive septic response. At this stage, autonomic control overrides peripheral vasodilation, and the patient manifests a

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general vasoconstriction with increased peripheral resistance. Arterial hypotension is severe, and hypooxygenation of all tissues worsens as a consequence of the septic hypotension, reduced cardiac output and peripheral vasoconstriction. The patient often falls into a coma and starts needing extensive supportive therapy to maintain the most basic functions of an increasing number of organ systems. Organ failure follows a prototypical sequence with the pulmonary system generally being the first one to fail and the renal system the last one, but individual variability among patients with septic response is considerable, and systems previously compromised or damaged by antecedent diseases may fail earlier. In retrospect, it is apparent that the technology for support of the patient's hemodynamics has considerably improved and that multiple organ failure has now become the fatal expression of severe infections (Fry, 1995).

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The Causative Agents

Following the observation that intensive antibiotic therapy could sometimes enhance the clinical symptoms (Shenep *et al.*, 1985; Hurley, 1995), attention was focused on a component of gram-negative bacterial cell wall: lipopolysaccharide (LPS). LPS can be liberated in large amounts into the circulation following bacterial lysis by therapeutic agents (Shenep and Mogan, 1984; Shenep *et al.*, 1988). The observation that mortality rate correlated well with blood concentration of LPS in patients suffering from gram-negative infection (Aasen, 1993), along with observations on animal models and healthy volunteers (Burrell, 1994), provided support for the role of LPS in the development of the multiple organ failure syndrome. LPS has now been implicated in the pathogenesis of gram negative sepsis, endotoxic shock, adult respiratory distress syndrome (ARDS) and multiple organ failure syndrome.

Gram Negative Bacteria and Lipopolysaccharide

In 1884, the histologist Christian Gram devised a method of staining bacteria in tissues now known as the "Gram stain." When bacteria are stained by this method, they are separated in two groups: the gram-positive bacteria are those which retain the primary dye despite the decoloration step, thus appearing deep violet, and the gram-negative bacteria are those which are readily decolorized and later stained pink by

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the counter-staining (Freeman, 1985). The reaction of bacteria to Gram staining has been correlated with a number of differences in physiology and structure, suggesting that the stain is reflective of essential differences between two classes of bacteria. It is now evident that the difference in staining comes from a fundamental difference in cell permeability between the two groups of bacteria. When the microorganism is stained with the crystal violet-iodine complex, the dye remains trapped within gram-positive bacteria and cannot easily be removed by the alcoholic solvent. In the case of gram-negative bacteria, the dye can be easily washed free. This difference underlines the different nature of the bacterial cell envelopes between those two classes of bacteria.

Gram-negative bacterial envelope

The bacterial cell is bounded by an integrated structure, the cell envelope, that consists in most cells of the cell wall and the underlying cytoplasmic membrane. Among bacteria there is a vast number of variations in the architecture, complexity and differentiation of the envelope. The cell envelope of gram-negative bacteria is considered the most complex and has received extensive attention due to its relationship with the pathogenic abilities of these micro-organisms (Figure 1.1). Typically, the envelope is made of two parallel, multilayer membranes that can be observed by electron microscopy.

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The inner, or cytoplasmic membrane encloses the cytoplasm of the cell. Its composition and structure are similar to other biological membranes: a phospholipid bilayer with a median hydrophobic zone traversed by proteins. Many of these proteins are involved in the active transport of small substances such as aminoacids or carbohydrates. The cytoplasmic membrane has little mechanical strength and does not contribute significantly to the maintenance of cell shape. Instead, it is forced against the cell wall by the internal hydrostatic pressure.

The shape and cellular rigidity of bacterial cells is almost entirely due to the presence of a large supporting structure just outside of the cytoplasmic membrane called the peptidoglycan layer. The peptidoglycan layer sits within the periplasmic zone, and can be envisioned as a single, large, bag-shaped molecule entirely surrounding the cytoplasmic membrane and composed of glycan strands cross-linked by short peptides. These glycan strands consist of repeating units of β-1,4-N-acetylmuramic acid, a chemical entity peculiar to bacterial walls.

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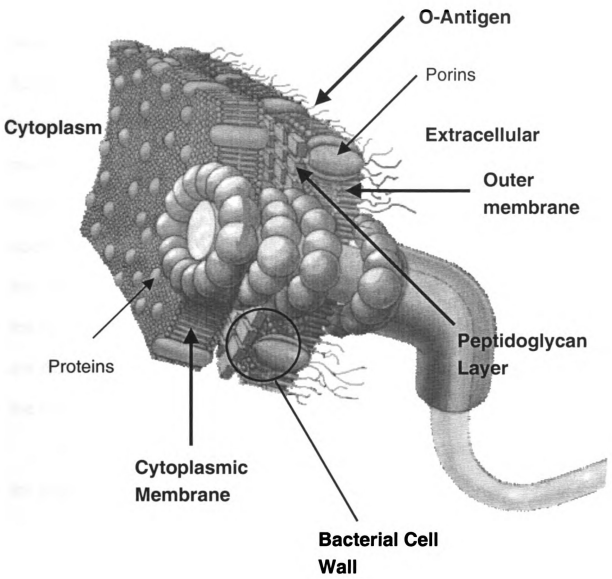

Figure 1.1 Model of a Gram-Negative Bacterial Envelope. The cytoplasmic membrane encloses the cytoplasm of the cell. The peptidoglycan layer sits just outside of the inner membrane and provides most of the rigidity of the cell wall. The outer membrane is the most external layer of the bacterium. The lipopolysaccharides form the major surface antigenic component, the O-antigen. Modified from (Whitfield, 1995).

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The outer membrane constitutes the external layer of most gram-negative bacteria and is similar in morphology and constitution to other biological membranes. Compared to the cytoplasmic membrane, it appears to have less phospholipid and fewer proteins, and it acts as an hydrophobic diffusion barrier against a variety of substrates. The membrane contains a variety of proteins, pore-forming proteins (porins), lipoproteins and others, which generally act to facilitate the permeation of solutes through the membrane. In conjunction with the peptidoglycan layer, the outer membrane lends structural integrity to the bacteria. Even though the cytoplasmic membrane and cell wall are two distinct structures, the two are closely affixed and evidence suggests some sort of adhesion between the two.

Embedded within the outer membrane is lipopolysaccharide (LPS), an important structural and functional component of the gram-negative bacterial cell surface. LPS molecules contain the major surface antigenic component, the O antigens, and mediate the toxic activity of many gram-negative bacteria. So far, only one gram-positive organism, *Listeria monocytogenes*, has been found to express an authentic LPS entity on its surface. LPS molecules are commonly called endotoxins to distinguish them from the heat-labile proteins secreted in the culture media by bacteria and called exotoxins.

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Structure of LPS

LPS molecules can be view as having three distinct regions: A fatty-acylated portion, termed lipid A, attached to a core oligosaccharide generally common within a genus. On the outer side of the core are attached polymeric carbohydrate side-chains called the O-specific chains, or O-antigens (Figure 1.2).

Lipid A is structurally the most highly conserved portion of the molecule among various families of gram-negative bacteria and is believed to mediate most of the biological effects of LPS. The primary structure of lipid A is diphosphorylated diglucosamine residues containing both ester- and amide-linked fatty acids (Wang and Hollingsworth, 1996). The ester-linked fatty acid chain generally contains 14 to 16 carbon residues, and the amide-linked fatty acid chains invariably contain an even number of carbon atoms, uniformly β-hydroxylated. These β-hydroxylated groups are frequently themselves esterified to fatty acids. Although the structure of lipid A is highly conserved, variants have been isolated depending on the organism and the medium on which it is grown. The presence of the intact lipid A structure (diphosphoryl lipid A) is associated with toxicity but precursors are not. However, the toxic and antigenic properties of lipid A appear to be determined not only by its primary structure, but also by the constituents to which it is coupled.

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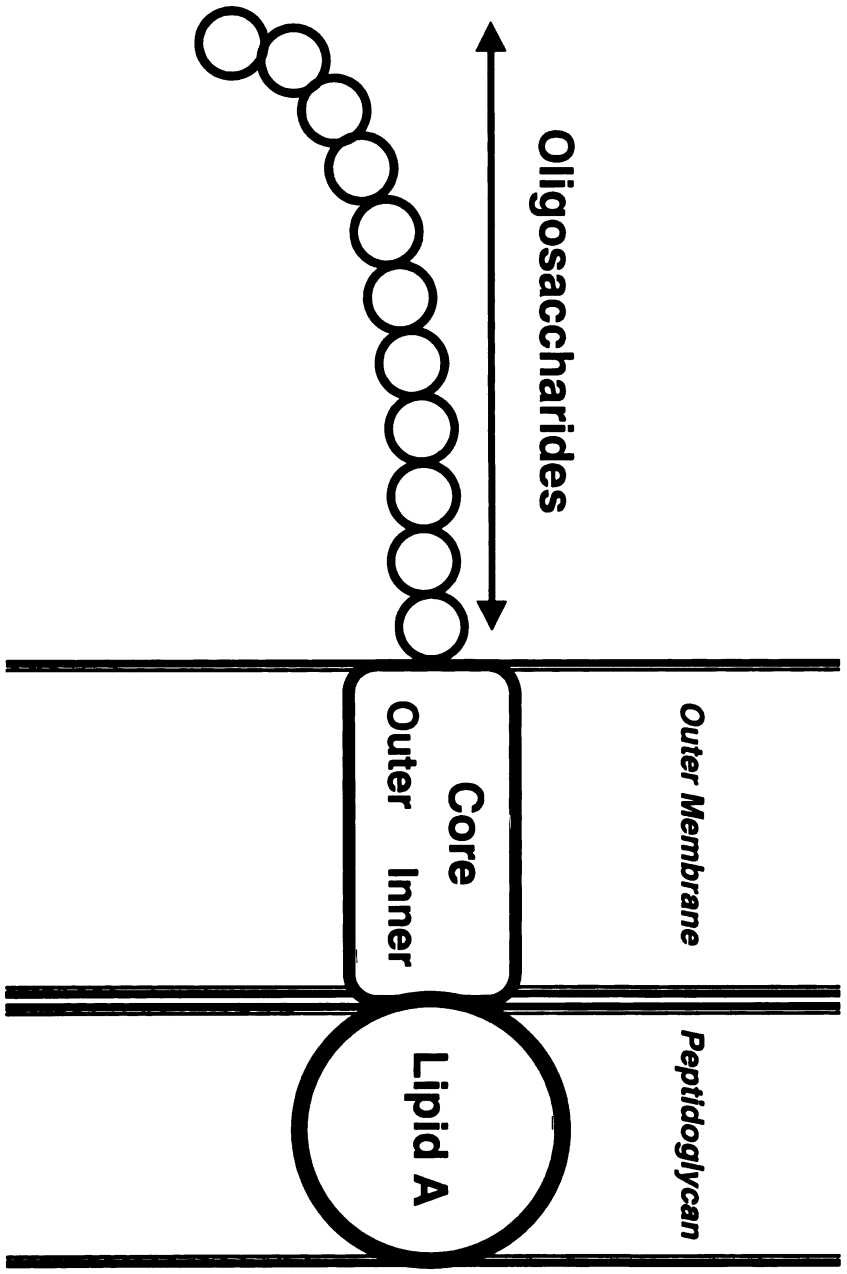

Figure 1.2 Structure of LPS. LPS molecules have three distinct regions. The fatty-acylated lipid A, the core and, on the outer side of the core, the polymeric carbohydrate side-chains called the O-antigen.

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This led to the hypothesis that the O-antigen polysaccharide part of the LPS molecule serves to solubilize lipid A in the aqueous environment, increasing the biological activity *in vivo*.

The core polysaccharide region of LPS links the O-antigen to the lipid A portion (Rietschel and Brade, 1992; Sonesson *et al.*, 1994). It is composed of ten to twelve saccharide residues, and itself is divided into three sub-regions: outer, intermediate and inner (deep) core. The outer region is composed of hexoses that are linked to the heptose forming the primary constituents of the intermediate core. The deep core is composed of three residues of 2-keto-3-deoxy-D-mann-octulosonic acid (KDO) which are linked to lipid A. Differences in the core composition between various gram-negative bacteria are primarily due to loss or substitution of hexoses in the outer core.

The O-antigen is the most variable region in the LPS molecule and is responsible for the heterogeneity in response among LPS from different strains of bacteria. It comprises repeating oligosaccharide units that are unique for each individual strain of bacteria. The bacteria that express O-antigen polysaccharides on their surface form colonies that are glossy and rounded in appearance, therefore called *smooth* type. Colonies of mutant bacteria lacking the O-antigen on their surface are crinkled in appearance and are commonly called *rough* bacteria. In parental smooth strains, the O-antigen is composed of 30-100 saccharide residues arranged in repeating

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Modes of exposure

The most common form of exposure to lipopolysaccharide results from a gram-negative bacterial infection. Gram-negative bacteria are widespread in nature and responsible for such dangerous diseases as plague, cholera and whooping cough (Table 1.1). However, the most common source of endotoxin are the usually harmless, enteric bacteria of the species *Escherichia* and *Salmonella* that colonize the lower part of our gastrointestinal tract.

LPS is an integral component of their bacterial cell walls, and it can be released into the general circulation during cell division or death. Local infections as well as gram-negative septicemia are frequently accompanied by a rise in levels of LPS in biological fluids.

Table 1.1: E
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Bacteria
Shigella sp.
Escherichia Coli
Campylobacter je
Helicobacter pylori
Yersinia sp.
Salmonella sp.
Vibrio cholerae
Neisseria gonorrhoe
Neisseria meningit
Bordetella pertussis
Pseudomonas aerug
Legionella pneumop
Borrelia sp.

Table 1.1: Examples of Pathogenic Gram-Negative Organisms and diseases commonly associated with their presence in the tissue.

Bacteria	Disease	Additional Toxins
Shigella sp.	Diarrhea, dysentery	Shiga toxin (blocks protein synthesis)
Escherichia Coli	Diarrhea, dysentery	Shiga-like enterotoxins
Campylobacter jejuni	Gastritis, enterocolitis, dysentery, diarrhea, septicemia	
Helicobacter pylori	Gastritis, ulceration	
Yersinia sp.	Ileitis, mesenteric lymphadenitis, arthritis, glomerulonephritis, pneumonia, black plague.	
Salmonella sp.	Gastroenteritis, sepsis	
Vibrio cholerae	Cholera	Cholera toxin (enterotoxin)
Neisseria gonorrhoeae	Gonorrhea	
Neisseria meningitidis	Meningitis	
Bordella pertussis	Whooping cough	Pertussis toxin
Pseudomonas aeruginosa	Pneumonia, sepsis, corneal keratitis, endocarditis, osteomyelitis, otitis	Exotoxin A, exoenzyme S, iron-containing toxins
Legionella pneumophila	Pneumonia	
Borrelia sp.	Fever, headache, MOFS, DIC, Lyme disease	

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For example, gram-negative bacterial meningitis has been associated with increased concentration of LPS in the cerebrospinal fluid in animal models (Tauber *et al.*, 1987) as well as in human patients (Berman *et al.*, 1976). Gram negative septicemia and its common treatment with antibiotics can produce a 50 to 2000-fold increase in the blood level of free LPS as a result of bacterial wall lysis (Shenep *et al.*, 1988; Shenep and Mogan, 1984). Thus, antibiotic therapy may, in some instances, increase exposure of the septic patient to endotoxin and lead to a paradoxical sudden worsening of the patient's condition.

A second frequent source of exposure to endotoxin is aspiration into the airways. LPS can be adsorbed on organic particles and released directly into the pulmonary circulation. This method of contamination has been implicated in several inhalation-related occupational diseases in agriculture, manufacturing, textile production and wastewater treatment (Burrell, 1997). While in most case of pulmonary exposure LPS remains localized to the lung with little effect on the systemic circulation (Ghofrani *et al.*, 1996), it has been shown that doses in excess of those required for neutrophil influx into the lung could cause the appearance of TNF-α into the general circulation three hours after administration (Turner *et al.*, 1993). At the intersection between infection and airway exposure we find the numerous pneumonias. Bacteria can penetrate into the lung following accidental aspiration of the oropharyngeal or intestinal flora, or more

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commonly following inhalation of aerosolized bacteria adsorbed on organic particles. The presence of infectious bacteria in the airways can lead to invasion of the systemic circulation by the pathogens and subsequent endotoxin release in the plasma.

Exposure to low doses of LPS has been hypothesized to be fairly frequent as the gastrointestinal tract, and more specifically the colon, contains an indigenous bacterial flora with a large number of gram-negative species (Engstrom *et al.*, 1992; Deitch, 1995). While some of them are pathogenic, most strains of *E. Coli* are constitutive and harmless inhabitants of the lower digestive tract. Disturbances of the gastrointestinal barrier can result in an influx of gram-negative bacteria first into the liver via the portal vein, and then to the general circulation (Sedman *et al.*, 1994; Mishima *et al.*, 1998; Deitch, 1994; van Goor *et al.*, 1994; Van Leeuwen *et al.*, 1994).

Transport and Clearance

The liver appears to play a critical role in preventing systemic endotoxemia by the activity of resident macrophages (Kupffer cells) that clear LPS from the portal circulation (McCuskey *et al.*, 1987). Quantitative measurement using labeled endotoxin has shown that the Kupffer cells were the major cells responsible for LPS uptake and degradation, and that a slow release could then occur transferring LPS back to other organs

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(Freudenberg *et al.*, 1982; Freudenberg *et al.*, 1985). As the lower intestinal tract appears to be the main source of LPS aside from infection, it is not surprising to find the liver as the main organ responsible for the clearance of this toxin. The hepatic portal vein drains the totality of the blood coming from the gut, and therefore the liver is the first organ to receive endotoxin and the one that experiences it in the highest concentration.

Several carrier proteins for LPS have been identified in the blood and on the cell surface (Table 1.2), but apparently only one is critical for the transport of endotoxin. The LPS-binding protein (LBP) is a 61 KDa glycoprotein (Su *et al.*, 1994; Halling *et al.*, 1992) that was discovered as a serum element capable of regulating the binding of LPS to high density lipoproteins (HDL) (Tobias *et al.*, 1988). It is normally present in the serum at a concentration of 3-10 µg/ml, but the concentration can rise more than 20 fold during the acute phase response to endotoxin (Wan *et al.*, 1995; Heumann *et al.*, 1992). LBP is an acute phase protein synthesized predominantly by the liver parenchymal cells (Su *et al.*, 1994). It binds endotoxin and presents it to cells expressing the membrane-bound CD14 receptor or transfers it to the soluble form of the CD14 receptor (Hailman *et al.*, 1994; Mathison *et al.*, 1992; Meszaros *et al.*, 1994). It can also opsonize gram-negative bacteria. LBP binds with a high degree of specificity ($K_d = 10^9$) to the lipid A portion of LPS.

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Table 1.2 Endotoxin-Binding Proteins found in the plasma or on the surface of cells.

Binding Moiety	*Description/Function*
Extracellular	
High-density Lipoproteins	*Initial detoxification/binding*
LPS-binding Protein (LBP)	*Facilitates CD14 binding*
Septins	*Facilitate target-cell binding*
Soluble CD14 (sCD14)	*Cell activation / Detoxification*
Cell Membrane Receptors	
Membrane CD14 (mCD14)	*Cell activation*
73 kDa Glycoprotein	*Binds LPS/Peptidoglycan*
CD11/18	*Phagocytic ligand*
BPI	*Lipid A binder*
Lectins	*Bind inner core region*

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The amino-terminal half of the protein is responsible for the specific binding to LPS, while the carboxyl-terminal half interacts with CD14 molecules (Su *et al.*, 1995).

Antibodies directed against LBP decrease LPS sensitivity by two to three orders of magnitude. In the presence of LBP, LPS sensitivity of monocytes is increased 1000 fold in respect to the production of TNF-α (Raetz *et al.*, 1991). LBP also facilitates the binding of LPS to high-density lipoproteins (HDL), helping in the removal of bacterial endotoxin from the general circulation.

Other than LBP, the bacterial permeability increasing protein (BPI) is the other well characterized, naturally occuring protein that binds specifically lipid A. BPI is not a serum protein, but rather is a 55-kDa membrane-associated protein released during degranulation of neutrophils in response to LPS, fMLP and cytokines (Marra *et al.*, 1992). BPI appears to have primarily LPS-neutralizing ability (Marra *et al.*, 1993) and is specifically cytotoxic for gram-negative bacteria. BPI is organized in two domains, with the carboxyl-terminus anchoring the protein into the membrane and the amino-terminus exhibiting several similarities with the sequence of LBP, suggesting that both proteins share a similar structure for binding LPS.

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The biological role of the soluble form of CD14 (sCD14) in the removal of LPS is not clear. Initially the hypothesis that it may act predominantly to neutralize LPS was proposed after examination of the activation of human monocytes by LPS. It was described that sCD14 was able to reduce in a dose-dependent manner the production of reactive oxygen species by monocytes in response to endotoxin (Schutt *et al.*, 1992). These results were later supported by the evidence that higher than normal levels of sCD14 could prevent the binding of labeled LPS to human and bovine monocytes (Grunwald *et al.*, 1993). However, both those studies used concentrations of sCD14 far greater than its physiological level, making the assumption that sCD14 is an LPS scavenger protein *in vivo* difficult to believe (Su *et al.*, 1995).

Finally, a wide variety of other proteins have been found to bind LPS opportunistically, facilitating its removal from the blood. Septins, lectins, HDL and CD11/18 are non-specific transporters involved in the process of detoxification.

LPS is primarily removed from the circulation through uptake by mononuclear phagocytes. At the present, two mechanisms independent of the cellular activation cascade have been identified. LPS or its complex with LBP can bind to the scavenger receptor, and it is assumed that the degradation of LPS follows a route similar to the degradation of acetylated LDL with which it competes. The LPS-sCD14 complex can also be taken

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up by endocytosis in phagocytic cells, in a process different from the signal transduction pathway (Gegner *et al.*, 1995). The kinetic of LPS removal is biphasic and dose-dependent. Intravenous administration of radiolabeled LPS results in a first rapid elimination phase (within 30 min), followed by a slower removal phase over hours to days depending on the dose (Mathison and Ulevitch, 1979).

Consequences of LPS exposure

Clinical Manifestations

The increase in LPS concentration in the blood leads to numerous pathophysiological alterations summarized in Figure 1.3. In all studies investigating the effects of LPS administration to healthy volunteers, a general, influenza-like feeling was reported, with headache and nausea occurring in nearly all subjects within one hour of administration (Burrell, 1994). Fever is a hallmark of endotoxin exposure, as LPS stimulates the release of endogenous pyrogens from monocytes (Heumann *et al.*, 1992; Nichols *et al.*, 1988). Administration of 4 ng/Kg LPS produces in humans a marked decrease in blood leukocyte count, which rebounds starting approximately 2 hours after administration and increases steadily until 6 hours (Elin *et al.*, 1981). At the same dose, the minute ventilation was found to be significantly greater 4 hours after infusion, and oxygen consumption and delivery was increased (Burrell, 1994).

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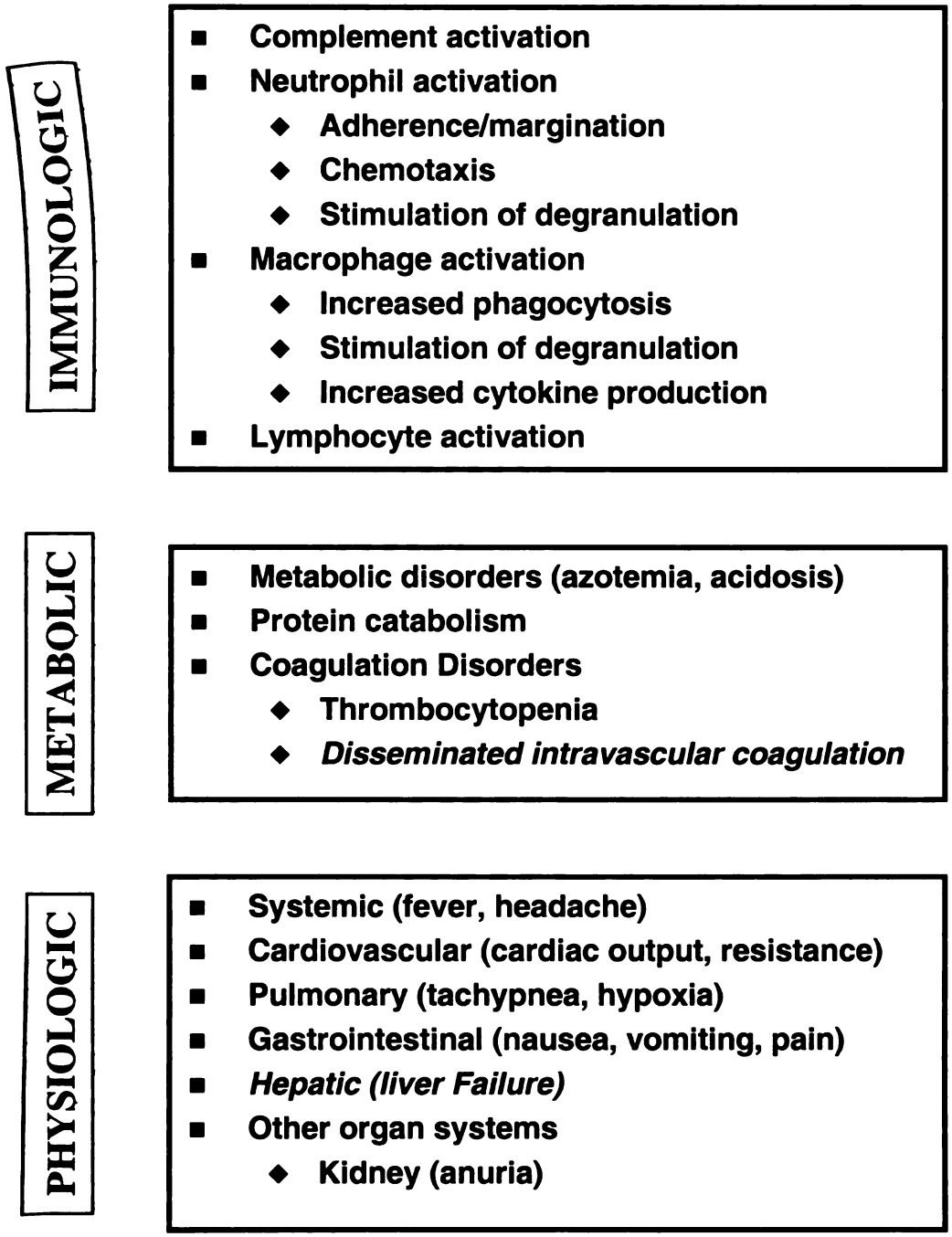

Figure 1.3 Physiological Alterations Due to LPS Exposure. Alterations in italics are directly relevant to this dissertation

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LPS administration to healthy volunteers results in increased heart rate and decrease in blood pressure within 2 hours after injection (Burrell, 1994). At this point, the combined increase in pulse rate coupled with decrease in diastolic pressure and vascular resistances translates into a large increase in vascular volume, reminiscent of the features seen in early stages of septic shock (Shoemaker *et al.*, 1993). This is followed in septic patients by a brief rebound period, the "hyperdynamic phase" (Casals-Stenzel, 1987), and a second sustained decrease in cardiac output more gradual in onset. The early changes appear to be mediated in part by metabolites of arachidonic acid (Hewett and Roth, 1993). The second phase is the result of a more severe decrease in myocardial performance that has been linked to alterations in the coronary blood flow, damage to the endocardium and edema (Gotloib *et al.*, 1992). The alterations in cardiac output are accompanied by perturbation in blood flow to most of the tissues. Marked decreases in blood flow to the heart, kidneys, pancreas and spleen have been reported (Somani and Saini, 1981; Breslow *et al.*, 1987; Hussain and Roussos, 1985), while the lungs (Olson *et al.*, 1990; Chang, 1992) and gastrointestinal tract (Hussain and Roussos, 1985) experience an increase in permeability leading to hemoconcentration.

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Tissue injury

The mechanisms by which LPS exposure results in cellular damage are complex and vary among tissues. Pulmonary alterations following LPS exposure bear a striking resemblance to those associated with the adult respiratory distress syndrome (Rabinovici *et al.*, 1993; Parsons *et al.*, 1989). In an early phase, mean pulmonary arterial pressure and pulmonary vascular resistance increase dramatically. After a short return to normal, these parameters follow a second, gradual increase accompanied by an augmentation of vascular permeability and neutrophil infiltration. LPS causes renal tubular necrosis (Ou *et al.*, 1994) associated with renal dysfunction. The hypotensive effects of LPS are believed to contribute to a marked decrease in renal blood flow and glomerular function. LPS-mediated hypotension has also been implicated in damage to the intestinal mucosa, increased microvascular permeability in the gastrointestinal tract and gastric bleeding (Boughton-Smith *et al.*, 1990). LPS-induced liver injury *in vivo* is characterized by a predominantly midzonal, multifocal hepatocellular necrosis with neutrophil accumulation in the lesion areas and formation of fibrin and platelet deposits (Hewett and Roth, 1993). A marked hyperbilirubinemia and an elevation of the plasma concentration of liver-specific enzymes reflects the increased inability of the organ to perform its normal functions (Czaja *et al.*, 1994).

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Cytokines Expression

The release into the general circulation of various cytokines in response to endotoxin has been well documented *in vivo* and *in vitro*. One of the most important mediators of septic shock appears to be tumor necrosis factor-alpha (TNF-α). Produced primarily by activated macrophages, lymphocytes and mast cells, TNF-α is detectable between 30 and 60 minutes after challenge with 2 ng/kg purified LPS in humans (Kuhns *et al.*, 1995; Dofferhoff *et al.*, 1991), peaks at approximately 90 minutes and returns to baseline with 3 to 5 hours (Michie *et al.*, 1988). TNF-α is a strong immunomodulator, able to induce production of interleukin-3 (IL-3) and interleukin-6 (IL-6), activation of T-cells and expression of interleukin-2 (IL-2) receptor. TNF-α also has effects on hematopoietic stem cells, B lymphocytes, neutrophils and endothelial cells. It shares many of its properties with interleukin-1 (IL-1), and the two cytokines act synergistically.

IL-1 is predominantly produced by neutrophils, fibroblasts, epithelial cells and endothelial cells, and can be detected in the plasma two hours after LPS administration (Doide and Steinman, 1987). Through its interaction with the endothelium, IL-1 promotes procoagulant production, thereby increasing adhesion of inflammatory cells to the vessel walls (Pugin *et al.*, 1995). It is also important in mediating the inflammatory response by stimulating the release of arachidonic acid metabolites from

the target cells and mediating the pyrogenic response of the organism (Jansen *et al.*, 1995; Movat, 1987).

The production of IL-6 can be stimulated by IL-1 and TNF-α as well as endotoxin from a variety of cells such as the lymphocytes and the macrophages/monocytes (Callery *et al.*, 1992). The human response appears very similar over a wide range of LPS concentration, peaking between 2 and 3 hour and returning to normal levels within 6 hours (van Deventer *et al.*, 1990). The role of IL-6 during inflammation appears linked to its ability to increase the synthesis of most major acute phase proteins, including fibrinogen. Il-6 modulates the response to inflammatory cytokines by, for example inducing the expression of IL-2. It also appears to play a role in the activation of the coagulation cascade frequently observed during sepsis (van der Poll *et al.*, 1994; Kruithof *et al.*, 1997).

Interleukin-8 (IL-8) appears in the plasma of LPS-challenged volunteers around 90 minutes, with peak levels occuring at 120 min post-infusion (Martich *et al.*, 1991). IL-8 is released in response to TNF-α, and it can activate neutrophils, enhancing various functions such as degranulation, chemotaxis and endothelial cell adherence. Interferon and IL-2 were not produced in sufficient quantities at the low level of exposure used in experimental studies on human volunteers to be detected.

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Coagulopathy

There have been few studies documenting activation of the coagulation and fibrinolytic systems in healthy volunteers (Burrell, 1994) despite the wealth of evidence that these systems are quite important in clinical endotoxemia and disseminated intravascular coagulation (Penner, 1998; Gando *et al.*, 1996; Vervloet *et al.*, 1998). Activation of the coagulation cascade was noted by an increase in prothrombin fragments appearing as early as two hours after administration, but becoming significant only at four hours, and the formation of thrombin-antithrombin complexes reaching a sharp peak after three hours (van Deventer *et al.*, 1990).

LPS can activate the intrinsic pathway of coagulation directly *in vitro* by initiating the conversion of factor XII (Morrison and Cochrane, 1974). It can also stimulate the release of tissue factor from endothelial cells, activating the extrinsic pathway of coagulation (Stern *et al.*, 1985; Stern *et al.*, 1991). During sepsis, disorders of coagulation are frequently observed either in animals models (Kruithof *et al.*, 1997; Ito *et al.*, 1990; Yoshikawa *et al.*, 1984; Yoshikawa *et al.*, 1981) or in human patients (Mammen, 1998a; Penner, 1998; Vervloet *et al.*, 1998; Bick, 1996; Gando *et al.*, 1996; Ito *et al.*, 1990; Yoshikawa *et al.*, 1984). They are often manifested by decreases in plasma fibrinogen and blood platelet concentrations, prolongation of both prothrombin and activated partial thromboplastin times

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and elevation in fibrin degradation products. This systemic thrombo-hemorrhagic disorder, showing evidence of simultaneous procoagulant and fibrinolytic activation, inhibitor consumption and end-organ damage has been termed disseminated intravascular coagulation (DIC).

Most physicians consider DIC to be a systemic hemorrhagic syndrome. However, it also involves profound microvascular thrombosis (Jourdain *et al.*, 1997; Regoeczi and Brain, 1969) and sometimes even large vessel thrombosis. The common theory is that the small and large vessel thrombosis, with impairment of blood flow, hypoxia and associated end-organ damage, is actually responsible for the morbidity and mortality (Bick, 1996). DIC is an important contributing factor to the mortality associated with severe exposure to LPS, and as such has been the target of numerous therapeutic attempts, none of them conclusively efficacious (Inthorn *et al.*, 1997; Fuse *et al.*, 1996; Gando *et al.*, 1996; Fourrier *et al.*, 1995; Fourrier *et al.*, 1993; Prager *et al.*, 1979). To this day, the complex cascade of interactions between the various mediators, including coagulation and fibrinolysis components, leading to the clinical manifestations of endotoxin exposure remains poorly understood.

Cellular Effects of LPS

The exact mechanisms by which endotoxin can affect cell functions is not yet fully elucidated. Two potential ways of LPS interactions with

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target cells will be considered: direct binding to the cell membrane and indirect effects through a broad spectrum of mediators.

Direct interaction

The CD14 protein functions as a receptor for endotoxin on cells (Wright *et al.*, 1990). It is a 55 kDa protein initially identified on the cytoplasmic membrane of monocytes. The mature CD14 membrane protein is composed of 356 amino acids with 4 sites for N-linked glycosylation. An additional 19 amino acid signal sequence is removed during processing. The receptor exists in two forms: a membrane-bound one found on the surface of cells (mCD14) and a soluble one (sCD14) identified in plasma (Bazil *et al.*, 1989). mCD14 is anchored to the cell surface by linkage to glycosylphosphatidylinositol (Simmons *et al.*, 1989; Haziot *et al.*, 1988). At least two soluble forms of CD14 (sCD14) have been described (Labeta *et al.*, 1993). One soluble form is produced by shedding the cell surface form, which results in an approximately 48 kDa molecule (Bazil *et al.*, 1986). A second soluble form is released from cells before addition of the GPI anchor, resulting in a higher molecular weight (Bufler *et al.*, 1995). The receptor is expressed strongly on the surface of monocytes (Marchant *et al.*, 1992) and weakly on the surface of granulocytes. It is also expressed by most tissue macrophages such as Kupffer cells. In mice stimulated with LPS, CD14 expression is detected in

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non-myeloid cell types (eg. hepatocytes and several other epithelial cell types)(Fearns *et al.*, 1995). The soluble forms of CD14 can be detected in serum and tissue culture supernatants of cells transfected with CD14.

Antibodies to CD14 block LPS-induced protein phosphorylation, the oxidative burst in neutrophils and T-cell proliferation. LPS upregulates the expression of CD14 in Kupffer cells (Matsuura *et al.*, 1994). Because CD14 responds to a broad spectrum of polysaccharide moieties with related structures, it has been proposed to be a "pattern recognition" receptor that may bind to microbial structures but not provide specific recognition. The soluble form of the receptor (sCD14) is also able to bind LPS (Frey *et al.*, 1992). The soluble receptor has been shown to mediate the response to endotoxin of cells lacking the membrane-bound form, such as endothelial and epithelial cells (Pugin *et al.*, 1993; Pugin *et al.*, 1994).

Binding of the LPS-LBP complex to membrane-bound CD14 can trigger an intracellular signaling cascade resulting in expression of immediate early genes. It appears that the final stages of intracellular signaling are shared with the pathways initiating from receptors such as TNF-α, interleukin-1 and various cellular stress factors (oxygen free-radicals, viruses, exotoxins). The cascade involves first binding of LPS or LPS-LBP to N-terminal portion of the membrane-bound CD14 receptor (Viriyakosol and Kirkland, 1996). The mCD14 receptor does not have an intracellular domain, and in some cells binding of LPS to its receptor leads

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to tyrosine phosphorylation of proto-oncogenes (ras, vav) (English *et al.*, 1997) and further down the second messenger cascade of the mitogen-activated protein kinases (MAPK). This results in activation of the activator-protein-1, and in turn expression of immediate-early genes.

Another pathway of cellular activation following binding of LPS to mCD14 involves nuclear factor kappa-B (NFκB) pathway (Muller *et al.*, 1993). NFκB is normally found in the cytoplasm tightly bound to an inhibitory protein called IκB. To release NFκB, the inhibitory protein must first be phosphorylated by a protein kinase (Baeuerle and Baltimore, 1996) and the phosphorylated IκB is subsequently degraded by a proteinase (Henkel *et al.*, 1993).

Different routes have been described for the activation of the IκB protein kinase. A common intermediate from a large number of incoming extracellular signals, including the CD14 receptor, appears to be a reactive oxygen, most likely H_2O_2 (Ziegler-Heitbrock *et al.*, 1993). The observation that activation of NFκB by LPS is suppressed by reducing agents such as dithiocarbamates support this assumption. However, the exact origin of the reactive intermediate as well as how it activates the protein kinase is currently unknown.

Another route of activation for the IκB protein kinase involves the ceramide signaling pathway (Haimovitz-Friedman *et al.*, 1997a). Binding of LPS to the mCD14 receptor leads, either directly or through the

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intermediate of phospholipase C and the production of diacylglycerol, to the activation of membrane-bound sphingomyelinase enzymes (SMase) (Mathias *et al.*, 1991). SMase catalyses the hydrolysis of sphingomyelin, yielding phosphocholine and ceramide (Haimovitz-Friedman *et al.*, 1997b). The free ceramide acts as an intracellular messenger, stimulating a ceramide-activated serine/threonine kinase (CAPK), which in turn phosphorylates IκB, resulting in degradation of the inhibitory protein and free NFκB (Machleidt *et al.*, 1994). Indeed, LPS and lipid A have been shown to enhance CAPK activity within seconds, and the effect is markedly enhanced by LBP (Joseph *et al.*, 1994).

In its free form, NFκB translocates to the nucleus were it binds to promotor sequences for the genes of various cytokines, immunoreceptors, and acute phase proteins (Pohlman and Harlan, 1997). The common result of cellular activation through the CD14 receptor is an increase in the production of inflammatory mediators and priming of the cell to LPS. This can take the form of cytokine expression, lipid mediator release and expression of CD14 or LBP (Jarvis *et al.*, 1994; Hannun, 1996). Recent results have demonstrated that the complex sCD14-LPS could increase activation of NFκB and expression of mRNA encoding for tissue factor (Read *et al.*, 1993) and that tolerance to LPS is determined by post-receptor mechanisms involving inactivation of the NFκB complex (Ziegler-Heitbrock *et al.*, 1994).

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In some specific cell types, the effect of LPS does not appear to be mediated by the membrane-bound CD14 receptor. Endothelial cells, for example, lack mCD14, and the effects of LPS are mediated by binding to the soluble CD14 receptor (Pugin *et al.*, 1993). In the proposed mechanism, sCD14 is able to displace the binding of LPS from its carrier protein, releasing free LBP in the circulation (Blondin *et al.*, 1997). It is the complex sCD14-LPS that binds to the surface of the endothelial cells, in turn activating the ceramide pathway (Wright *et al.*, 1990). The binding of LPS to sCD14 appears to compete for binding to mCD14, and the sCD14-LPS complex has no activity on cells expressing the membrane-bound receptor (Troelstra *et al.*, 1997). It has been hypothesized that the sCD14 could act as a "presenting agent" to cells lacking the mCD14 (Frey *et al.*, 1992).

Alternatively, higher concentrations of LPS can activate certain cells in the absence of CD14 binding. For example, leukocytes can be activated by the binding of LPS to the CD11b/CD18 receptor (Simms and D'Amico, 1994), and renal proximal tubular epithelium can be damaged directly by a mechanism dependent on the nitric oxide synthase. It is likely that many of the non-receptor mediated effects of LPS are due to lipid A, possibly mimicking ceramide (Wright and Kolesnick, 1995). The various signalling pathways of cell activation in response to LPS are summarized in Figure 1.4.

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Indirect interactions

The stimulation of cytokine secretion is an important mechanism through which LPS exerts its deleterious effects, and most of the signs of inflammation (redness, swelling, heat, pain and loss of function) are in fact the response to increased cytokine expression precipitated by LPS exposure.

As described previously, TNF-α is released early and appears to contribute to the manifestation of several LPS-mediated effects. Neutralization of TNF-α with antibodies prevents the decrease in blood pressure (Tobias *et al.*, 1988), lung damage (Caty *et al.*, 1990), hepatic necrosis (Hewett *et al.*, 1991) and lethality (Beutler *et al.*, 1985; Bodmer *et al.*, 1993). TNF-α stimulates the expression of adhesion molecules on the endothelium and on neutrophils (Klebanoff *et al.*, 1986), enhancing the accumulation of inflammatory cells within the microvasculature of various organs (Salyer *et al.*, 1990; Vadas and Gamble, 1990). TNF-α also stimulates these cells to produce various cytokines (IL-1, IL-6, IL-8) and provokes in Kupffer cells the induction of nitric oxide synthase (NOS). NOS activation is followed by a steady production of nitric oxide (NO). Endotoxin also induces the release of platelet activating factor (PAF), leukotrienes and prostaglandins. PAF in turn can activate platelets and neutrophils, initiating the release into the circulation of vasoactive amines. This contributes to vasodilation and increased vascular permeability.

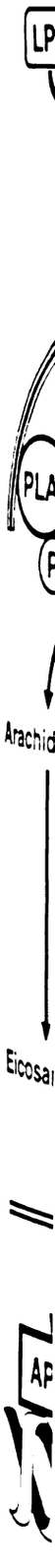
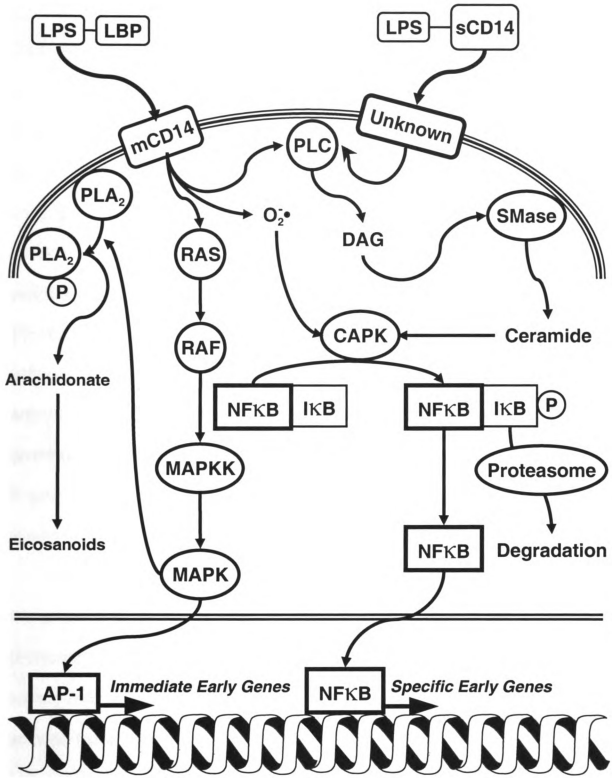


Figure 1.4. Signalling Pathways of Cell Activation in Response to LPS. mCD14, membrane-bound CD14 receptor; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; sCD14, soluble CD14 receptor; PLC, phospholipase C; DAG, diacylglycerol; SMase, sphingomyelinase; CAPK, ceramide-activated protein kinase; NFκB, nuclear factor kappa-B; IκB, inhibitory element kappa-B; MAPKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; PLA_2, phospholipase A_2. Modified from (Decker, 1997).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Experimental (Bengtsson *et al.*, 1993) and clinical (Bone, 1992c; Parsons *et al.*, 1989; Duchateau *et al.*, 1984) observations demonstrated that LPS is capable of directly activating the complement system. Clinical studies show decreased levels of C_3 and C_4 in patients undergoing shock during gram-negative bacteremia, with concurrent increases in products of complement degradation (Sprung *et al.*, 1986). LPS activates both the classical and alternate pathways of complement via a mechanism independent of immune-complex formation (Morrison and Kline, 1977). The O-antigen polysaccharide appears to activate primarily the alternate pathway, while lipid A activates the classical pathway by interacting directly with C_1 (Mey *et al.*, 1994). Because LPS also activates the coagulation cascade It has been suggested that the formation of occlusive fibrin thrombi and subsequent ischemia is responsible for tissue damage observed after LPS exposure (Shibayama, 1987; Nunes *et al.*, 1970).

Finally, by activating neutrophils and Kupffer cells, LPS can lead to the production and release of reactive oxygen products and proteases (Fittschen *et al.*, 1988; Guthrie *et al.*, 1984; Portoles *et al.*, 1994). These compounds can cause tissue damage, which in turn may call in and activate more inflammatory cells. The result is a self-amplifying process of cell destruction and most likely underlies organ injury. The variety of inflammatory mediators released by cells in response to LPS is summarized in Table 1.3.

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Table 1.3 Production of Cytokines, Inflammatory Mediators and Free Radicals by Cells Exposed to LPS. Modified from (Decker, 1997)

Mediators	***Possible Sources***
Peptides	
TNF-α	Kupffer Cells, Endothelial cells, Macrophages
IL-1β, IL-6, IL-8, IL-10	Kupffer Cells, Endothelial cells, Macrophages
Interferon α/β	Kupffer Cells
Transforming Growth Factor α/ β	Kupffer Cells
Catecholamines, endorphins	Neurons
Lipids	
Prostaglandin E_2, D_2, $F_{2\alpha}$,	Kupffer Cells, Endothelial cells, Macrophages, PMNs, Platelets
Prostacyclin (PG I_2)	Kupffer Cells,
Thromboxane A_2	Kupffer Cells, Endothelial cells, Macrophages, PMNs, Platelets
Leukotriene B_4, C_4, D_4, E_4	Kupffer Cells, Endothelial cells, Macrophages, PMNs, Platelets
Platelet Activating Factor	Kupffer Cells, Endothelial cells, Macrophages,
Complement Factors	
$C3_a$, $C4_a$	Plasma
Tissue Factor	Endothelial cells, Macrophages
Clotting Factors	
Thrombin, Protein C	Plasma
Free radicals	
Superoxide anion, Nitric oxide	Endothelial cells, Macrophages, Kupffer Cells, PMNs
Lysosomal Enzymes	
Proteases (Elastase, Cathepsin G)	Kupffer Cells, PMNs

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LPS-mediated Liver Injury

Liver structure

The liver consists mainly of hepatocytes and blood-filled sinusoidal capillaries (Figure 1.5). The liver lobule forms the smallest hepatic structural unit. The central vein lies in the center of the lobule. At the periphery of the lobule can be found the portal triads, comprising a combination of branches from the portal vein, the hepatic artery and the bile ducts. The blood enters the lobule from the portal triads and flows toward the central vein, eventually leaving the liver via the hepatic vein. The hepatic parenchymal cells (hepatocytes) form approximately 65% of the liver cells but account for over 90% of the cytoplasmic volume and for most of the hepatic functions (Guillouzo and Guguen-Guillouzo, 1986). The hepatocytes are arranged in the form of sheets one cell thick, which are fenestrated and branch. The sheets converge on the central vein and the spaces between them are filled with capillaries lined with phagocytic reticuloendothelial cells. Each hepatocyte has two surfaces that face the sinusoids and several others in contact with other cells. Near the center of the surface between two adjacent hepatocytes is formed a cylindrical channel: the biliary canaliculus (Hally and Lloyd, 1969). Bile canaliculi form a branching system in which the bile flows toward the periportal region of the lobule.

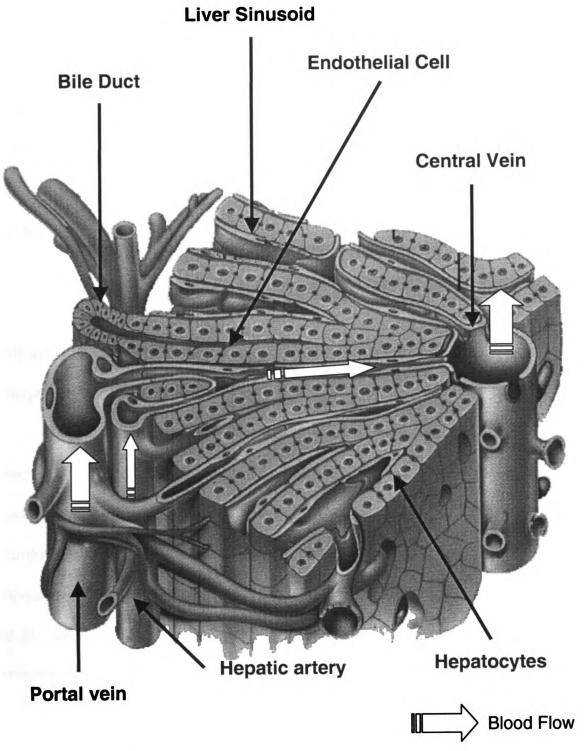

Figure 1.5 The Liver Lobule
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Fenestred endothelial cells lining the sinusoids allow contact between the blood and the hepatocyte surface. The liver resident macrophages, or Kupffer cells, are found also in the sinusoids where they phagocytose particulate debris, dead cells and invading pathogens. The remaining nonparenchymal cell in the sinusoids are the Ito cells (i.e. stellate cells). These contractile cells store fat and vitamin A and can evolve into collagen-producing cells during chronic liver injury (Shiratori *et al.*, 1986; Shiratori *et al.*, 1986).

Hepatic Alterations

Morphologic Changes

Varying degrees of hepatotoxicity have been reported by light and electron microscopy following injection of lethal or near-lethal doses of endotoxin in animal models. Within 15 minutes of intravenous LPS administration, the sinusoids appear congested, the space of Disse is moderately dilated and there is apparent swelling of the Kupffer cells (Levy *et al.*, 1968a). Platelets with intact secretatory granules can be observed within phagocytic vacuoles in the Kupffer cells (Levy *et al.*, 1968a; Levy *et al.*, 1968b). The endothelium shows swelling of the mitochondria and dilatation of the endoplasmic reticulum, associated with some vacuolization. Thirty minutes after exposure, a significant number of neutrophils can be observed within the sinusoids. At three hours, free fibrin

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is clearly visible in the microvasculature along with platelet microthrombi (McKay *et al.*, 1966). Hepatic parenchymal cells show little alteration before 2-4 hours. Some mitochondrial swelling, limited vacuolization and the presence of eosinophilic cytoplasmic granules have been reported (Durham *et al.*, 1990). The first evidence of hepatic parenchymal cell injury can be observed after approximately four hours. Hepatocellular lesions are characterized by multifocal, irregular areas of midzonal hepatocyte degeneration and necrosis. Dying cells appear pale and anucleated or with small pyknotic nuclei and often indistinct cytoplasmic borders (Hewett *et al.*, 1992). These lesions show neutrophil infiltration and mild hemorrhage. The lesions are dose-dependent but a tremendous variability in the response of animals to endotoxin has been observed (Hewett and Roth, 1993). Studies have reported similar injury with widely different doses, perhaps because of differences in the specific activity of the LPS used.

Functional Changes and Markers of Injury

LPS administration produces a transient hyperglycemia followed by hypoglycemia and a progressive depletion of glycogen stores in the liver. The initial hyperglycemia appears related to stimulation of glycogenolysis and release of glucose into the general circulation (Filkins and Buchanan, 1977). This effect is mediated through the release by Kupffer cells of prostaglandin D_2, which stimulates glycogenolysis in the parenchymal liver

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cells (Casteleijn *et al.*, 1988). The delayed hypoglycemic effect of endotoxin, though much less well understood, may be mediated by the inhibition of gluconeogenesis (Utili *et al.*, 1977a).

Studies *In vitro* suggest that LPS alters hepatic lipid metabolism. The addition of endotoxin to hepatocyte cultures increases cellular lipid and enhances lipid secretion into the medium (Victorov *et al.*, 1989). These alterations may explain in part the accumulation of lipids in the liver observed *in vivo* 24 hours after LPS injection (Levy *et al.*, 1968a; Levy *et al.*, 1968b). Endotoxin is also cholestatic, and some episodes of sepsis, noticeably in children, are associated with a significant bilirubinemia in the absence of overt increased activity in the plasma of transaminases. Results *in vitro* suggest that LPS can directly inhibit the secretion of bile into the canaliculi, sometimes refered to as the "salt-independent bile flow" process (Utili *et al.*, 1977b).

Between four and six hours after intravenous administration of LPS to animals, liver enzymes such as alanine aminotransferase (ALT or SGPT) or aspartate aminotransferase (AST or SGOT) are significantly elevated in the plasma, confirming the loss of hepatic parenchymal cell membrane integrity. Although some LPS can be found associated with the hepatic parenchymal cells (Freudenberg *et al.*, 1982), studies *in vitro* have shown that LPS by itself is only slightly toxic to hepatocytes (Wang *et al.*, 1995). LPS however does have a direct effect on liver parenchymal cells. The

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synthesis of LBP by hepatocytes is under the direct control of LPS (Wan *et al.*, 1995), and LPS treatment primes hepatocytes for subsequent responses to LPS, TNF, and IL-1 (Wan *et al.*, 1995). It is therefore possible that LPS exposure predisposes hepatocytes to the toxic effect of other inflammatory mediators.

Critical Mediators of Injury

It is now clear that LPS-induced liver injury is not the result of a direct toxicity of endotoxin to the hepatic parenchymal cell, but rather the result of a complex cascade of inflammatory mediators initiated by the arrival of the bacterial product in the liver circulation.

Animal Models

There are considerable differences among animal models of endotoxemia *in vivo*, depending on species sensitivity, route of administration, duration of exposure, dose administered, bacterial strain and specific activity of the LPS used. The model consistently used in this work consists of a single, intravenous injection of $96x10^6$ endotoxin units per kilogram (EU/kg) to male, Sprague-Dawley rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 250-350g. The specific activity of the endotoxin employed (*Escherichia coli*, serotype 0128:B12, $24x10^6$ EU/mg) was confirmed using a kinetic, chromogenic modification of

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the *limulus* amebocyte lysate (LAL) assay from BioWhittaker (Walkersville, MA). For the sake of clarity, we will limit our description of inflammatory mediators to those shown to play a critical role in this model, as it provides us with a clear time of initial exposure and an evolution that mimics the course of the human response to sepsis. By critical, we imply that inactivation or suppression of this mediator significantly decreases the amount of liver injury observed following administration of LPS as described above. The evolution of the critical markers related to toxicity in our model is described in Figure 1.6.

Kupffer Cells

Kupffer cells are phagocytic cells that contribute to the innate immune response by taking up and killing invading microorganisms as they transit through the liver microvasculature. The Kupffer cell bacterial killing process involves the production of reactive oxygen species by an NADPH oxidase, the action of lysosomal enzymes and the production of various inflammatory mediators such as TNF-α, lipoxygenase products and NO. Kupffer cells are considered to be the "gate keepers" of the portal circulation, filtering out of the blood foreign particles and neoplastic cells coming from the gastrointestinal tract. These cells express the membrane-bound CD14 receptor (Matsuura *et al.*, 1994) and respond to the binding of the LPS-LBP complex to this receptor through a cascade of intracellular

Figure 1.6. Temporal Effect of LPS Administration on Critical Mediators of Liver Injury. LPS ($96x10^6$ EU/kg) was administered intravenously at 0 hr. Results are normalized to the maximal value of each parameter, where a value of 1 (± standard error of the mean) corresponds to the following: 1253 ± 106 U/L for plasma ALT activity, 56.8 ± 3.7 percent of injected radioactivity for hepatic platelet accumulation, 388 ± 30 cells/mm^2 for neutrophils per area of liver, 160 ± 9.8 mg/dl for plasma fibrinogen concentration and 95.6 ± 13 ng/ml for plasma TNF-α concentration. Reproduced from (Pearson *et al*, 1995).

Figure 1.6

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messengers resulting in the increased uptake of LPS and the secretion of TNF-α, IL-1β, IL-1α, IL-6 and arachidonic acid metabolites (Callery *et al.*, 1992; Chensue *et al.*, 1991; Kawada *et al.*, 1992). Kupffer cells are presumably the first cell exposed to bacteria or endotoxin derived from the gut, and several studies demonstrate their critical involvement in the mechanism of liver injury. For example, inhibition of Kupffer cell function by gadolinium chloride ($GdCl_3$), a toxic compound forming insoluble precipitates phagocytosed by the macrophages (Dean *et al.*, 1988; Hardonk *et al.*, 1992; Roland *et al.*, 1996), attenuates the hepatotoxicity induced by LPS administration (Brown *et al.*, 1997; Iimuro *et al.*, 1994). Similarly, the increased number of activated macrophages in the liver after pretreatment with *Corynebacterium parvum* is associated with a dramatic increase in LPS hepatotoxicity (Arthur *et al.*, 1986).

Kupffer cells can release numerous cytotoxic mediators, and these have been suggested to mediate directly some of the hepatic parenchymal cell damage. Protein synthesis is inhibited in cultured hepatocytes in the presence of Kupffer cells (Billiar *et al.*, 1989a). This effect was later linked to activation of NOS in hepatocytes by a Kupffer cell factor (Billiar *et al.*, 1989b; Billiar *et al.*, 1989c; Freeswick *et al.*, 1994). However, direct inhibition of NOS during endotoxemia did not protect against LPS-induced liver damage, showing that *in vivo*, the mechanism of injury did not depend on NO production (Harbrecht *et al.*, 1992). Kupffer cells release other

inflammatory mediators, most notably the TNF-α (Chensue *et al.*, 1991) that rises after approximately 30 minutes of exposure to endotoxin (Michie *et al.*, 1988). Kupffer cells are considered by some to be the major source of TNF-α, yet inhibition by $GdCl_3$, although protective against liver damage, did not reduce the increase in TNF-α in plasma (Brown *et al.*, 1997).

Endothelial Cells

The endothelial lining of the vasculature was once seen as a passive barrier structure. Endothelial cell death caused by the inflammatory reactions associated with endotoxemia was held responsible for the increased permeability to fluid and macromolecules and the exposure of thrombogenic subendothelial surfaces (Gaynor *et al.*, 1970; Alper *et al.*, 1967). It is, however, now clear that the appearance of endotoxin in the microcirculation induces hepatic sinusoidal endothelial cell changes that promote inflammation (Pugin *et al.*, 1993; Deaciuc and Spitzer, 1996; Spolarics *et al.*, 1996) and coagulation (Arai *et al.*, 1995) long before any actual cell death.

Following LPS administration, investigators have reported accumulation of leukocytes in the vasculature, congestion of red blood cells in vessels, intense arteriolar constriction followed by extreme vasodilation and slowing of blood flow (Mazzoni and Schmid-Schonbein, 1996), hemorrhagic necrosis, congestion, edema and fibrin deposition in

the capillaries (McKay *et al.*, 1966). The change in vascular permeability is characterized by a continuous fluid transfer toward the extravascular compartment. This leakage affects organs differently and is independent of hydrostatic pressure (Mazzoni and Schmid-Schonbein, 1996). It has been linked to the loss of negative charges on the basement membrane and the separation of the tight junctions between endothelial cells, increasing the size of vascular pores (Gotloib *et al.*, 1992). At the same time, adherence of white blood cells to the surface of the capillaries slows blood flow and increases capillary pressure (Deaciuc and Spitzer, 1996). This effect is further enhanced by changes in viscoelastic properties of the red blood cell membrane in response to endotoxin (Hinshaw, 1996) which augment the time necessary for the erythrocytes to pass through capillary beds, and the opening of arterio-venous shunts which deprive the vascular beds of circulation (Piper *et al.*, 1996). The overall effect is hypoperfusion of the liver and gastrointestinal tract despite an increase in cardiac output (Mazzoni and Schmid-Schonbein, 1996). These findings have been attributed to direct and indirect effects of LPS on the endothelium (Pugin *et al.*, 1995).

The endothelial cells do not express the mCD14 receptor, but are activated by the binding of the sCD14-LPS complex to the cell membrane (Frey *et al.*, 1992; Arditi *et al.*, 1993; Pugin *et al.*, 1993). The second messenger system shares a common pathway with the one used by the

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IL-1 and TNF-α receptors and involves the ceramide-dependent activation of NFκB described previously (Witte *et al.*, 1989; Haimovitz-Friedman *et al.*, 1997a).

The consequences of endothelial activation are numerous. Endothelial cells release a vast array of soluble mediators including PAF, PG-I_2, PG-E_2, PG-$F_{2\alpha}$, plasminogen activating factor, IL-1, IL-6, IL-8, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF) (Mazzoni and Schmid-Schonbein, 1996). The endothelial cells alter their surface proteins. Various adhesion molecules such as P-selectin, intercellular adhesion molecule-1 (ICAM-1), endothelial-leukocyte adhesion molecule (ELAM-1) and vascular adhesion molecule (VCAM-1) are expressed on the cell membrane (Pohlman and Harlan, 1997). P-selectin expression is a key element responsible for neutrophil accumulation since antibodies directed against the protein completely block neutrophil infiltration in the sinusoids (Coughlan *et al.*, 1994). Simultaneously, the surface expression of tissue factor (TF) activates the extrinsic pathway of coagulation (Taylor, 1996). In concert with increased procoagulant properties, fibrinolytic activity is reduced by expression of tissue plasminogen activator inhibitor-1 (PAI-1). This activation of the endothelium in multiple vascular beds in the liver and other organs creates a large surface for initiation of coagulation, possibly leading to uncontrolled

coagulation and consumption of clotting factors.

The endothelium also responds to LPS by changes in cell shape involving increased fenestration, retraction of the cells and modifications of the basal membrane (Gotloib *et al.*, 1992). In addition, recent studies have shown that LPS exposure in conjunction with TNF-α was able to initiate programmed cell death in endothelial cells through a ceramide-mediated pathway (Eissner *et al.*, 1995; Haimovitz-Friedman *et al.*, 1997b). It is reasonable to suggest that the generalized activation of microvascular endothelium may account in part for the circulatory failure that is a major factor in the progression of the endotoxin response toward shock.

The Neutrophil

Polymorphonuclear phagocytes (PMNs) are components of the blood leukocyte cell population and normally form 20 to 30% of the total white blood cell population in the rat. They are critically involved in the defense of the host against bacteria, and their importance is clearly demonstrated by the increased susceptibility to infections in patients with genetic neutrophil deficiency (Tauber, 1981; Ricevuti and Mazzone, 1987; Rebora *et al.*, 1980; Bogomolski-Yahalom and Matzner, 1995).

The response of the neutrophils to Gram-negative bacterial infection involves a complex sequence of events resulting in the accumulation of the PMNs at the site of infection and the subsequent release of cytotoxic

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products (Smith, 1994). This normally requires four major process: chemotaxis, adherence, diapedesis and cell activation (Jaeschke *et al.*, 1996). Chemotaxis directs the PMNs toward the site of infection, following concentration gradients of inflammatory mediators. Diapedesis, or migration of the neutrophils from blood vessels into the infected tissue, usually starts with the expression of P-selectin on the surface of the endothelium. The P-selectin adhesion molecule interacts with L-selectin on the surface of the PMNs, slowing down the cells and making them "roll" on the surface of blood vessels (Essani *et al.*, 1998). As the β2-integrins (CD11b/CD18) on the PMNs come in contact with ICAM-1 adhesion molecules on the endothelium, the neutrophils stop and become firmly adhered to the vessel wall (Jaeschke *et al.*, 1991). After that stage, the PMNs start extending pseudopodia in the direction of the chemotactic agent, which can be bacterial peptides (e.g. N-formyl-methionyl peptides), complement components (e.g. C5a), leukotrienes (e.g. LTB_4) and/or cytokines (e.g. TNF-α, IL-1, IL-8) (Gruler, 1989; Zigmond, 1978). Neutrophils then migrate through the endothelium inside the tissue and become activated, for example as the result of the binding of LPS to the mCD14 receptor. The activation of the CD14 signal transduction pathway in humans leads to the release of reactive oxygen products (H_2O_2), nitric oxide and arachidonic acid metabolites. It triggers simultaneously the fusion of the alpha and dense granules in the PMNs with the plasma

membrane and the release in the vicinity of the target cell of the lysosomal contents. The combination of reactive oxygen species and proteases such as cathepsin G and elastase lyses the invading bacteria (Kubes *et al.*, 1993). The debris can then be phagocyted by the neutrophils .

Even through the neutrophils are clearly beneficial in the defense against bacteria, their action can damage host cells as well (Anderson *et al.*, 1991; Elgebaly *et al.*, 1984; Johnson and Ward, 1982). Perhaps the most compelling evidence of the ambiguous role played by neutrophils in the mechanism of LPS-induced liver injury is that neutrophil depletion with antibodies completely abolishes hepatic injury following endotoxin administration (Hewett *et al.*, 1992; Jaeschke *et al.*, 1991). The paradox is that neutrophils are necessary to limit the growth of gram-negative bacteria, and yet they contribute significantly to the pathogenesis of tissue injury associated with the infection (Smith, 1994).

The mechanism by which neutrophils accumulate in the liver is not entirely clear, but it appears related to the direct effect of LPS on endothelial cells and the expression of adhesion molecules (Jaeschke *et al.*, 1996; Jaeschke and Smith, 1997a). Similarly, the exact mechanism of activation once the PMNs have entered the liver parenchyma is poorly understood. It has been hypothesized that the expression of ICAM-1 in response to endotoxin on the surface of the hepatocytes, where it is normally absent, could result in the binding of PMNs to the hepatic

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parenchymal cells and activation (Fujita *et al.*, 1994; Kvale and Brandtzaeg, 1993). In support of this theory, blockage of ICAM-1 with an antibody did protect against LPS-induced liver injury but did not inhibit the hepatic accumulation of neutrophils (Essani *et al.*, 1995). Many other inflammatory mediators released during LPS exposure such as TNF-α can prime neutrophils and thereby enhance superoxide generation and degranulation (Klebanoff *et al.*, 1986). Reactive oxygen species produced during the oxidative burst might initiate lipid peroxidation and damage the hepatocyte membrane (Jaeschke and Smith, 1997b). Indeed, the administration of antioxidants such as catalase, superoxide dismutase and α-tocopherol reduces liver damage in endotoxin-treated mice (Sugino *et al.*, 1987) and rats (Lalonde *et al.*, 1997; Suntres and Shek, 1996). In addition, neutrophils can kill hepatocytes in culture through the release of cathepsin G and elastase (Ganey *et al.*, 1994; Sauer *et al.*, 1996; Ho *et al.*, 1996), suggesting that products released during degranulation could also participate in liver injury.

The Platelet

Platelets prevent bleeding when the integrity of the blood vessel wall is disrupted. Upon activation, these small cells degranulate, project pseudopodia, express P-selectin and produce vasoactive, pro-inflammatory products and epithelial growth factors. The receptors P-

selectin and integrin GPIIIa expressed on the activated cell surface create platelet aggregates which plug sites of damaged endothelium. However, these platelet clumps can also be released and produce thromboemboli in blood vessels. Platelets are able to modulate neutrophil responses and functions. They increase PMN aggregation in response to N-formyl-met-leu-phe peptide (fMLP) or activated complement (Redl *et al.*, 1983) and increase neutrophil-mediated bacterial killing. Platelets stimulate superoxide production by PMN in response to thrombin (Moon *et al.*, 1990). The primary agonists for platelet activation include thrombin (Davey and Luscher, 1967; Martin *et al.*, 1975) which is formed after activated endothelium or platelets convert the prothrombinase complex (Billy *et al.*, 1997), and cathepsin G released by neutrophil degranulation (LaRosa *et al.*, 1994; Molino *et al.*, 1995).

Several animal studies suggest a role for platelets in the mechanism of LPS-induced liver injury, and platelet trapping is considered an early sign of shock and multiorgan failure in SIRS patients (Sigurdsson *et al.*, 1992). Within two hours of intravenous endotoxin injection in the rat, blood platelet number decreases significantly (Pearson *et al.*, 1995). LPS causes a rapid, transient accumulation of platelets into the lungs, followed shortly by a redistribution of the cells toward the liver vasculature (Gutmann *et al.*, 1987; Sostman *et al.*, 1983).

Electron microscopic examination demonstrates platelet accumulation within the sinusoids and in Kupffer cell phagocytic vesicles (McKay *et al.*, 1966). Depletion of circulating platelets with antibodies attenuates liver damage and prevents the initiation of coagulation cascade (Kramer and Muller-Berghaus, 1977; Pearson *et al.*, 1995). The depletion treatment had no effect on neutrophil accumulation in the liver. Similarly, blockage of the coagulation cascade by heparin prevented the onset of liver damage, but it had no effect on either platelet or neutrophil accumulation (Pearson and Roth, 1995).

Activated platelets produce cyclooxygenase metabolites and lipid mediators. Studies conducted with PAF receptor antagonists and 5-lipoxygenase inhibitors showed no effects on liver injury (Pearson *et al.*, 1997). Blockage of the cyclooxygenase pathway attenuated LPS-induced hepatocellular damage, but the thromboxane synthase inhibitor dazmegrel had no effect (Pearson *et al.*, 1996). These results demonstrate the importance of platelets in the pathogenesis of endotoxin-mediated liver injury, however, the exact role of these cells is still poorly understood.

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LPS and the coagulation system

Various studies have documented the activation of the coagulation system either in septic patients (Mammen, 1998b; Penner, 1998; Vervloet *et al.*, 1998; Aihara *et al.*, 1997; Bick, 1996; Gando *et al.*, 1996; Bell, 1994; Colman, 1994; Marder *et al.*, 1994; Bell, 1993; Levi *et al.*, 1993; Gando *et al.*, 1992; Prager *et al.*, 1979; Allen and Glottzer, 1964) or following LPS administration in animal models (Kruithof *et al.*, 1997; Taylor, Jr. *et al.*, 1991; Freund *et al.*, 1990; Ito *et al.*, 1990; Emerson, Jr. *et al.*, 1987; Yoshikawa *et al.*, 1984; Yoshikawa *et al.*, 1983; Yoshikawa *et al.*, 1981; Ishikawa *et al.*, 1980; Nowak and Markwardt, 1980; Margaretten *et al.*, 1967). Evidence for activation of the coagulation system includes an increase in prothrombin and partial thromboplastin times (Gurewich *et al.*, 1976; Yoshikawa *et al.*, 1981) and a decrease in plasma fibrinogen concentration (Prager *et al.*, 1979; Hewett and Roth, 1995; Pearson *et al.*, 1995), likely resulting from the proteolytic action of thrombin. Additional evidence includes the appearance of fibrin thrombi in the microvasculature of the kidneys, lungs, and liver (Levy *et al.*, 1968a; Koth *et al.*, 1980; Yoshikawa *et al.*, 1981) and elevations in fibrin degradation products following exposure to gram-negative bacterial LPS (Gomez *et al.*, 1989). In the rat model of LPS-induced liver injury employed in our laboratory, circulating plasma fibrinogen concentration falls within 2 to 3 hours after intravenous injection of LPS. This is accompanied by the appearance of

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fibrin clots within the liver sinusoids (Pearson *et al.*, 1995).

The importance of this activation of the coagulation system in the pathogenesis of liver injury (Mammen, 1994) is further emphasized by the ability of anticoagulants to abolish hepatotoxicity in septic patients (Mammen, 1998b; Inthorn *et al.*, 1997; Fuse *et al.*, 1996; Gabriel, 1994; Fourrier *et al.*, 1993; Bone, 1992b) and animal models. In rats, pretreatment with warfarin or heparin prevents the decrease in plasma fibrinogen concentration and offers complete protection against LPS-induced liver injury (Margaretten *et al.*, 1967; Hewett and Roth, 1995), despite the fact that other markers of LPS activity, such as increased TNF-α in the plasma and accumulation of neutrophils and platelets in the sinusoids, are still present (Pearson *et al.*, 1995). The observation of the protective effect of anticoagulant drugs as well as the decrease in plasma fibrinogen and the accumulation of platelets in the liver that normally follow LPS administration suggest that the coagulation system is intricately linked to the hepatotoxicity (Pearson *et al.*, 1994). This was further confirmed by the ability of thrombin to mimic LPS toxicity when infused in the portal vein (Shibayama, 1987).

The mechanism usually suggested to explain the involvement of the coagulation cascade in tissue injury is the formation of insoluble fibrin clots in the microvasculature (Suzuki *et al.*, 1988; Shibayama *et al.*, 1991). By blocking blood flow to certain areas of the liver for example, the

platelet-fibrin clots are hypothesized to create ischemic conditions, hypoxia and the resultant death of hepatocytes. However, pretreatment with ancrod resulted in an 89% decrease in plasma fibrinogen and yet did not prevent LPS-induced liver injury (Hewett and Roth, 1995). Ancrod is a thrombin-like enzyme extracted from the venom of the Malayan pit viper (*Agkistrodon rhodostoma*). It cleaves fibrinopeptide A but not fibrinopeptide B from fibrinogen (Pizzo *et al.*, 1972; Chang and Huang, 1994). Because ancrod does not activate Factor XIII, it does not lead to the formation of normal insoluble fibrin clots as does thrombin (van Pelt-Verkuil *et al.*, 1989; Cole *et al.*, 1993). The cleavage products are rapidly cleared from the vasculature, effectively preventing further clot formation without inhibiting any other components of the coagulation system (e.g. thrombin) (Berglin *et al.*, 1976; Bell, 1982). Pretreatment with ancrod did not protect against LPS-induced liver necrosis, suggesting that the role of the coagulation system is independent of the circulating fibrinogen level and the ability to generate insoluble fibrin clots and that the decrease in plasma fibrinogen concentration observed after LPS administration is merely a marker of the activation of a system responsible for the onset of liver injury.

The recent demonstration that hirudin, a small peptide with specific antithrombin activity, could prevent the onset of injury up to two hours after LPS administration pointed strongly to the involvement of thrombin as critical and distal mediator of the toxicity (Pearson *et al.*, 1996). Heparin

and warfarin do inhibit thrombin, but along with other elements of the coagulation cascade (Fasco and Principe, 1982; Tyrrell *et al.*, 1995). By contrast, hirudin has been shown to bind specifically to the active site of thrombin to prevent its cleaving activity (Fenton *et al.*, 1991). Thus, the demonstration that hirudin protects against LPS-induced liver injury, together with the observation that ancrod had no protective effect, pointed strongly toward an involvement of thrombin independent of its ability to cleave fibrinogen and form insoluble clots. This was further emphasized by the ability of antithrombin III, the physiologic inhibitor of thrombin, to reduce the symptoms and improve the survival rate of patients with septic shock (Fourrier *et al.*, 1993; Fuse *et al.*, 1996). Thrombin appears to be an important mediator of the LPS-induced hepatic damage that acts after the accumulation of inflammatory cells in the liver and the release of certain inflammatory mediators. However, the mechanisms of its action and how it interacts with other cellular or soluble mediators remain to be elucidated.

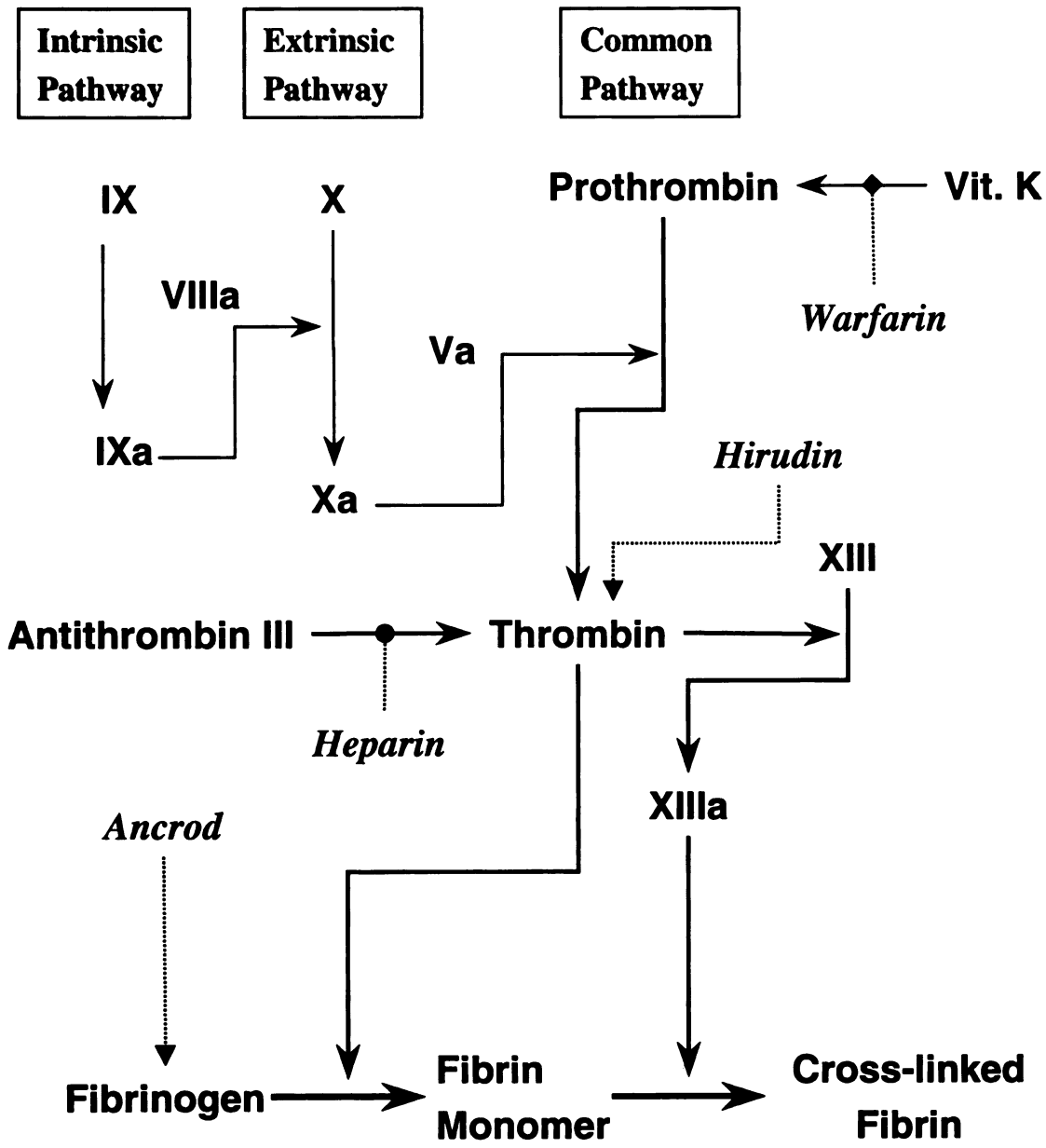

Figure 1.7 The Coagulation Cascade and its Blockage

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Thrombin and LPS-induced Liver Injury.

Thrombin Formation

Thrombin is not a normal constituent of the blood. It is generated by the catalytic cleavage of its plasma precursor, prothrombin (FII), by activated Stuart factor (FXa). Activation of FX to FXa is catalysed either by the complex of FIXa and FVIIIa or by the complex of tissue factor and FVIIa on membrane surfaces. FXa and its cofactor FVa form on the surface of platelets and endothelial cells the prothrombinase complex, converting prothrombin to thrombin (see Figure 1.7).

Structure of Thrombin

Thrombin is a glycoprotein formed by two peptide chains of 36 and 259 aminoacids linked by disulfide bonds. Three important sites have been identified on the surface of the enzyme (Tulinsky, 1996): The *catalytic site* confers to the molecule its serine protease activity, *exosite I* is responsible for the binding of the substrate (fibrinogen or thrombin receptor) and the *exosite II* is responsible for the binding of antithrombin III (Fenton and Bing, 1986).

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Clot Formation

The earliest identified function of thrombin is the cleavage of fibrinogen into fibrin monomers and the activation of the fibrin-stabilizing factor (factor XIII) and protein C (Davie and Ratnoff, 1964). Clotted blood is a meshwork of insoluble fibrin threads that traps blood cells. Thrombin has the property of activating factor XIII to form covalent links between the carboxyl and amino groups of two different fibrin monomers, creating an insoluble meshwork of fibrin strands and enhancing the strength of the clot (Glusa, 1992).

Platelet activation

Thrombin is, however, more than a simple plasma enzyme. It is the most potent stimulator of platelets (Davey and Luscher, 1967; Shuman, 1986) causing them to spread, aggregate and release the contents of their alpha and dense granules (Holmsen and Day, 1970; Venturini and Kaplan, 1992). The components released by the granules modify vascular tone and promote further platelet aggregation (MacIntyre *et al.*, 1977). The stimulated platelets also present a procoagulant surface which promotes assembly of the prothrombinase complex and subsequent formation of active thrombin (Bevers *et al.*, 1991). In addition, platelets can also protect factors XIa and Xa from proteolytic inactivation (Walsh, 1981; Walsh *et al.*,

1993; Walsh and Griffin, 1981), thereby prolonging the activity of that complex. They also express receptors that promote platelet adhesion to endothelium and the subendothelial matrix, as well as to other platelets and fibrin in forming a platelet thrombus (Marsh Lyle *et al.*, 1995).

Thrombin binding to platelets displays specificity and affinity typical for receptor interaction (Tollefsen *et al.*, 1974). Two types of binding sites were described: a small number of high-affinity sites and a larger number of low-affinity sites. Studies with proteolytically inactivated thrombin showed that the catalytic site was not required for the binding of the enzyme to platelets, but that this site was necessary for activation of the platelets (Martin *et al.*, 1975; Davey and Luscher, 1967). A specific thrombin receptor was then cloned, revealing a novel member of the seven transmembrane domain receptor family closely resembling receptors for small peptides like substance P (Vu *et al.*, 1991; Chen *et al.*, 1994).

Endothelial Cells Activation

Thrombin also has numerous effects on other cells that are involved in the development of LPS-induced liver injury: For example, it alters the synthesis, expression and release of proteins from endothelial cells. This results in increased production of PAF (Prescott *et al.*, 1984), fibronectin (Galdal *et al.*, 1985a), thromboplastin (Galdal *et al.*, 1985b), tissue-type plasminogen activator (Levin *et al.*, 1984), von Willebrand factor (Hattori *et*

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al., 1989), and adenine nucleotides (Pearson and Gordon, 1979). Some of these agents (eg. PAF) are unnecessary for LPS-induced hepatocellular injury (Pearson *et al.*, 1997) and others (eg, thromboplastin, tissue-type plasminogen activator) are probably involved primarily in the formation of thrombin.

Expression of Adhesion Molecules

One of the important actions of thrombin is its ability to promote adhesion of PMNs to endothelial cells (Watanabe *et al.*, 1991; Lorant *et al.*, 1991; Zimmerman *et al.*, 1985; Zimmerman *et al.*, 1986; Carveth *et al.*, 1992). The enzyme stimulates a rapid translocation of P-selectin from storage granules and upregulation of ICAM-1 on the endothelium, resulting in the transient, reversible adherence of neutrophils to endothelial cells (Sugama *et al.*, 1992). This effect was shown to occur through activation of the thrombin receptor (Sugama and Malik, 1992) and to be P-selectin-dependent (Sugama and Malik, 1992; Lorant *et al.*, 1993). However, P-selectin is not expressed on sinusoidal endothelial cells of the liver and is not induced on these cells after endotoxin injection (Essani *et al.*, 1995; Essani *et al.*, 1998). Moreover, P-selectin is not required for LPS-induced liver injury (Essani *et al.*, 1998), and modulation of P-selectin expression by thrombin probably does not contribute to LPS-induced liver injury. By contrast, ICAM-1 and VCAM-1 are expressed on hepatic sinusoidal

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endothelial cells and are elevated during sepsis (Sessler *et al.*, 1995) and important for LPS-induced liver injury. Inactivation of these adhesion molecules with antibodies inhibits PMN transmigration from the sinusoid into the parenchyma and attenuates LPS-induced liver injury (Essani *et al.*, 1997). Thrombin can induce the expression of ICAM-1 on endothelial cells *in vitro* (Sugama *et al.*, 1992; Nagy *et al.*, 1996; Ishizuka *et al.*, 1994). These results suggest that thrombin may contribute to LPS-induced liver injury by maintaining the expression of endothelial adhesion molecules or by enhancing TNF-α and IL-1-induced expression of endothelial adhesion molecules.

Neutrophil transmigration

Thrombin induces chemotaxis in human neutrophils (Bizios *et al.*, 1986) and promotes the release of inflammatory components (Bar-Shavit *et al.*, 1983a; Cohen *et al.*, 1991). Catalytically inactive thrombin and thrombin lacking the fibrinogen binding site have similar effects, suggesting that the chemotactic properties of thrombin require neither cleavage nor substrate binding (Bizios *et al.*, 1986). Hirudin and antithrombin III do, however inhibit the chemotaxis (Morin *et al.*, 1990; Morin *et al.*, 1991), suggesting a mediation through the binding of Exosite II.

Thrombin induces neutrophil transendothelial migration. Intradermal injection of thrombin *in vivo* triggers local neutrophil accumulation and can

act synergistically with the chemoattractant effects of TNF-α or IL-1α (Drake and Issekutz, 1992).(Moser *et al.*, 1990) and enhances PMN transmigration stimulated by IL-1α or TNF-α through human umbilical vein endothelial cells (Drake and Issekutz, 1992). It has been proposed that interaction between the integrin adhesion molecules on PMNs (Mac-1, LFA-1, and VLA-4) with ligands on the sinusoidal endothelial cells (ICAM-1 and VCAM-1) is required for transendothelial migration of PMNs in the liver (Jaeschke and Smith, 1997a). By promoting the transmigration of PMNs from the liver sinusoids into the tissue in addition to increasing the expression of adhesion molecules, thrombin could mediate neutrophil-dependent liver injury. The observation that infusion of thrombin into the portal vein produces liver injury which is associated with the infiltration of PMNs into the liver (Shibayama, 1987) is consistent with this hypothesis. Although this study did not determine if the injury produced by thrombin was dependent upon PMNs, the lesions produced by thrombin were very similar to LPS-induced liver injury which is PMN-dependent (Jaeschke *et al.*, 1991; Hewett *et al.*, 1992).

Interactions with Neutrophils

There are complex interactions between thrombin and the neutrophils. First neutrophils are able to bind FXa and convert prothrombin to thrombin. A receptor for FXa, called effector cell protease receptor-1

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(EPR-1) has been identified on their surface (Altieri and Edgington, 1990), and binding of FXa to EPR-1 induces procoagulant ability (Gillis *et al.*, 1997). Althrough thrombin binds specifically and with high affinity to neutrophils (Sonne, 1988), the thrombin receptor has not been identified on these cells. On the other hand, it has been reported that cathepsin G, a serine protease released during neutrophil activation, has the ability to activate the thrombin receptor on platelets and endothelial cells (Molino *et al.*, 1995). This suggests that neutrophil activation at the site of injury may mimic or amplify the effect of thrombin on various cellular mediators of liver injury. Finally, thrombin appears capable to modulate the inflammatory response of neutrophils: incubation of sheep neutrophils with thrombin resulted in the generation of thromboxane (Bizios *et al.*, 1987), and thrombin can promote the synthesis and release of kallikrein from human neutrophils (Cohen *et al.*, 1991). Thrombin has also been shown to promote the release of superoxide anions *in vitro* when added to a suspension of neutrophils (Moon *et al.*, 1990) in the presence of platelets.

Interactions with Macrophages

Thrombin is a potent chemotaxin for macrophages and can alter their production of cytokines and arachidonic acid metabolites (Bar-Shavit *et al.*, 1983b). Little is known of thrombin action on the liver resident macrophages (Kupffer cells), but high affinity binding sites for thrombin

have been identified on rat Kupffer cells (Kudahl *et al.*, 1991). However, studies have shown that the thrombin receptor is not expressed on Kupffer cells in human livers (Marra *et al.*, 1998). Many of the factors secreted by Kupffer cells that are necessary for LPS-induced liver injury such as TNF-α, IL-1, and IL-6 can be elicited directly by LPS (Decker, 1990). Although there has been no reported effect of thrombin on cytokine secretion from Kupffer cells, the enzyme is only a weak stimulant for IL-6, and TNF-α secretion from monocytes (Kranzhofer *et al.*, 1996). In addition, after endotoxin administration the plasma levels of these factors (TNF, IL-1, IL-6) increase prior to activation of the coagulation pathway (Ohira *et al.*, 1995), and some of the factors secreted from macrophages after injection of LPS are actually required for activation of the coagulation system during endotoxemia (van der Poll *et al.*, 1994 and 1997). This suggests that the interactions between thrombin and Kupffer cells may play a limited role in the mechanism of liver injury.

Interactions with Hepatocytes

The direct effect of thrombin on hepatic parenchymal cells is poorly understood. High-affinity binding sites for thrombin have been identified on primary rat hepatocytes (Weyer *et al.*, 1988), but such binding has not been associated with any particular functional change. It has been suggested that chemotactic factors derived from hepatocytes during

endotoxemia are necessary to promote transendothelial migration of neutrophils from the liver sinusoids into liver tissue and for adhesion-dependent killing by PMNs (Jaeschke and Smith, 1997b). After hepatic ischemia-reperfusion in rats, inhibition of the coagulation cascade attenuated the production of chemotactic factors (Yamaguchi *et al.*, 1997) and inhibited PMN activation and liver injury. Therefore, thrombin might play a role during endotoxemia by stimulating the release of chemotactic molecules from hepatocytes necessary for the transmigration of PMNs into the hepatic parenchyma.

In conclusion, thrombin has numerous effects on many of the mediators critically involved in the mechanism of LPS-induced liver injury (Table 1.4). First, it converts fibrinogen into fibrin, leading to the formation of vascular clots. Secondly, thrombin activates blood platelets, causing aggregation and secretion, as well as neutrophils, macrophages and endothelial cells. Thirdly, thrombin modulates the expression of adhesion molecules as well as chemotactic factors involved in the migration of PMNs from the vasculature into the tissue. And finally, thrombin appears to bind with high affinity on each of the cells involved in LPS-induced liver damage, even through the effect of that binding has been poorly characterized. Most of these properties of thrombin rely on its action as serine proteinase, since modifications (either chemically or by mutation) which destroy the catalytic site result in a loss of the biological activity

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(Grand *et al.*, 1996). These findings were recently explained by the characterization of a novel, widely expressed family of cellular receptors which are activated by proteolytic cleavage rather than ligand binding.

Table 1.4 Effects of Thrombin Potentially Involved in the Mechanism of LPS-induced Liver Damage.

Action of Thrombin	*Site of Action*
Promotion of Coagulation	
Cleavage of fibrinogen	*Plasma*
Activation of FXIII	*Plasma*
Assembly of prothrombinase complex	*Platelets, PMNs*
Cell Activation	
Production of PAF, fibronectin, thromboplastin, TPA,Von Willebrand factor, adenine nucleotides	*Platelets*
Degranulation	*PMNs, Platelets*
Stimulation of proinflammatory response	*Macrophages, PMNs*
Cell migration	
Expression of adhesion molecules	*Platelets, PMNs, Endothelial cells*
Aggregation and arrest in the vasculature	*Platelets, PMNs*
Transmigration through endothelium	*PMNs*
Direct chemotaxis	*PMNs, Macrophages*
Release of chemotactic factors	*Hepatocytes*
Cell Death	
Activation of apoptosis	*Astrocytes*

The Protease-activated Receptors

The mechanism by which thrombin activates platelets or other cells was until recently unknown, and in cases for which traditional binding studies had identified thrombin-binding proteins, the signal transduction pathway remained mysterious. In 1991, a thrombin receptor on platelet was cloned (Vu *et al.*, 1991). This revealed a new mechanism of receptor activation. The aminoacid sequence of the human thrombin receptor comprises 425 aminoacids forming seven, helical, hydrophobic, transmembrane domains connected by three intra- and three extra-cellular loops. The C-terminal intracellular tail contains a large number of potential phosphorylation sites, and the long N-terminal extracellular domain contains a thrombin cleavage site near a sequence thought to bind thrombin at the anion-binding exosite-1 (Figure 1.8). The receptor sequence exhibits a number of structural features observed in many other seven-transmembrane domain, G-protein-coupled receptors such as neuropeptide and glycoprotein hormone receptors. After binding to its receptor, thrombin cleaves the extracellular tail between residues Arg-41 and Ser-42, unmasking a new amino terminus with the sequence SFLLRN in humans. The new end functions as the receptor ligand, binding to the second extracellular loop and part of the exodomain located near the start of transmembrane domain 1.

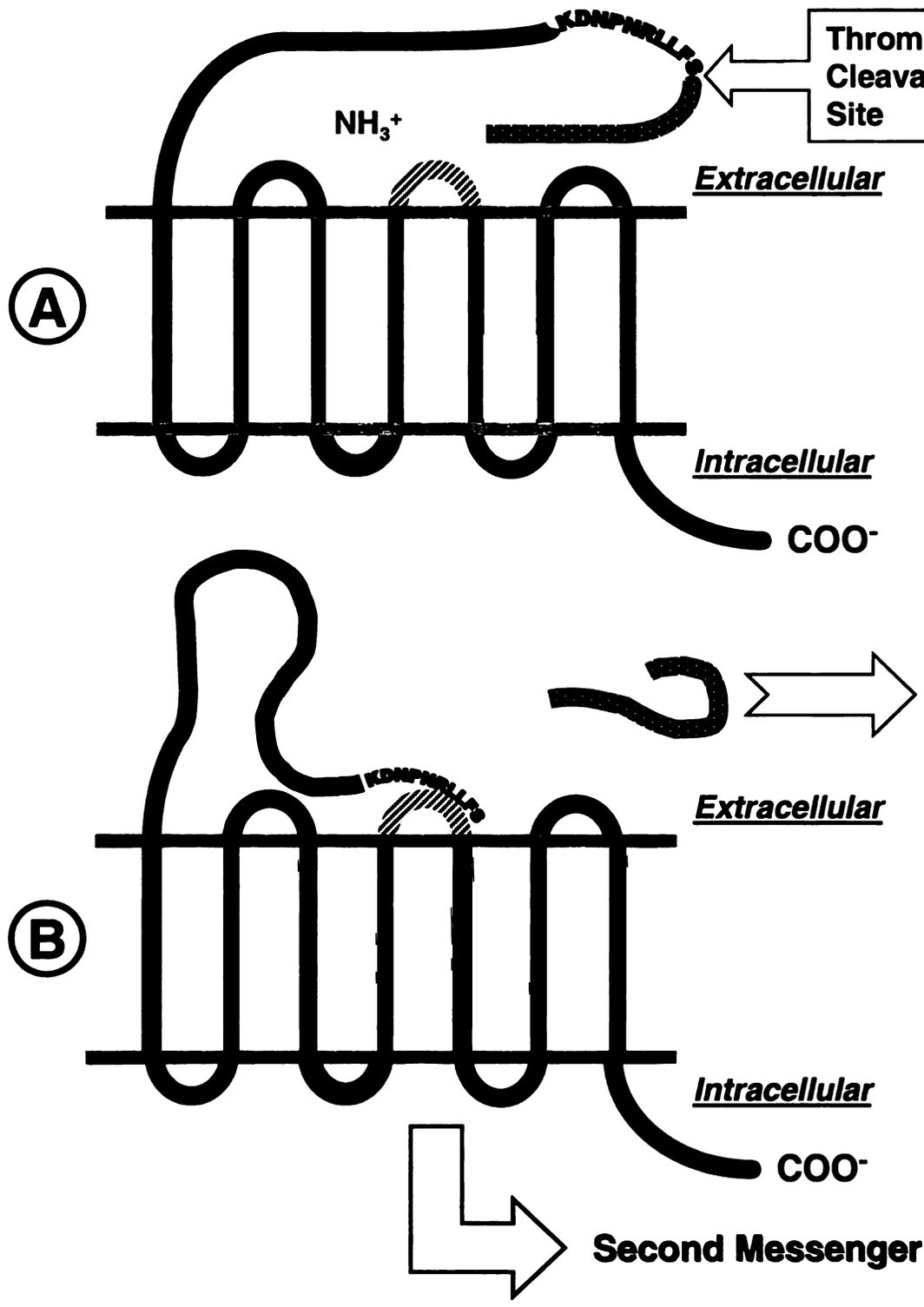

Figure 1.8 The Protease-Activated Receptor-1.
A: quiescent B: activated. ▨ sites of interaction with tethered ligand

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This binding activates a second messenger system. The sequence capable of self-activation was named the "tethered ligand". This receptor was recently renamed protease-activated receptor-1 (PAR-1).

Various peptides similar to the sequence of the tethered ligand have now been synthesized (Gerszten *et al.*, 1994), and their abilities to bind and activate the thrombin receptor have been demonstrated (Uchiyama *et al.*, 1992; Hui *et al.*, 1992). In essence, the agonist peptide bypasses the need for thrombin-mediated receptor proteolysis and activates the receptor independently of thrombin cleavage. These PAR-1 activating peptides, originally called "thrombin receptor activating peptide" (TRAP) act as complete agonists, and amino-acid permutation studies have shown that the minimum sequence required to mimic thrombin effects resided in the first six residues SFLLRN. Neutral, hydrophilic aminoacids such as glycine, alanine, threonine and cysteine can substitute for the serine in position 1 with only limited loss of activity, but an aromatic side-chain in position 2 appears essential for receptor activation, since replacing the phenylalanine with alanine completely destroys the activity. The 14-amino acid peptide SFLLRNPNDKYEPF stimulates human platelet aggregation at concentrations of 10 μM, and shorter peptides of five, six and eight amino acids require larger concentrations (respectively, 50, 25 and 20 μM) to achieve a full aggregation response, (Chao *et al.*, 1992). By comparison, a similar level of activation is achieved at a concentration of 1 nM thrombin

(Lasne *et al.*, 1995). The ability of thrombin or TRAP to activate the PAR-1 receptor has since been demonstrated on cell types other than platelets (Hung *et al.*, 1992).

Following the discovery of the thrombin receptor, a second protease-activated receptor was cloned from a mouse genomic library (Nystedt *et al.*, 1994). Named Protease-activated Receptor-2 (PAR-2), this receptor is activated by trypsin rather than thrombin, but follows the same mechanism of activation. Trypsin cleaves the N-terminal extracellular domain of PAR-2 to generate the tethered ligand SLIGRL. Apparently, in PAR-2 as in PAR-1, the identities of the amino acids following the first five or six are of less importance for peptide interaction. However, in PAR-2, it is the first serine which is essential for signaling by the agonist peptide (SLIGRL/SLIGKV) (Blackhart *et al.*, 1996). Since the PAR-2 agonist peptide shows some homology with TRAP (SLIGRL versus SFLLRN, respectively) and both have a serine in position 1, it has been suggested that these agonist peptides may not be entirely specific for each receptor. Indeed, when receptors expressed in *Xenopus* oocytes were tested for their possible ligands, PAR-1 was found to respond to thrombin and TRAP (SFLLRN), but neither to trypsin nor SLIGRL. However, PAR-2 was shown to respond to trypsin, its agonist peptide SLIGRL and TRAP, but not to thrombin (Lerner *et al.*, 1996; Blackhart *et al.*, 1996). This raises the possibility that some of the cellular effects of TRAP that are not reproduced by thrombin

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may be mediated by the activation of PAR-2 (Jenkins *et al.*, 1995).

Disruption of the gene encoding for the thrombin receptor in knockout mice revealed that platelets from the PAR-1-defective mutant mice still strongly responded to thrombin, whereas the fibroblasts had lost that ability (Connolly *et al.*, 1996). In 1997, a second thrombin receptor, designated protease-activated receptor-3 (PAR-3), was cloned and identified (Ishihara *et al.*, 1997). PAR-3 exhibits 27% amino-acid sequence similarity with PAR-1, and 28% with PAR-2. The extracellular domain of the receptor contains a thrombin cleavage site followed by a sequence identical to the thrombin-binding sequence found on the anticoagulant hirudin. Several observations suggest that PAR-3, like PAR-1, uses the fibrinogen-binding exosite of thrombin for receptor recognition. The receptor exhibits a strong response to thrombin and appears relatively insensitive to other serine proteases such as trypsin, chymotrypsin, elastase and cathepsin G. Thus, PAR-3 appears as specific for thrombin as PAR-1, and the tethered ligand domain (TFRGAP) is required for signalling, with Phe-40 in PAR-3 being analogous to the critical Phe-43 in the tethered ligand of PAR-1 (SFLLRN) (Ishihara *et al.*, 1997). Surprisingly, synthetic peptides mimicking the tethered ligand domain of PAR-3 (TFRGAP or TFRGAPPNS) as well as TRAP have been unable to activate the receptor, raising the possibility of a slightly different activation mechanism (Ishihara *et al.*, 1997).

Finally, in June of 1998, a fourth protease-activated receptor (PAR-4) was identified (Xu *et al.*, 1998) and cloned, joining the family of G protein-coupled receptors activated by proteolysis and binding of a tethered ligand (Coughlin, 1994). The receptor is a seven transmembrane protein of 385 amino acids exhibiting a 33% amino-acid sequence identity with PAR-1,2 and 3. The protease cleavage site appears accessible to either thrombin or trypsin. Moreover, a peptide representing the newly exposed tethered ligand from PAR-4 (GYPGQV) was able to activate PAR-4-transfected cells and caused secretion and aggregation of PAR-3-deficient mouse platelets (Kahn *et al.*, 1998). Since mice do not express the PAR-1 receptor on their platelets, the thrombin response of mouse platelets appears to be mediated by a dual receptor system involving PAR-3 and PAR-4 (Kahn *et al.*, 1998). Studies with PAR-4 activating peptide suggest that PAR-4 is also functional on human platelets, and PAR-4 mRNA was found in a number of human tissues, with especially high levels in the lungs, pancreas, thyroid and small intestine.

Cellular expression and functions

There is evidence that protease-activated receptors exist on most of the cells critical to LPS-induced liver injury. However, the cellular distribution, functions and signaling pathways of those receptors have not yet been well delineated, and significant differences have been noted

between humans and rodents (Connolly *et al.*, 1994). Nevertheless, the presence of these receptors on critical cells and the importance of thrombin in LPS-induced liver injury suggests that activation of these receptors may participate in the mechanism of liver damage. The synthetic peptides which mimic the action of the tethered ligands can provide relatively specific tools to investigate the effects and functions of these receptors.

Incubation of HEP G2 cells, a transformed hepatocyte cell line, with thrombin increased the expression and secretion of plasminogen activator inhibitor type-1 (PAI-1), indicating that thrombin can evoke a functional change in this cell type (Hopkins *et al.*, 1992). A recent study did not find expression of PAR-1 on human hepatocytes from adult livers. However, following either chronic hepatitis or fulminant hepatitis, activated hepatocytes in the areas of regeneration and in culture after a few days displayed a strong immunostaining for PAR-1 (Marra *et al.*, 1998). Therefore, thrombin-induced secretion of PAI-1 from hepatocytes may not be mediated through activation of PAR-1, or expression of the receptor is dependent on the cell state: absent or very low during quiescent state and upregulated during active state. In that respect, the transformed HEP G2 cells may be closer to activated cells. This does not rule out either the possibility that thrombin may act on hepatocytes through a different receptor such as PAR-3 or PAR-4. Indeed, some effects of thrombin on

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other cell types are mediated through specific receptors which are not PAR-1 (Kinlough-Rathbone *et al.*, 1993). Therefore, thrombin may mediate an effect during LPS-induced liver injury through direct interactions with high-affinity receptors on hepatocytes.

Despite the occurrence of the thrombin receptor on certain types of macrophages (Kudahl *et al.*, 1991), Kupffer cells were not specifically identified by the PAR-1 immunostaining (Marra *et al.*, 1998), even through they express high affinity binding sites for thrombin. This does not rule out the possible effect of other protease-activated receptors on these cells or a differential expression of PAR-1 in activated Kupffer cells.

The existence of the PAR-1 receptor was demonstrated on human platelets (Ramachandran *et al.*, 1997; Vu *et al.*, 1991), and in these cells it appears to mediate most of the effects of thrombin (Lau *et al.*, 1994). However, other species such as rodents exhibits response to thrombin that can not be reproduced by TRAP (Connolly *et al.*, 1994), suggesting that the activity of thrombin is not mediated through PAR-1 (Kinlough-Rathbone *et al.*, 1993). Indeed, it was later confirmed that PAR-3 is the main platelet receptor for mice, and that its action is complemented by PAR-4 (Kahn *et al.*, 1998). PAR-3 has now been identified on human platelets (Ishihara *et al.*, 1997), but its role appears secondary.

Endothelial cells express both functional PAR-1 and PAR-2 (Molino *et al.*, 1997). However, only PAR-2 could be strongly induced during inflammatory processes (Nystedt *et al.*, 1996). Human neutrophils also express PAR-2 (Howells *et al.*, 1997) and PAR-3, and the paradoxical response of these cells to TRAP (Jenkins *et al.*, 1995) but not to thrombin has been explained by cross-reactivity between TRAP and the PAR-2 activating peptide (Blackhart *et al.*, 1996; Lerner *et al.*, 1996). For now, the physiological significance of PAR-2 awaits the identification of the endogenous ligand. It is interesting to note that even though PAR-1 has not been identified on isolated neutrophils, in patients with chronic hepatitis, infiltration of portal tracts and lobules was associated with the appearance of inflammatory cells showing strong staining for PAR-1 (Marra *et al.*, 1998). This observation raises the possibility that PAR-1 expression could be upregulated during activation of inflammatory cells.

In conclusion, the protease-activated receptors have been identified on many of the different cells critically involved in the mechanism of LPS-induced liver injury (Table 1.5). However, little is still known of their specific cellular effects and regulation of their expression during inflammatory process.

Table 1.5. Expression of Protease-Activated Receptors on Human Cells Critical to LPS-Induced Liver Injury.

Cellular Localization	PAR-1	PAR-2	PAR-3	PAR-4
Platelets	+	?	+	+
Neutrophils	-	+	?	?
Hepatocytes	-	?	?	?
Endothelium	+	+	?	?
Kupffer Cells	-	?	?	?

+: Identified by immunohistochemistry
-: Absent by immunohistochemistry
?: No reported studies

Summary and Objectives

Despite more than thirty years of research, the mechanisms of LPS-induced liver toxicity remain incompletely understood. It is now clear that they involve a complex and sequential series of interactions among cellular and humoral mediators. In the same period of time, gram-negative systemic sepsis and its sequelae have become a major health concern, yet our therapeutic interventions have met with little success. The discovery of the importance of LPS and endogenous cytokines in the pathogenesis of the multi-organ failure syndrome led to numerous pharmacologic attempts to block the process. However, clinical trials of anti-endotoxin monoclonal antibodies have not shown them to have therapeutic benefit (McCloskey *et al.*, 1994), and anti-IL-1 (Fisher *et al.*, 1994) and anti-TNF-α therapies (Dhainaut *et al.*, 1995; Abraham *et al.*, 1995; Cohen and Carlet, 1996) have demonstrated efficacy when administered preventively in animal models, but not clinically in human trials (Boom *et al.*, 1993; Baue and Chaudry, 1980; Baumgartner, 1992).

One of the reasons currently invoked to explain the lack of efficacy in the clinic of techniques that were successful in laboratory animals is the delay between the actual exposure to LPS and the time of admission in the critical care unit. From a medical viewpoint, the first exposure to LPS and the rise in TNF-α or IL-1 in the patient's circulation are not in close enough temporal apposition to the appearance of the first clinical symptoms to be

useful targets for therapeutic intervention. In essence, when TNF-α peaks, around 90 min after exposure to a critical dose of LPS, the patient is still in stage 1 of SIRS, and the flu-like symptoms usually do not warrant consultation of a physician or transport to intensive care unit. Only after several hours does the onset of sepsis become clearly visible, and at that time most of the early events, including the presence of LPS into the circulation, the rise in cytokine concentrations and the influx of inflammatory cells are either finished or well on their way. For these reasons, it seems a clinical priority to target critical events occurring shortly before the onset of multiple organ failure, but well after the appearance of the SIRS symptoms.

Recent evidence in animal studies suggest that thrombin is a critical mediator of liver injury and that its involvement closely precedes the onset of hepatic damage. Given that thrombin action does not appear to be dependent on the formation of insoluble fibrin clots as previously suggested, this work will focus on explaining the mechanisms by which it contributes to hepatocellular necrosis following LPS administration.

The overall purpose of this thesis research is to understand the mechanisms by which thrombin leads to liver injury following exposure to LPS. The hypothesis is that thrombin acts on inflammatory cells present in the liver after LPS administration through the activation of a specific receptor and this event initiates a cascade of cellular interactions ultimately resulting in the death of liver parenchymal cells.

The specific objectives are:

1/ To confirm the importance of thrombin as a late but critical mediator of LPS-induced liver toxicity and its role independently of the formation of fibrin clots.

2/ To determine whether the damaging effect of thrombin is related to the activation of a thrombin receptor.

3/ To determine which cell types need to be present in the liver prior to thrombin activation in order for the liver to be damaged.

It is our hope that this work will contribute to a better understanding of the events closely preceding the onset of organ injury, and by doing so unmask new directions toward a better treatment of endotoxemia.

Thrombin involvement in the pathogenesis of respiratory distress following endotoxin has also been reported, and it is now believed to mediate some of the symptomes of SIRS (Gando *et al.*, 1997; Mammen, 1998b). Anti-thrombin agents also prevent the lethal consequences of

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Gram-negative bacterial infections in rats (Emerson, Jr. *et al.*, 1987) and other animal models (Gomez *et al.*, 1989; de Boer *et al.*, 1993). Thus, despite the focus of this thesis to the study of hepatotoxicity, it is reasonable to envision a broader implication of thrombin in the mechanism of endotoxin-induced multiple organ failure syndrome. Despite our knowledge of the beneficial effects of anticoagulants in sepsis, it is often impossible for physicians to completely block coagulation during the treatment of critically ill or traumatized septic patients, since the risk of massive hemorrhage and subsequent death is extremely high. If the mechanism of thrombin action in LPS-induced liver injury could somehow be separated from its role in clot formation, it could be possible to target the onset of organ failure while maintaining efficient coagulation.

CHAPTER 2

THROMBIN IS A DISTAL MEDIATOR OF LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY IN THE RAT

Introduction

Exposure to gram-negative bacterial lipopolysaccharide (LPS) results in a myriad of pathophysiologic alterations including shock, disseminated intravascular coagulation and injury to several organs, including the liver (Hewett and Roth, 1993). The intravenous administration of *E. coli* LPS to rats produces neutrophil infiltration with multifocal, primarily midzonal, hepatocellular necrosis (Pearson *et al.*, 1995; Hewett *et al.*, 1992). This hepatocellular injury is dependent on numerous cellular and soluble mediators of inflammation, including neutrophils (Shibayama *et al.*, 1995; Jaeschke *et al.*, 1991), Kupffer cells (Iimuro *et al.*, 1994), tumor necrosis factor-*alpha* (Hewett *et al.*, 1993), platelets (Pearson *et al.*, 1994) and an activated coagulation system (Margaretten *et al.*, 1967; Hewett and Roth, 1995). The mechanism of LPS-induced liver injury remains unclear, but it is likely that these inflammatory components interact in the manifestation of tissue damage.

Evidence for activation of the coagulation system during LPS exposure includes a pronounced decrease in plasma fibrinogen (Prager *et al.*, 1979), an increase in the prothrombin and partial thromboplastin times (Yoshikawa *et al.*, 1981) and elevations of plasma fibrin degradation products (Gomez *et al.*, 1989). In rats, these alterations in the coagulation system begin 2-3 hrs after the intravenous administration of LPS and occur prior to the onset of liver injury, which begins between three and four hours

(Pearson *et al.*, 1995; Hewett and Roth, 1995). The coagulation system appears to contribute to LPS-induced hepatocellular damage, since pretreatment with anticoagulants such as warfarin or heparin at doses large enough to reduce thrombin activity attenuates liver injury (Pearson *et al.*, 1996; Margaretten *et al.*, 1967).

The mechanism by which the coagulation system contributes to hepatic parenchymal cell injury has not been elucidated. Because one of the LPS-induced changes is deposition of fibrin in the microvasculature of the liver (Endo and Nakamura, 1993; McKay *et al.*, 1966), it has been hypothesized that occlusion of the microcirculation by insoluble fibrin clots and consequent ischemia are responsible for the liver necrosis (Shibayama, 1987; Suzuki *et al.*, 1988). Recent results, however, have raised doubts about this theory. For example, treatment of rats with ancrod, a thrombin-like enzyme that cleaves fibrinopeptide A but not fibrinopeptide B from fibrinogen (Pizzo *et al.*, 1972) and does not cross-link the resulting fibrin monomers, depleted the animals of circulating fibrinogen but failed to protect against the liver injury (Hewett and Roth, 1995). This result suggested that the formation of insoluble clots in the microvasculature is not a critical factor in the pathogenesis of liver damage.

Since both heparin and warfarin result in reduced thrombin activity *in vivo*, the hepatoprotection by these agents raised the possibility that

thrombin may be involved critically in liver pathogenesis during endotoxemia. The recent finding that hirudin, a highly selective thrombin inhibitor, prevented LPS-induced liver injury supported this contention (Pearson *et al.*, 1996). In this chapter, we provide further support for the hypothesis that thrombin is a critical mediator of LPS-induced liver injury. In addition, several heretofore unanswered questions about the role of thrombin are addressed with studies *in vivo* and in the isolated, perfused rat liver. These include the following:

[1] Within an hour after LPS exposure, critical blood cells such as neutrophils and platelets accumulate in liver, and TNF-α appears in the plasma (Shibayama *et al.*, 1995; Pearson *et al.*, 1994). Is the action of thrombin in liver pathogenesis also an early event, or does thrombin act distally relative to these other critical events?

[2] Does thrombin require other plasma components to produce hepatocellular necrosis?

[3] Acting independently of its role in fibrin clot formation, can thrombin injure healthy livers, or are hepatic inflammatory alterations initiated during LPS exposure required to produce liver damage?

Materials and Methods

Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12), heparin (Type II, disodium salt), ancrod from *Agkistrodon rhodostoma*, bovine serum albumin, trypan blue dye and Kit 59 for determination of alanine aminotransferase (ALT) activity were purchased from Sigma Chemical Company (St. Louis, MO). Bovine *alpha*-thrombin and Data-Fi® fibrinogen determination kits were purchased from Baxter Scientific Products (McGraw Park, IL). Recombinant hirudin (HBW-023) was a generous gift from Behring (Marburg, Germany).

Animals

Female, Sprague-Dawley rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 200-250 g were used in all studies *in vivo*. Male, Sprague-Dawley rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 200-250g and 300-350g were used as liver and blood donors respectively, for studies in the isolated, perfused livers. The animals were maintained on a 12 hr light/dark cycle under controlled temperature (18-21^0 C) and humidity (55 ± 5%). Food (Rat chow, Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out according to the humane guidelines of the AALAS and the University Laboratory Animal Research Unit at MSU.

In vivo studies with heparin or hirudin

To elucidate when the coagulation system contributes to the pathogenesis of LPS-induced liver injury, animals were treated with heparin (2000 U/kg, iv) or its saline vehicle either 1 hr before or 1.5 or 2.5 hrs after administration of LPS (4 mg/kg, iv) or its saline vehicle . Six hours after the administration of LPS, animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), blood was drawn into sodium citrate (0.38% final concentration) from the descending aorta and plasma samples were assayed for plasma fibrinogen concentration in a BBL Fibrometer (Becton, Dickinson and Company, Hunt Valley, MD) and for ALT activity. Liver sections were also collected. Liver injury was assessed from increased activity of ALT in plasma and by histologic evaluation.

Preliminary studies conducted to determine a dose and route of administration of hirudin that would effectively inhibit thrombin showed that subcutaneous administration of recombinant hirudin produced a three-fold elevation in plasma activated partial thromboplastin time that lasted for 2 hrs. This dose also completely inhibited the decrease in plasma fibrinogen induced by LPS administration (data not shown). To test the hypothesis that thrombin is a distal mediator of liver injury, animals were treated with LPS (4 mg/kg, iv) or its saline vehicle, and were given r-hirudin (36,000 U/kg, sc) or its saline vehicle 2.5 and 4.5 hrs later. Six hours after the administration of LPS, animals were anesthetized as described above, and

blood and liver sections were collected for the determination of liver injury.

Isolation and perfusion of rat livers

As described previously (Ganey *et al.*, 1988), liver donors were anesthetized with pentobarbital sodium, and a midline laparotomy was performed. An inflow cannula (PE 190 polyethylene tubing, Clay Adams, Parsippany, NJ) with medium flowing through it was inserted into the portal vein and secured with two ligatures. The abdominal section of the caudal vena cava was then sectioned, and the gastrointestinal tract was removed. After thoracotomy, an outflow cannula (PE 240 polyethylene tubing, Clay Adams, Parsippany, NJ) was inserted into the thoracic portion of the caudal vena cava and secured with sutures to the walls of the vessel and the diaphragm. The abdominal section of the vena cava was occluded with a ligature, and the liver was removed from the abdominal cavity. The liver was then placed on a plexiglass block inside of a thermostatic chamber.

The perfusion circuit for the isolated livers was as follows: two reservoirs (2000 ml used for the single pass perfusion and 40 ml used in the recirculating circuit) maintained at a temperature of 38.5ºC were connected via a three-way stopcock to tubing connected to a Masterflex pump (Model 7553-80, Cole-Parmer Instrument Company, Niles, IL). The medium was pumped from the reservoir to an artificial lung (12 ft of coiled Silastic tubing, model 602-235, Dow Corning, Midland, MI) saturated with

95% O_2/5% CO_2 and then to a bubble trap. It then passed through a fine-mesh filter to the inflow cannula. A Gould P23 pressure transducer connected downstream from the filter was used to monitor inflow pressure. The oxygen concentration in the effluent perfusion medium was recorded by a Clark-type, platinum electrode (Model 125/05, Instech, Plymouth Meeting, PA) connected to a DOM-2 Oxygen Meter (University of Pennsylvania, Philadelphia, PA). The perfusion medium was then directed to a waste container during the initial single pass perfusion, or back to the 40 ml reservoir for recirculation.

Flow was maintained constant during the perfusion at a value of 0.14 ml/min/g body weight. The temperature of the perfusion medium was maintained at 38.5ºC, and the pH (7.4) was controlled by an in-line electrode connected to a pH controller (Chemtrix Inc., Hillsboro, Oregon). Experiments were performed using two identical systems, allowing simultaneous perfusion of treated and control livers.

Liver perfusion with heparin-treated blood

The blood used in the perfusion medium and the livers isolated for perfusion were obtained from different animals. Two rats were used as blood donors for each liver. Each blood donor was anesthetized with pentobarbital sodium, and a midline laparotomy was performed. Heparin (1000 U) was injected into the inferior vena cava, and 10 seconds later 10

ml of blood were drawn from the aorta into a silicon-treated glass syringe. The blood was diluted 50% with Krebs-Henseleit buffer containing 2% bovine serum albumin and stored at 38.5ºC until used in the recirculating perfusion.

Liver donors received LPS (4 mg/kg) or its saline vehicle (2 ml/kg) as a single injection in the tail vein two hours before pentobarbital anesthesia. Figure 2.1 depicts the status of the livers in LPS-treated rats at the time of isolation. Within the first two hours after LPS injection, hepatic accumulation of both platelets and neutrophils occurs, and TNF-α activity appears in plasma (2). Neither ALT activity nor fibrinogen concentration in the plasma is altered during this period. Livers were removed, positioned as described above and perfused in a single-pass manner for 10-15 minutes with Krebs-Henseleit solution (pH 7.4) containing 2% bovine serum albumin. Livers were then perfused with the blood-containing solution in a recirculating manner for two hours, and outflow samples of 250 μL were taken every 15 min and stored on ice for determination of ALT activity in the sample. At the end of the experiment, livers were perfused again with Krebs-Henseleit solution for ten minutes, and then fixed with 10% buffered formalin.

Liver perfusion with ancrod-treated blood

The blood used for the perfusion medium and the livers isolated for perfusion were obtained from different animals. Two rats were treated as blood donors for each liver. Blood donors received 60 units of ancrod 6 hours before blood collection, and 40 units 2 hours later. Preliminary experiments confirmed that this treatment protocol reduced plasma fibrinogen to a level below the detection limit of the assay (50 mg/dl) 4 hours after the second injection and that the blood remained effectively anticoagulated for the duration of the experiment, as evaluated by the absence of fibrin clots in the tubing of the perfusion apparatus or on the inflow filter and by the constancy of the inflow pressure during the time of perfusion. Two hours before blood collection, the animals received LPS (4 mg/kg) or its saline vehicle (2 ml/kg) as a single injection in the tail vein. At the time of exsanguination, animals were anesthetized with pentobarbital sodium, and a midline laparotomy was performed. Ten milliliters of blood were drawn from the aorta into a silicon-treated, glass syringe. The blood was then diluted 50% with Krebs-Henseleit solution and stored at 38.5ºC until used in the recirculating perfusion. Liver donors were treated as described above, receiving either LPS or its saline vehicle. The protocol for perfusion was as stated above for the heparin perfusion studies. Thus, the experiment was conducted as a 2x2 factorial design: Animals were randomly assigned to groups in which blood donor rats were treated with

either LPS or saline, and liver donors were treated with either LPS or saline. During experiments, livers were randomly assigned to the two perfusion apparatus.

Liver perfusion with buffer containing thrombin.

Liver donor rats received LPS (4 mg/kg) or saline vehicle (2 ml/kg) as a bolus injection in the tail vein two hours before anesthesia. Their livers were removed and perfused for 10-15 minutes in a single pass manner as described above. The livers were then perfused in a recirculating manner with Krebs-Henseleit buffer containing 2% bovine serum albumin and either thrombin (4.15 U/ml) or its buffer vehicle. Perfusate samples (250 µL) were taken before perfusion and every 15 min thereafter for determination of ALT activity. After two hours of recirculating perfusion, livers were perfused for ten minutes with trypan blue dye (0.4 mM) dissolved in Krebs-Henseleit solution (Bradford *et al.*, 1986), followed by Krebs-Henseleit solution for ten more minutes, and subsequently with 10% buffered formalin for 10 min, all in a single pass manner. The livers were then stored in formalin until processing for histologic evaluation.

The experiment was conducted according to a 2x2 factorial design. Liver donors were randomly assigned to four treatment groups: livers from LPS-treated donors perfused with buffer containing thrombin, livers from LPS-treated donors perfused with buffer only, livers from saline-treated

donors perfused with buffer containing thrombin or livers from saline-treated donors perfused with buffer only. Livers were randomly assigned to the two perfusion systems.

Assessment of hepatic injury

Hepatic injury was evaluated by measuring the activity of ALT in the plasma or the perfusion medium (Sigma kit 59-UV). In experiments involving perfusion with a blood-containing medium, a sample was taken before the start of the recirculating perfusion, and ALT activity in the plasma of this sample was subtracted from all subsequent samples.

Sections of liver were fixed in 10% neutral buffered formalin and processed for histopathologic evaluation. Paraffin-embedded sections were cut at 6 μm and stained with hematoxylin and eosin. For the studies *in vivo*, the slides were coded, randomized and evaluated by a pathologist. The severity of each lesion was graded as described in the footnote to table 1. Isolated livers perfused with trypan blue were dehydrated directly in 100% ethanol, embedded in paraffin, cut at 6 microns and stained with eosin. The resultant sections were used for identification and localization of cells with nuclear staining.

Statistical Analysis.

A 2 X 2 multifactorial, completely random analysis of variance was used to evaluate the data from studies *in vivo*. Data with non-homogeneous variances were square root- or log-transformed to restore homoscedasticity prior to analysis. Comparisons among groups were performed with Tukey's omega test (Steel and Torrie, 1980). In the heparin and hirudin posttreatment studies, comparisons between groups were performed with the Student's t-test (Steel and Torrie, 1980). Results are presented as the mean ± standard error of the mean (S.E.M.). In the isolated liver studies, time-dependent changes in ALT activity, oxygen consumption and perfusion pressure were analyzed using a two-way, repeated measurements analysis of variance, or the Kruskal-Wallis ANOVA on ranks when variances remained heterogenous. Multiple comparisons were performed using the Student-Newman-Keuls test when the data were homogeneous, or a non-parametric multi-comparison test (Conover, 1980) when they were not. For all studies, criterion for significance was $p < 0.05$.

Results

Post-treatment with inhibitors of thrombin. Previous studies have shown that liver injury, as marked by increases in liver-specific enzyme activity in plasma, begins 3 to 4 hours after i.v. administration of 4 mg/kg LPS and becomes prounounced by 6 hours (Pearson *et al.*, 1995; Hewett and Roth, 1995). Decreased plasma fibrinogen concentration, a marker of coagulation system activation, occurs before the onset of liver injury but after the onset of other critical events, such as hepatic accumulation of inflammatory cells (Pearson *et al.*, 1995) and the appearance of plasma TNF-α activity, as summarized in Figure 2.1. Pretreatment with heparin protected rats against LPS-induced liver injury evaluated either histopathologically or as increased plasma ALT activity (Table 2.1). This observation confirms previous reports (Margaretten *et al.*, 1967; Hewett and Roth, 1995). To test the hypothesis that the critical action of thrombin is a late event in the genesis of LPS-induced liver injury in the rat, heparin or its saline vehicle was administered either 1 hr before or 1.5 or 2.5 hrs after the administration of LPS, and liver injury was evaluated at 6 hr. Heparin treatment prevented the LPS-induced decrease in plasma fibrinogen, thus confirming its effectiveness in inhibiting activation of the coagulation system (Figure 2.2).

Table 2.1. Protection from LPS-Induced Hepatotoxicity by Administration of Heparin. Heparin (2000 U/kg, iv) or its saline vehicle was administered to rats 1 hr before the administration of LPS (4 mg/kg, iv) or its saline vehicle. Six hours after the administration of LPS, liver sections were collected and fixed in 10% neutral buffered formalin and prepared for analysis by light microscopy. The severity of hepatic injury was graded on a scale of 0 - 5 reflecting the frequency and size of the lesions. 0 = no evidence of inflammation; 1 = sinusoidal neutrophilia only; 2 = sinusoidal neutrophilia with multifocal single cell necrosis; 3 = multifocal, acute, mild hepatocellular necrosis with sinusoidal neutrophilia; 4 = multifocal, acute, moderate hepatocellular necrosis with sinusoidal neutrophilia; 5 = multifocal, acute marked hepatocellular necrosis with sinusoidal neutrophilia. Values indicate the percent of animals presenting each score. ALT activity (U/L) is expressed as mean ± SEM, N=8-12 per group.

*: significantly different from SAL/SAL group.

#: significantly different from SAL/LPS group.

Table 2.1 Protection from LPS-Induced Hepatotoxicity by Administration of Heparin

Treatment	Percent of Animals with Histopathologic Score						
	0	1	2	3	4	5	ALT
Saline/Saline	100	-	-	-	-	-	50±7
Heparin/Saline	100	-	-	-	-	-	62±7
Saline/LPS	-	17	8	41	17	17	889±381 *
Heparin/LPS	-	75	25	-	-	-	110±20 #

Figure 2.1. Status of Livers Isolated from LPS-Treated Rats. Livers taken for perfusion from LPS-treated rats have experienced substantial accumulation of neutrophils and platelets and exposure to TNF-alpha released into blood; however, at the onset of perfusion two hours after LPS administration, plasma fibrinogen concentration and ALT activity are normal. Data summarized from (Pearson *et al.*, 1995).

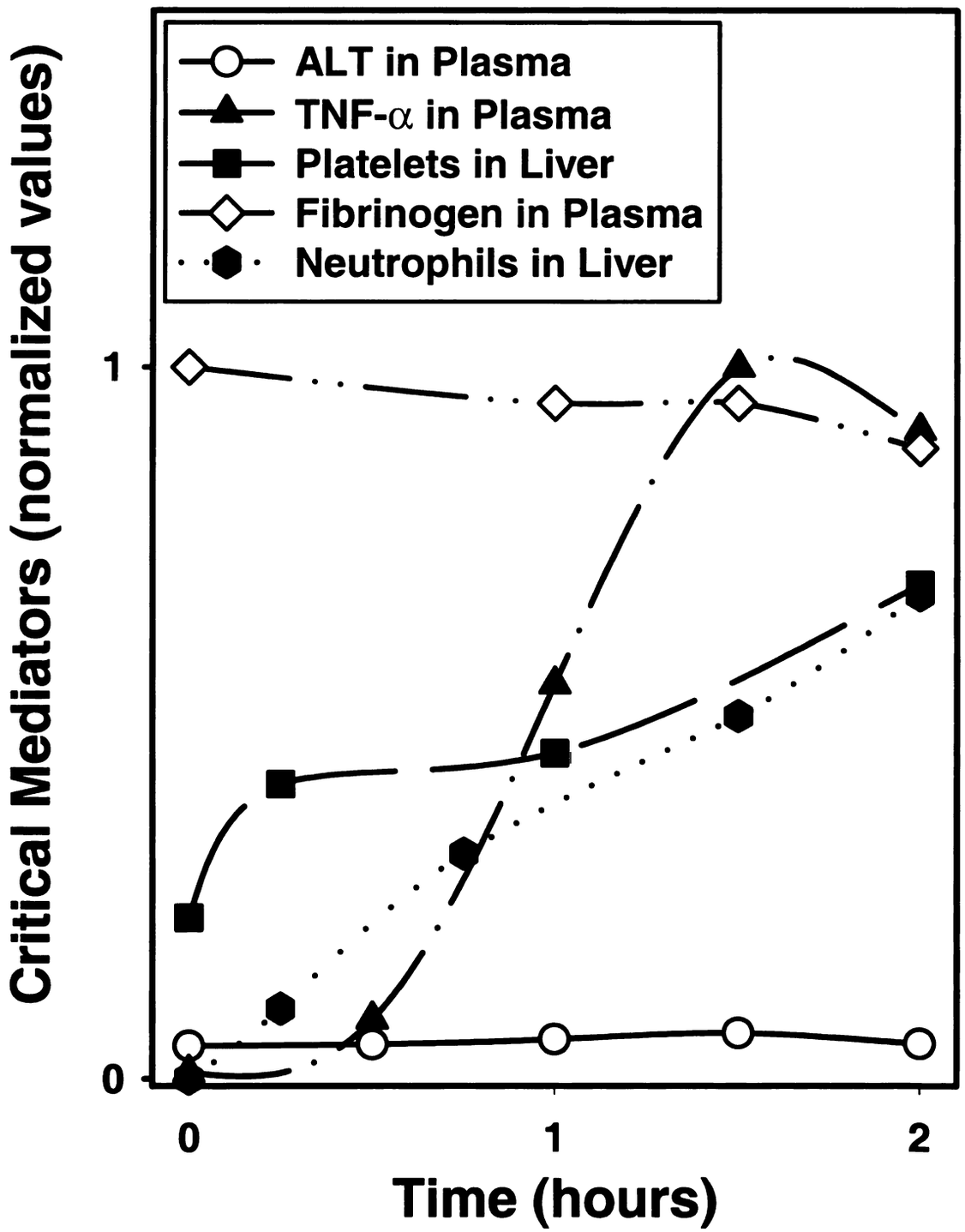

ALT in Plasma
TNF-α in Plasma
Platelets in Liver
Fibrinogen in Plasma
Neutrophils in Liver
Critical Mediators (normalized values)
1
0
0
1
2
Time (hours)

Figure 2.1

Figure 2.2. The Effect of LPS and Heparin on Plasma Fibrinogen Concentration. Animals were treated with heparin (HEP, 2000 U/kg, iv) or its saline vehicle (SAL) 1 hr before the administration of LPS (4 mg/kg, iv) or its SAL vehicle. Six hours after LPS administration, blood samples were collected for measurement of plasma fibrinogen concentration as described in Methods. Results are expressed as mean ± SEM, N=6-12 per group.

*, significantly different from SAL/SAL group

#, significantly different from SAL/LPS group

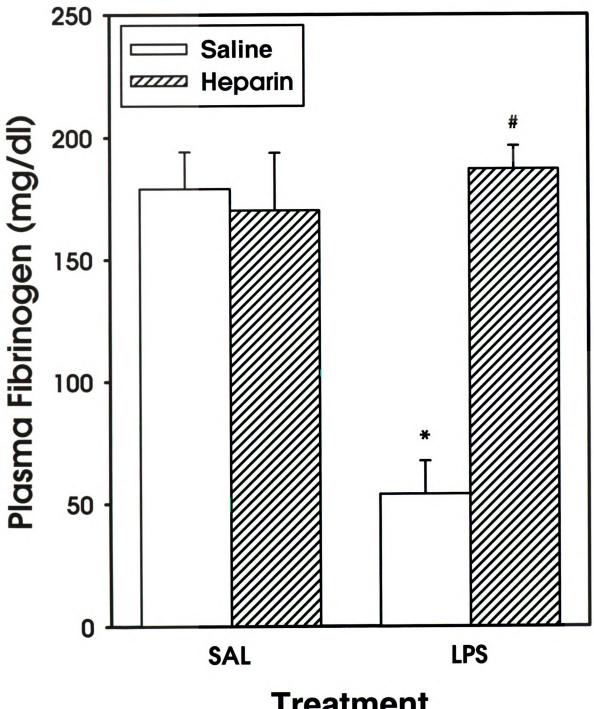

250
200
150
100
50
0
Plasma Fibrinogen (mg/dl)
Saline
Heparin
#
*
SAL
LPS
Treatment

Figure 2.2

Treatment with LPS resulted in liver injury, as measured by plasma ALT activity, and the administration of heparin either 1 hour before or 1.5 or 2.5 hrs after LPS similarly reduced liver injury (Figure 2.3). In this experiment, the degree of histopathologic change caused by LPS and the salutary effect of heparin were similar to those reported in Table 2.1. The histopathologic scores generally reflected the plasma ALT values except at 2.5 hours, at which time the effect of heparin on the LPS-induced ALT increase was more obvious than its effect on liver histology. In a separate experiment, treatment with heparin 3.5 hrs after LPS did not significantly alter the increase in plasma ALT activity (data not shown).

Heparin inhibits thrombin but also has several other actions that could influence LPS hepatotoxicity. Accordingly, a similar study was conducted with hirudin, a highly selective inhibitor of thrombin's catalytic activity (Fenton *et al.*, 1991). The treatment regimen employed prolongs activated partial thromboplastin time by approximately 3 fold and prevents the LPS-induced decrease in plasma fibrinogen (Pearson *et al.*, 1996). Hirudin administration to vehicle-treated animals had no effect on plasma ALT activity. As with heparin, the administration of hirudin 2.5 hr after LPS markedly reduced liver injury (Figure 2.4).

Figure 2.3. The Effect on Liver Injury of Heparin Administered Before or After LPS. Animals were treated with heparin (2000 U/kg, i.v.) or its saline vehicle (SAL) 1 hr before (-1hr) or 1.5 or 2.5 hrs after the administration of LPS (4 mg/kg, i.v.). Six hours after the administration of LPS, blood was collected for assessment of liver injury as marked by increased plasma ALT activity. Results are expressed as mean ± SEM, N=3-9 per group.

*, significantly different from respective group in the absence of heparin.

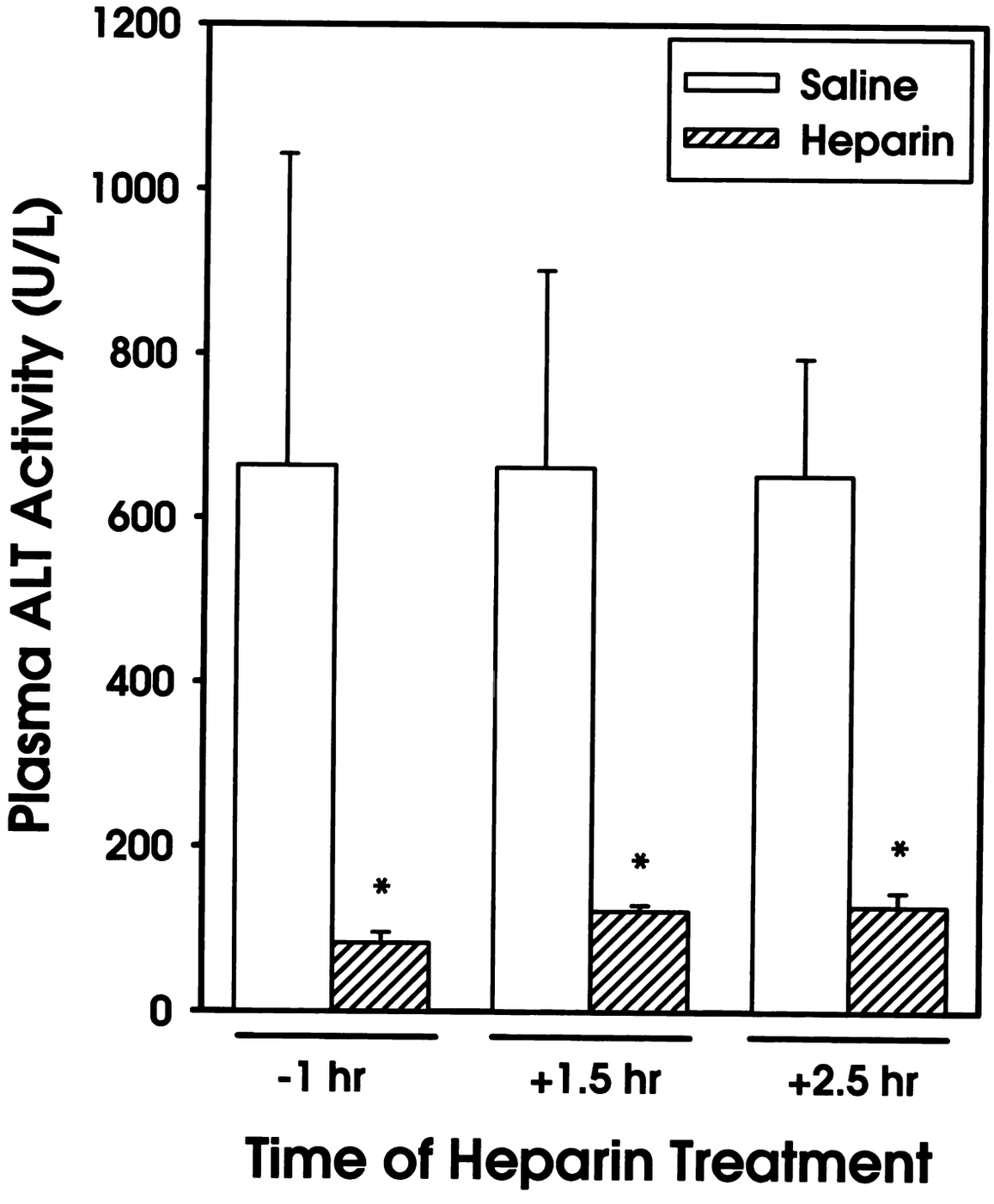

1200
1000
800
600
400
200
0
Plasma ALT Activity (U/L)
Saline
Heparin
*
*
*
-1 hr
+1.5 hr
+2.5 hr
Time of Heparin Treatment

Figure 2.3

Figure 2.4. The Effect on Liver Injury of Hirudin Administered After LPS. Animals were treated with LPS (4 mg/kg, iv) or its saline vehicle (SAL). Hirudin (36,000 U/kg, s.c.) or its saline vehicle was administered twice, at 2.5 and 4.5 hrs after LPS. Six hours after the administration of LPS, blood was collected for assessment of liver injury as measured by plasma ALT activity. Results are expressed as mean ± SEM, N=6-9 per group.

*, significantly different from respective group in the absence of LPS.

#, significantly different from respective group in the absence of hirudin.

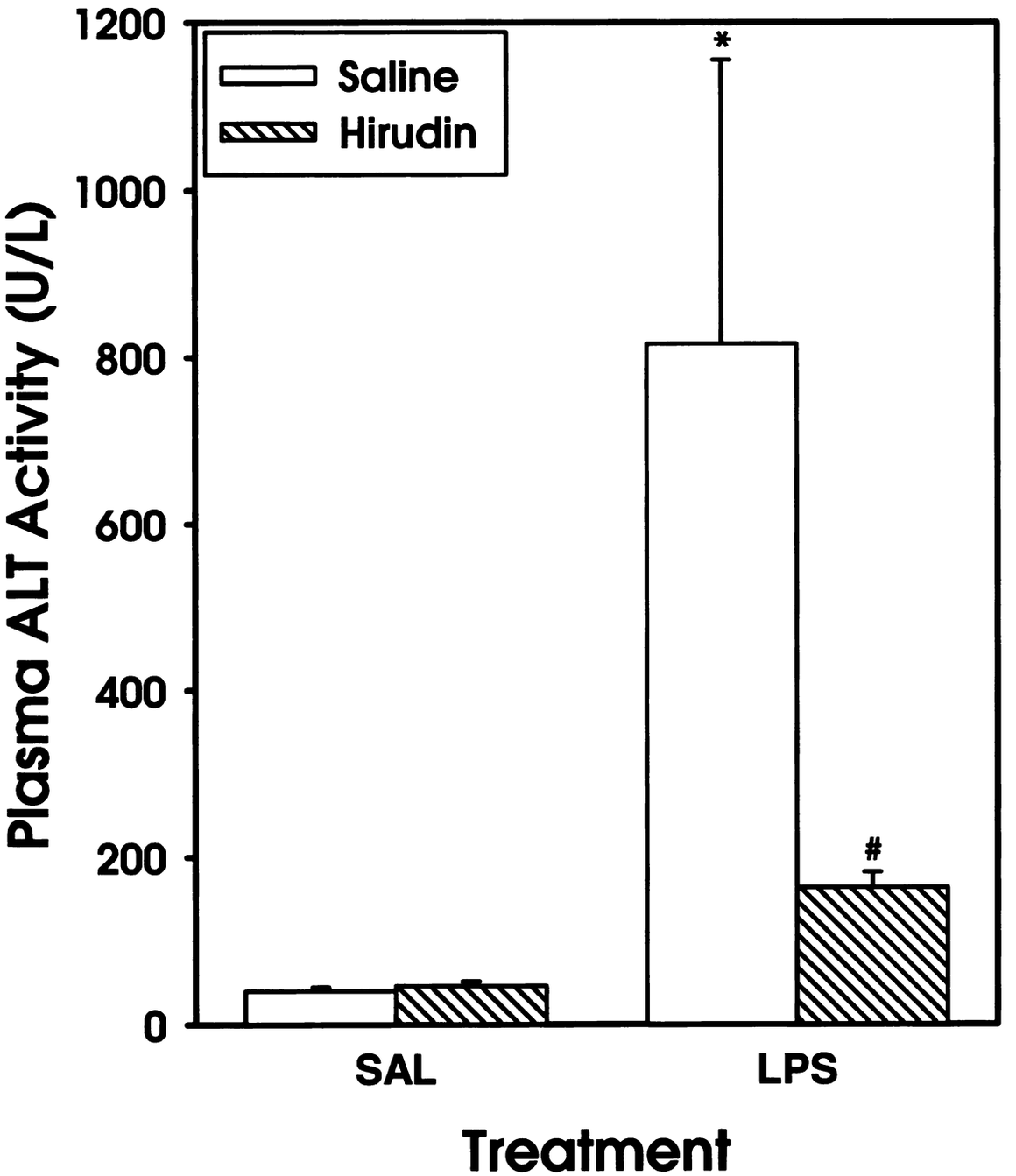

1200
1000
800
600
400
200
0
Plasma ALT Activity (U/L)
Saline
Hirudin
*
#
SAL
LPS
Treatment

Figure 2.4

Studies in isolated, perfused livers. We examined injury in livers from LPS-treated rats perfused with blood taken from animals treated with ancrod, which prevents coagulation by depleting blood of fibrinogen but does not inhibit thrombin (Pizzo *et al.*, 1972; Bell, 1982). Livers for perfusion were taken from donor animals treated with LPS or saline vehicle two hours before isolation. As reported previously (Hewett and Roth, 1995), the ancrod treatment regimen depletes fibrinogen *in vivo* and in this study prevented the formation of visible clots on the inflow filters of the liver perfusion apparatus, suggesting effective anticoagulation. Figure 2.5 shows the cumulative release of ALT in the medium during two hours of recirculating perfusion with ancrod-treated blood. ALT activity in the perfusion medium from livers taken from saline-treated rats was modest and unchanged during the two-hour perfusion, indicating that ancrod-treated blood by itself was without toxic effect. By contrast, perfusion of livers from donors treated with LPS resulted in a time-dependent increase in ALT activity in the perfusion medium. This increase occurred irrespective of treatment of blood donors with LPS. Oxygen consumption and inflow perfusion pressure in livers from saline-treated rats averaged 103±14 μmol/hr/g liver and 35±4 mm of Hg, respectively, at the onset of perfusion and did not change significantly with time of perfusion. In addition, none of the treatments significantly altered either oxygen consumption or perfusion pressure (Figures 2.8 and 2.9).

Figure 2.5. ALT Activity in the Perfusion Medium of Isolated Livers Perfused with Blood from Ancrod-Treated Rats. Rats used as blood donor received 60 units of ancrod 6 hours before blood collection and 40 units 2 hours later. Two hours before blood collection, the animals received either LPS (4 mg/kg) or its saline vehicle (2 ml/kg) as a single injection in the tail vein. The collected blood was diluted 50% with Krebs-Henseleit buffer containing 2% bovine serum albumin to form the perfusion medium. Liver donors received either LPS (4 mg/kg) or saline vehicle (2 ml/kg) as a single injection in the tail vein two hours before liver isolation. Livers were perfused as described in Methods. Samples of the perfusion medium were collected every 15 minutes for two hours, and ALT activity in the medium was determined. Results are expressed as mean ± S.E.M. N=8-11 per group.

*, significantly different from saline liver control at respective time.

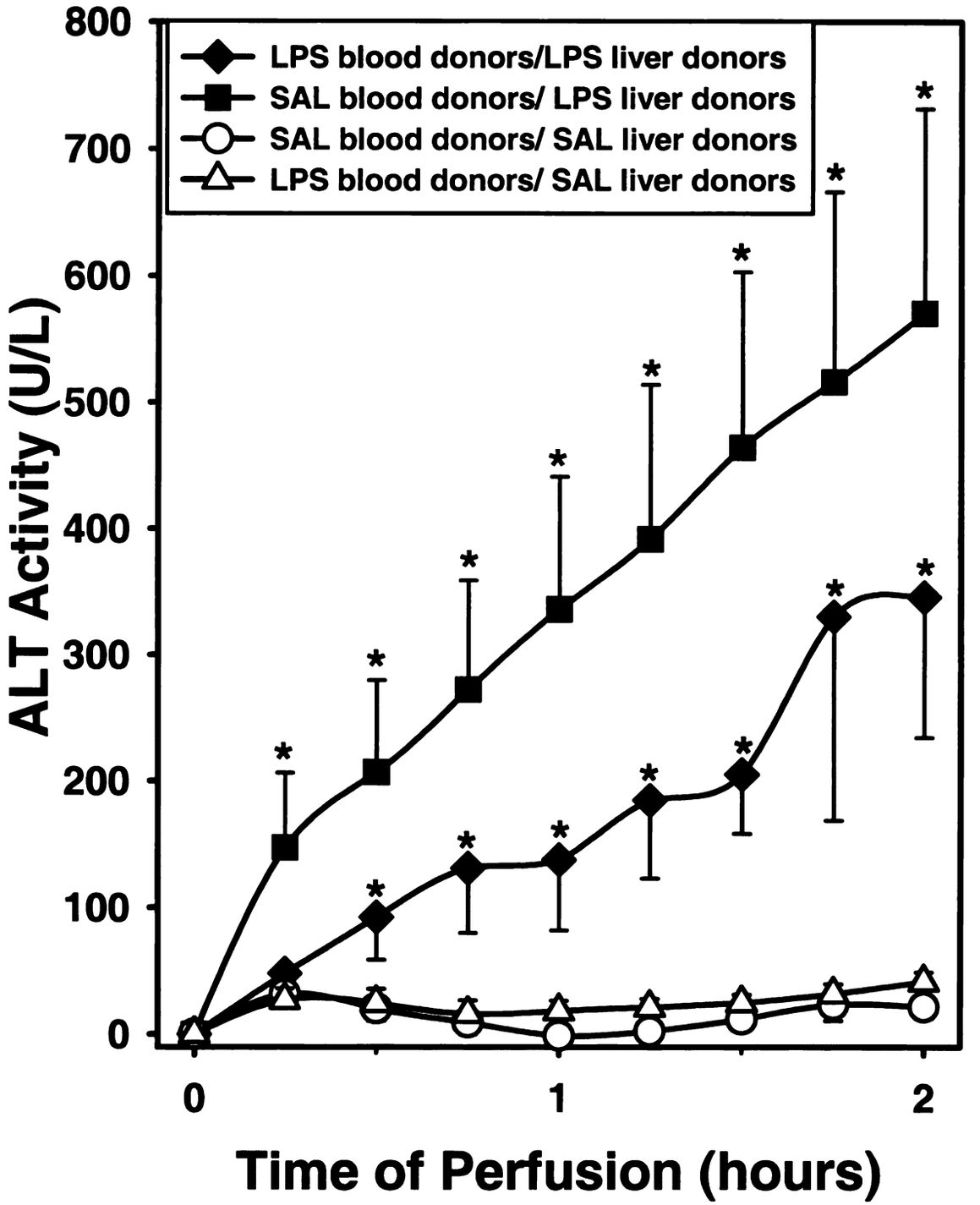

LPS blood donors/LPS liver donors
SAL blood donors/ LPS liver donors
SAL blood donors/ SAL liver donors
LPS blood donors/ SAL liver donors
ALT Activity (U/L)
800
700
600
500
400
300
200
100
0
0
1
2
Time of Perfusion (hours)

Figure 2.5

Consistent with the moderate release of ALT during the limited period of perfusion, histopathologic evaluation at the conclusion of perfusion revealed no striking morphologic changes due to LPS exposure. Perfusion with medium containing trypan blue resulted in uptake of dye in hepatic parenchymal cells in midzonal areas in livers from LPS-treated donors.

In a separate study, livers from saline- or LPS-treated rats were perfused with blood taken from rat donors treated with heparin. Unlike the results with blood anticoagulated with ancrod, in livers perfused with heparin-treated blood ALT activity in the perfusion medium was not elevated by LPS-pretreatment of liver donors (Figure 2.6). There were no time- or treatment-dependent alterations in either oxygen consumption or perfusion pressure in this study (Figure 2.8).

The results described above suggested that the expression of injury in isolated livers from LPS-treated rats requires thrombin in the perfusion medium. As a further test of this hypothesis, isolated livers from LPS-treated rats were perfused with buffer with or without added thrombin. A time-dependent increase in ALT activity occurred in medium of livers perfused with thrombin but not in those perfused with buffer alone (Figure 2.7). By contrast, no increase in ALT activity occurred in medium from livers from saline-treated animals, irrespective of the addition of thrombin. There were no time-dependent changes in either oxygen consumption or perfusion pressure in any of the groups (Figure 2.8).

Figure 2.6. ALT Activity in the Perfusion Medium of Isolated Livers Perfused with Blood Anticoagulated with Heparin. Rats used as blood donors were treated with heparin (1000 U) 10 seconds before blood collection from the aorta into a silicon-treated glass syringe. The blood was diluted 50% with Krebs-Henseleit buffer containing 2% bovine serum albumin to form the perfusion medium. Liver donors received either LPS (4 mg/kg) or saline vehicle (2 ml/kg) as a single injection in the tail vein two hours before liver isolation. Livers were perfused as described in methods. Samples of the perfusion medium were collected every 15 minutes for two hours, and ALT activity in the medium was determined. Results are expressed as mean ± S.E.M. N=5 per group. No significant differences between the groups were observed.

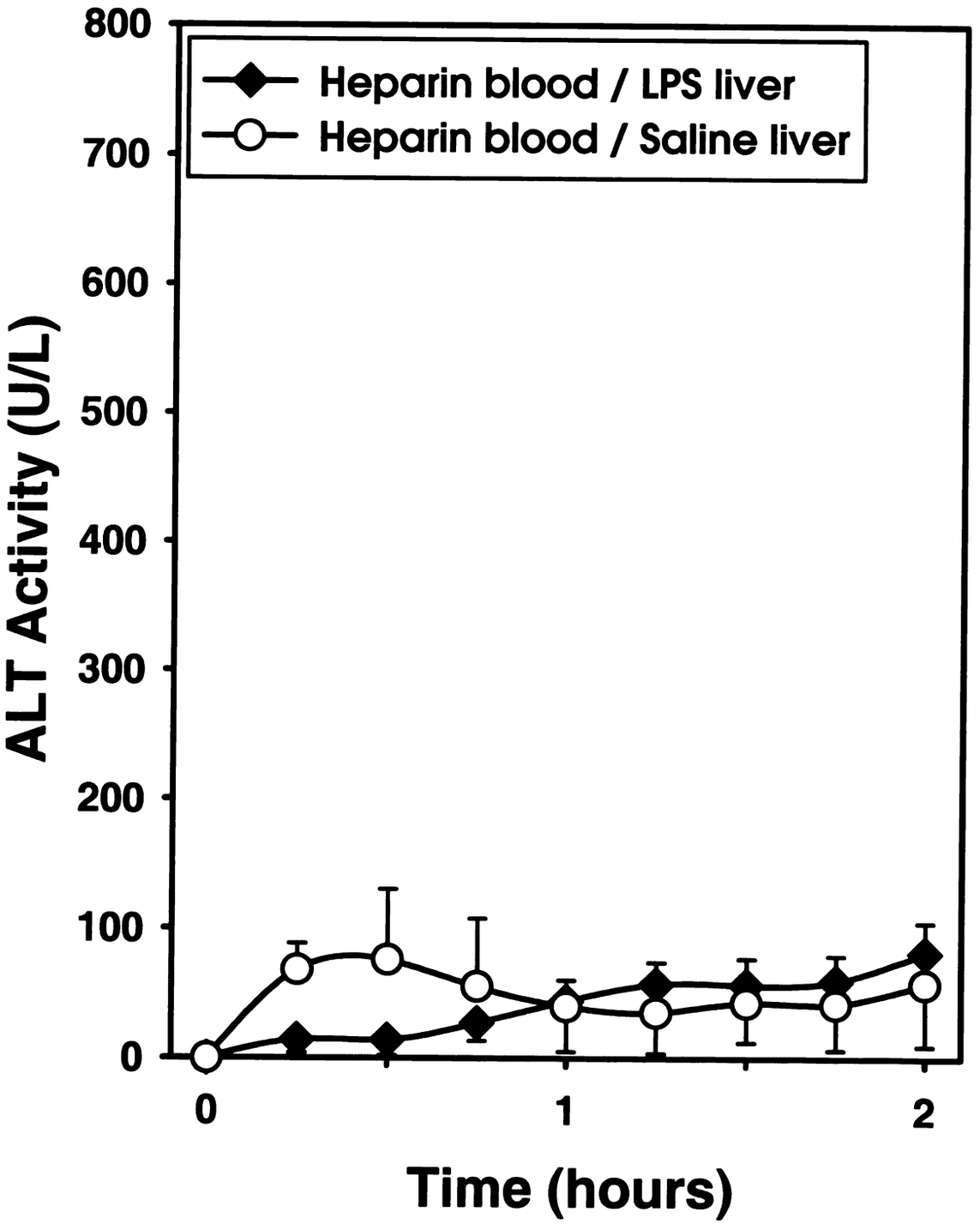

800
700
600
500
400
300
200
100
0
ALT Activity (U/L)
Heparin blood / LPS liver
Heparin blood / Saline liver
0
1
2
Time (hours)

Figure 2.6

Figure 2.7. Release of ALT from Isolated Livers Perfused with Thrombin. Liver donor rats received either LPS (4 mg/kg) or saline vehicle (2 ml/kg) as a bolus injection in the tail vein two hours before liver isolation. The livers were then perfused with Krebs-Henseleit buffer containing 2% bovine serum albumin and either bovine □-thrombin (4.15 U/ml) or its buffer vehicle. Samples of the perfusion medium were collected every 15 minutes for two hours, and ALT activity in the medium was determined as described in Methods. Results are expressed as mean ± S.E.M. N=5 per group.

*, significantly different from control medium without thrombin.

Thrombin.

e (2 ml/kg)

lation. The

taining 2%

U/ml) or its

d every 15

ermined as

M. N=5 per

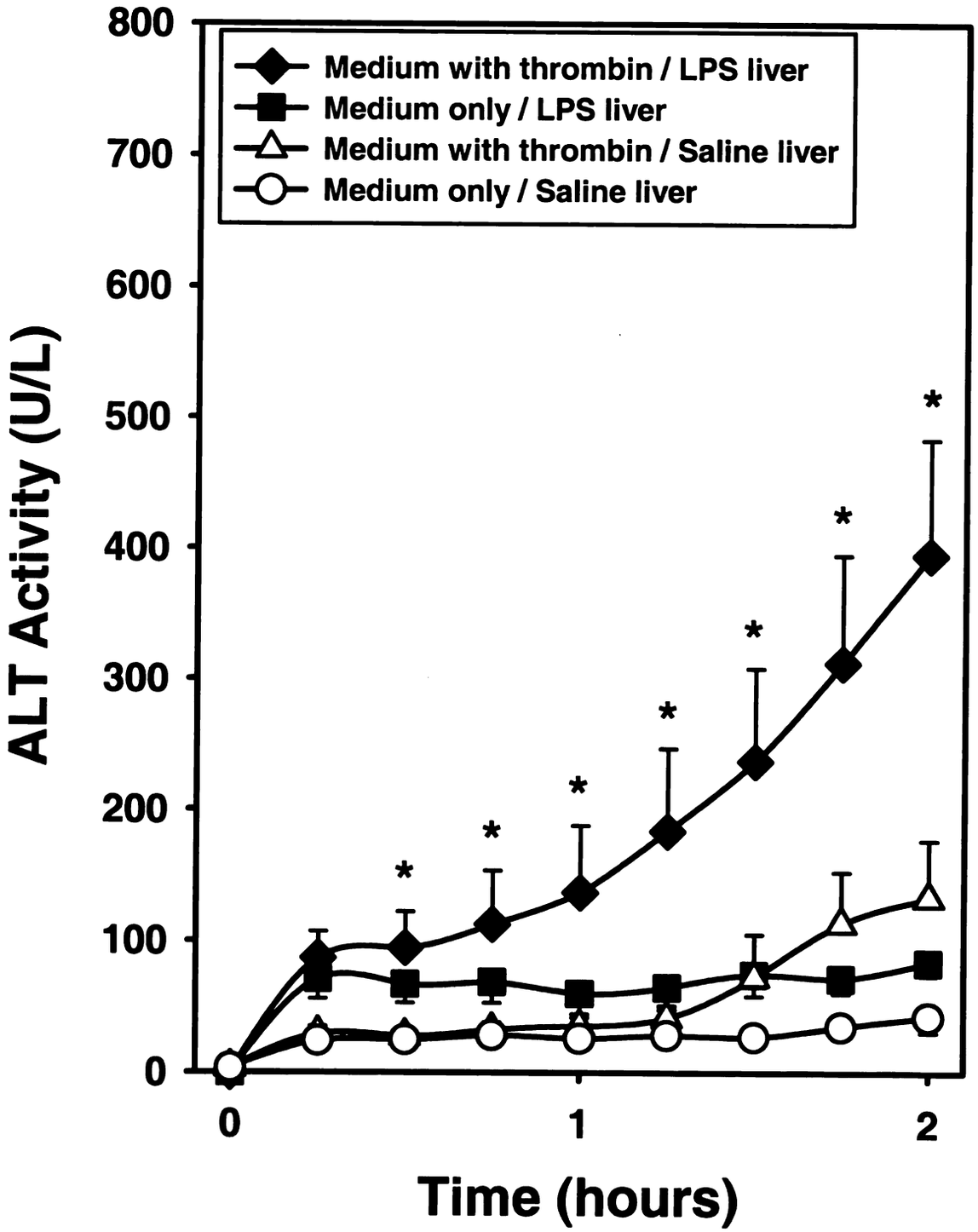
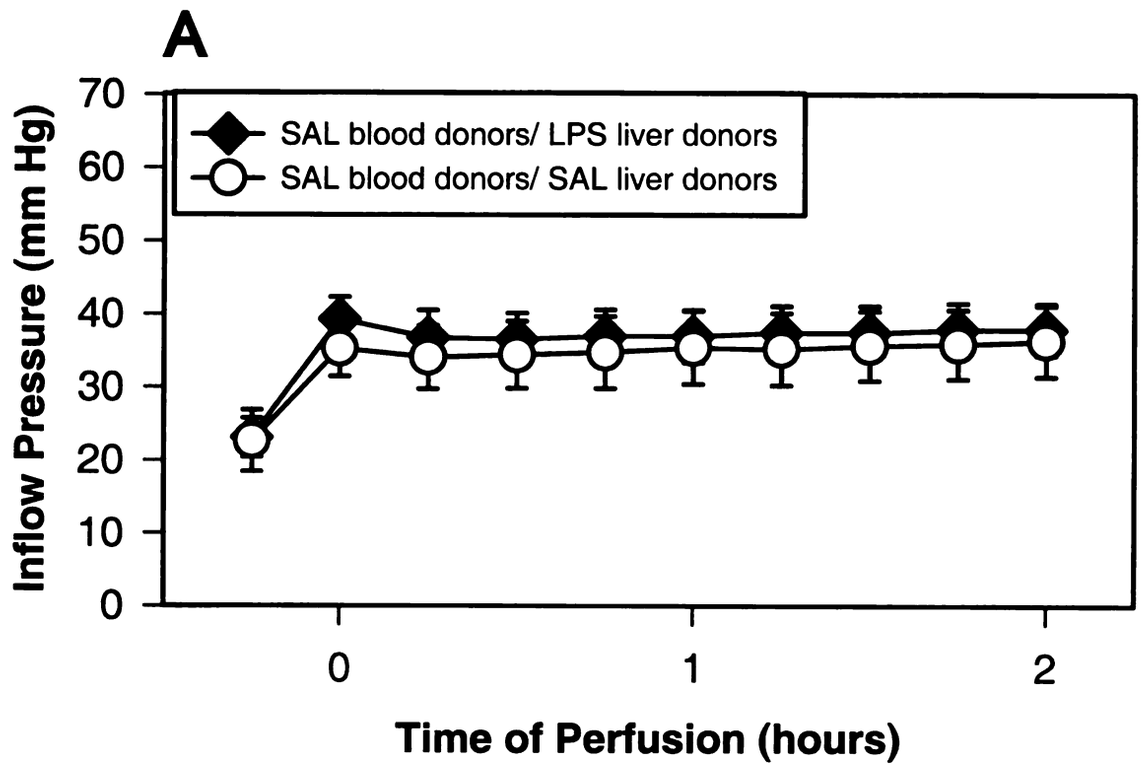


Figure 2.7

Figure 2.8. Inflow Pressures in Isolated Livers Perfused with Different Media. Liver donors and blood donors were treated as described in the legends to Figure 2.5 (A), Figure 2.6 (B) or Figure 2.7 (C). Pressure was monitored continuously with a Gould P23 pressure transducer. Values represent mean ± SEM, N = 5-10.


A
70
60
50
40
30
20
10
0
Inflow Pressure (mm Hg)
SAL blood donors/ LPS liver donors
SAL blood donors/ SAL liver donors
0
1
2
Time of Perfusion (hours)

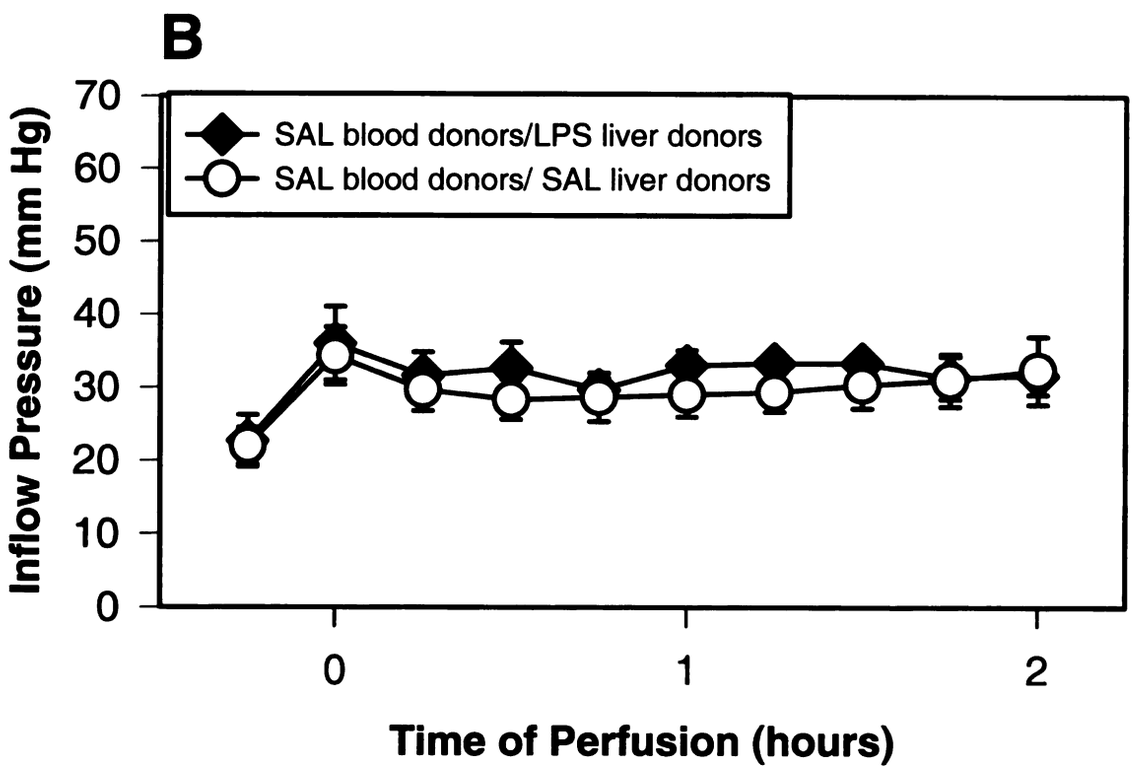

B
70
60
50
40
30
20
10
0
Inflow Pressure (mm Hg)
SAL blood donors/LPS liver donors
SAL blood donors/ SAL liver donors
0
1
2
Time of Perfusion (hours)

Figure 2.8

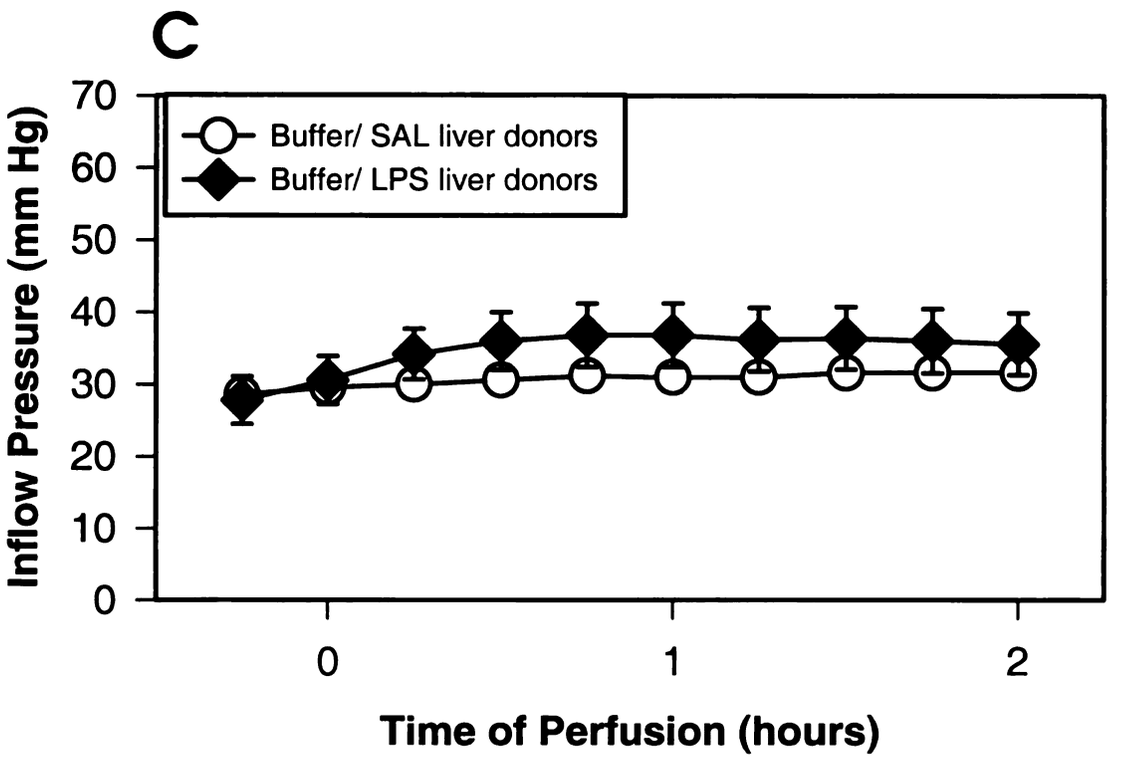


Figure 2.8 (cont'd)

Figure 2.9. Oxygen Consumption from Livers Perfused with Ancrod-Treated Blood. Liver donors and blood donors were treated as described in the legend to Figure 2.5. Oxygen concentration in the effluent perfusate was recorded by a Clark-type platinium electrode, and oxygen uptake was calculated from inflow minus effluent concentration. Values represent mean ± SEM, N = 8-10.

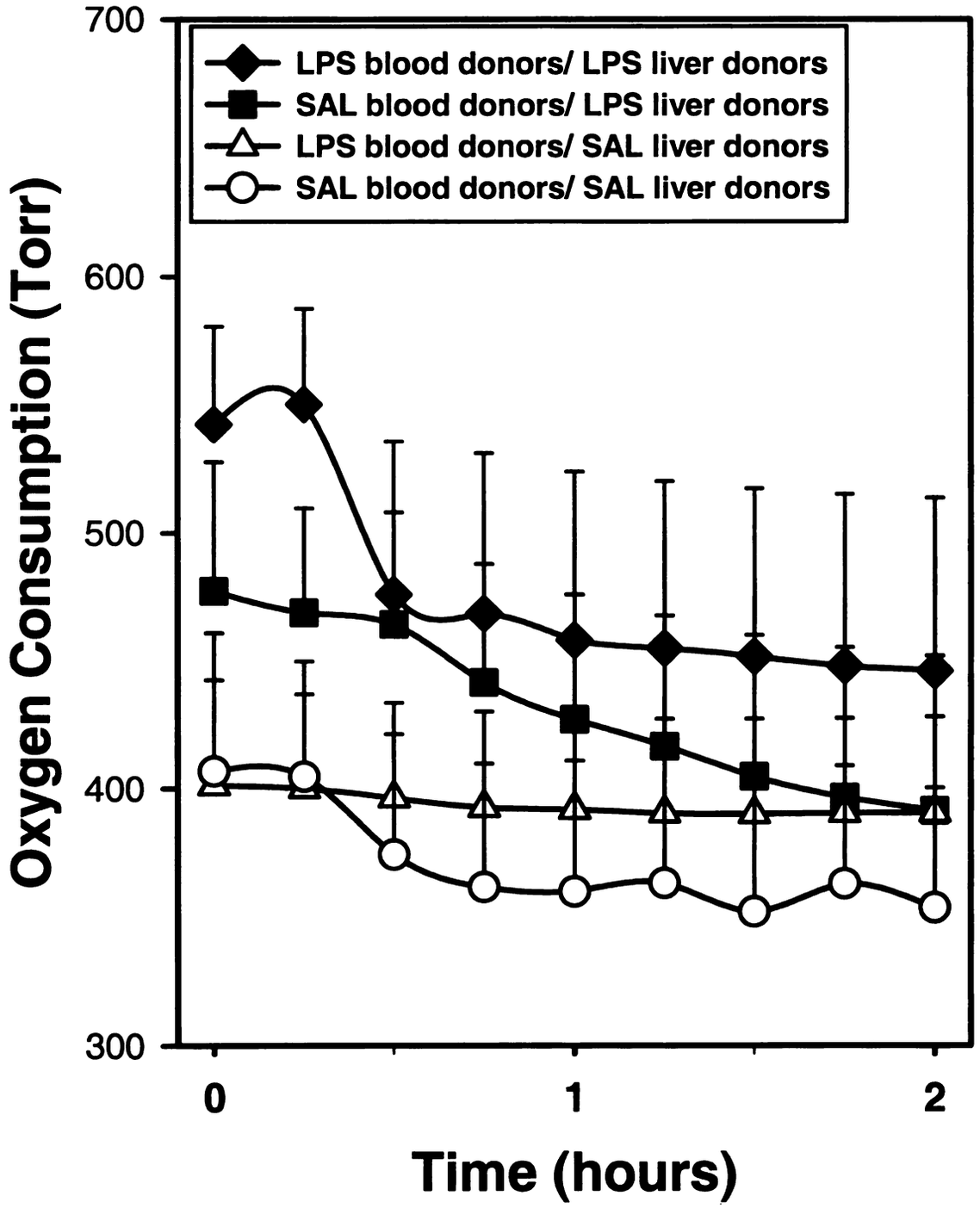

LPS blood donors/ LPS liver donors
SAL blood donors/ LPS liver donors
LPS blood donors/ SAL liver donors
SAL blood donors/ SAL liver donors
Oxygen Consumption (Torr)
700
600
500
400
300
0
1
2
Time (hours)

Figure 2.9

Discussion

Numerous studies have documented activation of the coagulation system both in septic patients (Prager *et al.*, 1979; Lorente *et al.*, 1993) and during LPS exposure in various animal models (Pearson *et al.*, 1995; Hewett and Roth, 1995; Colman, 1994). In the rat, circulating plasma fibrinogen concentration falls dramatically after intravenous injection of LPS (Pearson *et al.*, 1995; Hewett and Roth, 1995), and there is morphologic evidence of fibrin deposition within the liver sinusoids (Endo and Nakamura, 1993; Levy *et al.*, 1968a). Results demonstrating that anticoagulant drugs, like heparin or warfarin, prevent LPS-induced liver injury (Hewett and Roth, 1995) corroborate the importance of activation of the coagulation cascade in the pathogenesis of tissue damage. It has been hypothesized that the coagulation system contributes to tissue injury through the formation of occlusive fibrin thrombi that result in decreased tissue perfusion and ischemia. However, the midzonal location of the lesion in LPS-treated rats argues against ischemia as the basis for injury, inasmuch as studies of hepatic ischemia/reperfusion in the rat have shown that resulting damage is characterized by centrilobular tissue injury (Jaeschke *et al.*, 1990; Hughes *et al.*, 1992). Furthermore, prevention of the formation of insoluble fibrin clots in the sinusoids by pretreatment of rats with ancrod did not protect against LPS-induced liver toxicity (Hewett and Roth, 1995).

These results suggest that the role of the coagulation system in the mechanism by which LPS causes liver injury is independent of its ability to form insoluble fibrin clots.

An alternative mechanism by which activation of the coagulation system may play an important role in liver damage is through activation of thrombin. Thrombin, a serine protease, has long been known for its role in the coagulation system, and activation of either the intrinsic or extrinsic pathways of coagulation by LPS culminates in the generation of thrombin from prothrombin. In rats, infusion of thrombin into the portal vein induces damage similar to that observed after administration of LPS (Shibayama, 1987), and antithrombin III attenuates the effects of LPS (Emerson, Jr. *et al.*, 1987). The observations that either heparin or warfarin attenuates LPS-induced liver damage but ancrod is without effect support the hypothesis that thrombin is important in the pathogenesis of liver injury after exposure to LPS because, unlike heparin or warfarin, ancrod does not inhibit the activity of thrombin. The current study was undertaken to characterize further the role of thrombin in LPS-induced liver injury.

Is thrombin a temporally distal mediator of LPS-induced liver injury?

Results indicating that heparin or hirudin protects against liver injury even when administered up to 2.5 hrs after LPS (Figures 2.3 and 2.4) suggest that the critical actions of thrombin occur later than other events

after LPS exposure, such as hepatic neutrophil and platelet accumulation and release of TNF-α into plasma (see Figure 1.6). Thrombin's critical actions occur sometime between 2.5 and 3.5 hrs after LPS treatment, just prior to the appearance of liver injury, which begins 3-4 hrs after LPS administration in this model. Thus, there is a relatively short period of time between involvement of thrombin and the onset of liver injury, a finding that refutes further an ischemia-based mechanism, which requires a longer duration of reduced blood flow to cause injury to the liver (Jaeschke *et al.*, 1990; Hughes *et al.*, 1992; Jaeschke and Farhood, 1991). These results support the contention that thrombin is an important contributor to LPS-induced injury to liver and that it is a distal mediator of this tissue damage.

Results in isolated, blood-perfused livers support this interpretation: when livers were isolated from rats treated 2 hrs earlier with LPS (i.e., before the onset of injury), only blood anticoagulated with ancrod, in which activity of thrombin was preserved, produced injury (Figure 2.5). Thus, thrombin was able to exert an effect after 2 hrs to damage liver parenchyma. This requirement for thrombin to produce liver damage may explain why, although effects of LPS on some liver functions have been demonstrated using isolated, perfused liver preparations (Gaeta and Wisse, 1983; Utili *et al.*, 1976), previous efforts have failed to reproduce the hepatocellular destruction (eg, ALT release) observed *in vivo*. All earlier studies employed as perfusion medium either blood containing

anticoagulants that target thrombin (e.g., heparin) or buffer devoid of thrombin.

It is tempting to compare results in isolated livers with those observed *in vivo*. In doing so, it is important to consider that plasma ALT activity *in vivo* begins to increase about 3-4 hrs after LPS administration and continues to increase through 6 hours. In the isolated, perfused liver studies, perfusion began 2 hrs after LPS treatment and lasted only 2 hrs; thus, the total time after LPS administration was only 4 hrs, a time before a pronounced degree of liver injury develops *in vivo*. When perfused with ancrod-treated blood, livers from LPS-treated animals released ALT in an amount and at a time comparable to what is observed *in vivo* and exhibited a similar midzonal distribution of lesions. Thus, this preparation appears to be a useful model for studying LPS-induced liver injury.

Does thrombin require other plasma components to produce liver injury after LPS exposure?

Anticoagulation of blood with ancrod does not prevent activation of any of the components of the coagulation cascade proximal to fibrinogen; therefore, results of experiments with ancrod did not preclude the possibility that these other components could contribute to the injury observed in isolated livers. However, the observation that injury results from the addition of thrombin to buffer used to perfuse livers from LPS-

treated rats (Figure 2.7) suggests that other components of the coagulation system or plasma are not required for this effect.

Does thrombin-induced liver injury require LPS exposure?

Although plasma constituents other than thrombin are not required for hepatocellular injury in isolated livers, treatment with LPS is necessary. Only livers taken from rats treated earlier with LPS were damaged upon perfusion with buffer containing thrombin; livers from control animals were not injured (Figure 2.7). This result suggests that events that occur during the first 2 hrs after treatment with LPS "sensitize" the liver to thrombin-induced injury. Early events after treatment with LPS include activation of Kupffer cells and endothelial cells (Herbert *et al.*, 1992; Prajgrod and Danon, 1992), accumulation in liver tissue of neutrophils and platelets, and release of TNF-α. Each of these events plays a critical role in LPS-induced liver injury *in vivo* since inactivation or elimination of any one of them abrogates injury. However, it is not known which of these events is linked critically to thrombin.

Thrombin is a glycoprotein on which three important sites have been identified (Fenton, II and Bing, 1986). The catalytic site confers to the enzyme its serine protease activity, exosite I is responsible for binding to substrate (fibrinogen or thrombin receptor) and exosite II binds to antithrombin III. The earliest identified function of thrombin was the

cleavage of fibrinogen into fibrin monomers and the activation of the fibrin-stabilizing factor (factor XIII) and protein C (Glusa, 1992); however, its actions extend beyond those involved directly in coagulation. Thrombin activates receptors on a wide variety of cells (Hung *et al.*, 1992). Its capacity to induce platelet spreading and degranulation are well documented (Siess, 1989; Venturini and Kaplan, 1992). Numerous effects have been reported on other cell types, including cells involved in the mechanism of LPS-induced hepatotoxicity. For example, thrombin alters the synthesis and release of various proteins from endothelial cells (de Groot *et al.*, 1987; DeMichele and Minnear, 1992) ultimately resulting in an enhanced adhesion of inflammatory cells. It induces chemotaxis in neutrophils (Bizios *et al.*, 1986) and promotes the release of inflammatory components from these cells (Bar-Shavit *et al.*, 1983; Drake and Issekutz, 1992; Cohen *et al.*, 1991). Thrombin is a potent chemotaxin for macrophages (Bar-Shavit *et al.*, 1983) and modulates their production of cytokines and arachidonic acid metabolites. Little is known of the actions of thrombin on Kupffer cells or hepatocytes, but it is probable that thrombin affects these cells because high affinity, thrombin binding sites have been identified on their surfaces (Weyer *et al.*, 1988; Kudhal *et al.*, 1991).

Although thrombin can influence cell adherence (Garcia *et al.*, 1986), it does not appear to promote the accumulation of platelets in the liver since hepatic platelet number was unaffected by pretreatment with

hirudin at a dose that prevented activation of the coagulation system (Pearson *et al.*, 1996). Other studies have shown that heparin does not alter neutrophil sticking to mesenteric venules in response to LPS (Suzuki *et al.*, 1988). These results suggest that the critical action of thrombin in this model does not entail recruitment of inflammatory cells into the liver.

The fact that only livers from LPS-treated animals could be damaged by the addition of thrombin in the perfusion buffer (Figure 2.7) confirms the importance of the events preceeding the activation of the coagulation cascade, mainly the release of cytokines and the influx of inflammatory cells, and suggests that the mechanism by which thrombin causes hepatocellular damage during LPS exposure involves an indirect effect mediated through an inflammatory cell that has accumulated in the liver or through increased susceptibility of hepatic parenchymal cells. Activation of specific receptors by thrombin appears as a seducing hypothesis to explain its involvement in the pathogenesis of liver injury during LPS exposure; however, much remains unknown about the cells activated by thrombin and the relation between the activation of a thrombin receptor and the death of hepatocytes. A mechanism by which thrombin stimulates inflammatory cells already present within the sinusoids would be consistent with the findings that LPS-induced liver injury is dependent upon platelets and neutrophils (Pearson *et al.*, 1995; Hewett *et al.*, 1992; Jaeschke *et al.*, 1991) as well as thrombin. It is also possible that neutrophils and platelets

alter hepatic parenchymal cells in a way that makes them susceptible to injury by thrombin. Of potential interest in this regard is the recent observation that thrombin-activated platelets can increase the release of cytotoxic factors from neutrophils *in vitro* (Moon *et al.*, 1990). Whether thrombin acts via such a mechanism during LPS exposure *in vivo* remains to be determined.

That thrombin is a distal link in the chain of events that result in liver injury may prove useful in the clinical setting. In experimental studies, drugs that interfere with cellular and soluble mediators have usually been administered to animals prior to the administration of endotoxin. In this study, we have discovered agents that afford protection when administered after LPS exposure and in close temporal apposition to the onset of liver injury. In endotoxemic patients, for whom prophylactic treatment is not practical, this quality has considerable importance. Indeed, treatment of patients already in septic shock with antithrombin III led to a reduction of the duration of disseminated intravascular coagulation and a trend toward improved survival (Fourrier *et al.*, 1993).

In summary, these studies demonstrate that thrombin is a critical and distal mediator of LPS-induced liver damage and contributes to liver injury through a mechanism independent of clot formation. The results indicate that events triggered by LPS exposure that occur before the activation of the coagulation system are necessary for thrombin-induced

injury. Accordingly, thrombin may contribute to liver injury by stimulation of inflammatory cells present within the liver tissue. Further exploration of this hypothesis may provide insight into critical interactions among cellular and soluble inflammatory mediators during the development of tissue injury.

CHAPTER 3

THROMBIN-INDUCED LIVER INJURY FOLLOWING LPS EXPOSURE REQUIRES STIMULATION OF A PROTEASE-ACTIVATED RECEPTOR

Introduction

Sepsis resulting from gram-negative bacterial infection remains a major clinical problem with nearly 500,000 cases occuring annually in the United States (Perl *et al.*, 1995; Brun-Buisson *et al.*, 1995; Proulx *et al.*, 1996). It has been shown that many of pathophysiological effects of gram-negative bacterial sepsis, which include fever, leukopenia, tachycardia, hypotension, disseminated intravascular coagulation, and multi-organ failure, are mediated by LPS. LPS is a major component of the cell wall of gram-negative bacteria and can be released into the blood during septicemia. In rats, intravenous administration of *E. coli* LPS produces platelet and neutrophil infiltration in the liver with multifocal, primarily midzonal, hepatocellular necrosis (Nunes *et al.*, 1970; Pearson *et al.*, 1995).

It is now clear that many of the adverse effects of LPS are dependent on cellular and soluble mediators. A multitude of inflammatory mediators has been implicated, including platelets (Pearson *et al.*, 1995), neutrophils (Hewett *et al.*, 1992; Jaeschke *et al.*, 1991), Kupffer cells (Iimuro *et al.*, 1994), cytokines such as TNF-α (Hewett *et al.*, 1991), and components of the coagulation cascade (Hewett and Roth, 1993).

Numerous studies have shown that the coagulation system is activated in septic patients (Lorente *et al.*, 1993; Levi *et al.*, 1993; Thijs *et al.*, 1993) and in animal models following LPS administration (de Boer *et*

al., 1993; Hauptman *et al.*, 1988). In rats, circulating plasma fibrinogen concentration falls within 2 to 3 hours after intravenous injection of LPS (Hewett and Roth, 1995). The importance of this activation of the coagulation system in the pathogenesis of liver injury is emphasized by the ability of anticoagulants to abolish LPS hepatotoxicity (Margaretten *et al.*, 1967; Hewett and Roth, 1995). In light of an activated coagulation system and the observation of fibrin in the liver vasculature (McKay *et al.*, 1966), it has been hypothesized that occlusion of the microcirculation by insoluble fibrin clots and consequent ischemia is responsible for the liver necrosis (Shibayama, 1987; Suzuki *et al.*, 1988). Recent results, however, cast doubt over this theory. For example, inhibition of clot formation was not sufficient to prevent LPS-induced liver injury *in vivo* when active thrombin was present (Hewett and Roth, 1995). Moreover, the ability of thrombin to mediate LPS toxicity independently of fibrin clot formation was confirmed by the genesis of liver damage after addition of the enzyme to the buffer perfusing isolated livers from LPS-treated rats (see Chapter 2). Thus, it appears that thrombin participates in LPS-induced hepatocellular necrosis independently of the formation of insoluble fibrin clots. Furthermore, thrombin acts after the accumulation of inflammatory cells in the liver and the release of certain inflammatory mediators, as evidenced by the ability of heparin or hirudin to prevent liver damage up to two hours after LPS administration. However, the mechanisms of its action and how it interacts

with other cellular or soluble mediators remain to be elucidated.

One possible mechanism is a direct toxic effect of thrombin on hepatic parenchymal cells. Thrombin is a serine protease, and other serine proteases, such as cathepsin G and elastase, damage hepatocytes *in vitro* (Hill and Roth, 1998; Ho *et al.*, 1996; Sauer *et al.*, 1996). Another possibility is an indirect mechanism involving activation by thrombin of one or more critical inflammatory cells. Thrombin has marked effects on cells implicated in the development of LPS-induced liver injury, including endothelial cells (de Groot *et al.*, 1987; DeMichele and Minnear, 1992), platelets (Holmsen and Day, 1970; Venturini and Kaplan, 1992; Lasne *et al.*, 1995; Molino *et al.*, 1995), neutrophils (Bizios *et al.*, 1986; Drake and Issekutz, 1992; Bar-Shavit *et al.*, 1983) and macrophages (Bar-Shavit *et al.*, 1983). High affinity binding sites have also been identified on rat Kupffer cells (Kudhal *et al.*, 1991) and hepatic parenchymal cells (Weyer *et al.*, 1988).

In 1991, a receptor for thrombin (PAR-1) was cloned (Vu *et al.*, 1991), and a new mechanism of receptor activation discovered. After binding to this receptor thrombin cleaves the extracellular domain to expose a new N-terminal sequence that binds to the third extracellular loop of the receptor. This "tethered ligand" activates the receptor. Various thrombin receptor activating peptides (TRAPs) with sequences similar to the tethered ligand (Hui *et al.*, 1992) and the ability to bind to and activate

the receptor (Gerszten *et al.*, 1994) have now been synthesized. In essence, TRAP bypasses thrombin-mediated receptor proteolysis and activates the receptor directly. This mechanism of activation has since been demonstrated on cell types other than platelets (Hung *et al.*, 1992). Since this early discovery, other receptors with the same mechanism of activation have been discovered, forming the family of protease-activated receptors (Coughlin, 1994).

In this study, we examined further the role of thrombin in the mechanism of liver injury that follows LPS exposure. Specifically, we tested whether thrombin mediates its effects through PAR-1 and whether platelets are necessary for the damaging effect of thrombin.

Materials and Methods

Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12, $24x10^6$ EU/mg), bovine serum albumin and Kit 59 for determination of alanine aminotransferase (ALT) activity were purchased from Sigma Chemical Company (St. Louis, MO). The specific activity of the LPS was confirmed using a kinetic chromogenic modification of the *limulus amebocyte* lysate (LAL) assay from BioWhittaker (Walkersville, MA). Human α-thrombin (3048 NIH u/mg) was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Rat thrombin receptor activating peptide (TRAP, SFFLRN) and reverse sequence peptide (NRLFFS) were purchased from Multiple Peptide Systems (San Diego, California) with a purity >97 %. Collagenase type B was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Williams' medium E and gentamicin were purchased from Gibco (Grand Island, NY) and fetal calf serum from Intergen (Purchase, NY). Triton X-100 was purchased from Research Products International (Mount Prospect, IL).

Animals

Male, Sprague-Dawley rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 250-350g were used as hepatocyte and liver donors. The animals were maintained on a 12 hr light/dark cycle under

controlled temperature (18-21^0 C) and humidity (55 ± 5%). Food (Rat chow, Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out according to the guidelines of the American Association of Laboratory Animal Sciences and the University Laboratory Animal Research Unit at Michigan State University.

Preparation and culture of isolated hepatocytes

Liver parenchymal cells were isolated from male rats according to the method of Seglen (Seglen, 1973) as modified by Klaunig (Klaunig *et al.*, 1981). Hepatocyte donors were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and a cannula was inserted into the portal vein. The liver was then perfused with approximately 150 ml of Mg^{2+}-free, Ca^{2+}-free Hanks' balanced salt solution followed by 250 ml of collagenase type B (0.5 mg/ml), and the liver digest was collected and filtered through gauze. The digestion product was subsequently centrifuged at 50xg for two minutes. Cells from the pellet fraction were resuspended in Williams' medium E containing 10% fetal calf serum and 1% gentamicin and plated in six-well plates at a density of $5x10^5$ cells per well. After a three-hour attachment period, the medium with unattached cells was removed, and fresh medium without fetal calf serum was added. Using this isolation procedure, 98% of the cells in the final preparation was hepatic parenchymal cells. The remaining cell population constituted lymphoid

cells, neutrophils and macrophages. The viability of the hepatocytes using this method of isolation was routinely greater than 90%.

Thrombin administration to isolated hepatocytes

At the end of the cell attachment period, human α-thrombin was added in fresh medium (0, 0.04, 0.4, 4 and 40 nM final concentrations). In one set of experiments, the cells were incubated with various concentrations of LPS (0, $12x10^4$, $12x10^6$, $12x10^8$ EU/ml) for two hours before the addition of thrombin. Toxicity was assessed 3, 6 and 16 hours after thrombin addition from the release of ALT into the medium. ALT in the medium was measured using Sigma kit 59-UV and expressed as a percentage of total cellular ALT activity obtained after cell lysis with 1% triton X-100. Previous studies have shown that ALT is a sensitive and selective indicator of hepatic parenchymal cell damage (Loeb, 1989; Kodavanti and Mehendale, 1991) in cultures of hepatocytes and correlates well with cell death measured by other methods (Ganey *et al.*, 1994).

Isolation and perfusion of rat livers

The recirculating perfusion system used in these experiments was described in detail previously (see Chapter 2). Under pentobarbital anesthesia (50 mg/kg, i.p.), the abdominal cavity of the rat was opened, and the portal vein was cannulated with polyethylene tubing (PE 190, Clay

Adams, Parsippany, NJ). Perfusion was started immediately so that the period of ischemia was less than 15 seconds. The perfusion medium comprised Krebs-Henseleit bicarbonate buffer containing 2% bovine serum albumin and was saturated with 95% O_2 / 5% CO_2. Flow was constant at 0.14 ml/min/g body weight. After thoracotomy, an outflow cannula (PE 240 polyethylene tubing, Clay Adams, Parsippany, NJ) was inserted into the thoracic portion of the inferior vena cava and secured to the vessel wall. The liver was removed from the abdominal cavity and transferred into a temperature-controlled perfusion cabinet maintained at 37ºC. Livers were allowed to stabilize for 15 min under conditions of single-pass perfusion. At the end of the stabilization period, a sample of the outflow was taken and analyzed for ALT activity. Livers for which this initial sample contained more than 20 U/L ALT were considered damaged during the surgical procedure and discarded. For the remaining livers a sample of the perfusion solution was then taken from the reservoir before passage through the liver (time 0), and the system was switched to recirculating perfusion. Samples (350 μL) of the recirculating solution were taken every 15 min thereafter for determination of ALT activity. The total volume of recirculating solution was 50 ml. The temperature of the perfusion solution was maintained at 38.5ºC, and the pH (7.4) was monitored by an in-line electrode connected to a pH controller (Chemtrix Inc., Hillsboro, OR). Experiments were performed using two identical systems, allowing

simultaneous perfusion of treated and control livers. For all studies, liver donors were randomly assigned to the various treatment groups and to the two perfusion systems.

Thrombin dose-response in the isolated, perfused liver

Donor rats received LPS ($96x10^6$ EU/kg) as a bolus injection in the tail vein two hours before anesthesia. Studies *in vivo* have shown that this dose of endotoxin leads to significant liver injury within four hours after injection as monitored by the release of ALT in the plasma and histological examination of organ samples. Livers were removed as described above and perfused in a recirculating manner with Krebs-Henseleit buffer containing 2% bovine serum albumin and one of several concentrations of human α-thrombin (0, 0.04, 0.4, 4 and 40 nM). Samples of perfusion medium were taken as described above. After two hours of recirculating perfusion, livers were perfused for ten minutes with 10% buffered formalin in a nonrecirculating manner and then stored in formalin until they were processed for histologic evaluation.

Liver perfusion with buffer containing TRAP

Liver donor rats received LPS ($96x10^6$ EU/kg) as a bolus injection in the tail vein two hours before anesthesia. Their livers were removed and perfused as described above. Either rat thrombin receptor activating

peptide (SFFLRN) or the inactive, reverse sequence peptide (NRLFFS) was added to the perfusion medium (final concentration, 10 μM). Perfusate samples (350 μL) were taken before perfusion and every 15 min thereafter for determination of ALT activity.

Effect of platelet depletion on thrombin-mediated injury in the isolated perfused liver

Liver donors received anti-platelet serum (APS) or control serum (0.5 mL, i.p.) 22 hours before LPS ($96x10^6$ EU/kg, i.v.) administration. This dose of APS has been shown previously to reduce the number of circulating platelets to less than 5% of control and to protect against LPS-induced platelet accumulation and injury in the liver (Pearson *et al.*, 1995). A sample of blood was collected into 0.38% sodium citrate at the time of sacrifice for measurement of circulating platelet and white blood cell numbers. Platelets and white blood cells were counted using a system 9000 cell counter (Serono Baker Diagnostics, Allentown, PA). Livers were isolated for perfusion 2 hours after LPS administration. Perfusion medium comprised Krebs-Henseleit buffer with 2% BSA with or without thrombin (10 nM). Plasma ALT activity was measured in the recirculating medium over time.

Statistical Analysis.

Results are presented as the mean ± standard error of the mean (SEM). For all studies, N represent the number of repetitions of the experiment, each experiment using cells or a liver from a different rat. In cell culture experiments, data expressed as a percentage of total enzyme release were subjected to arcsin transformation and analyzed by Student's *t*-test. In the isolated liver studies, time-dependent changes in ALT activity were analyzed using a two-way, repeated measurements analysis of variance. Multiple comparisons were performed using the Student-Newman-Keuls test. For all studies, the criterion for significance was $p<0.05$.

Results

Effect of thrombin on cultured hepatocytes. Addition of thrombin (40 nM) to buffer perfusing isolated livers from LPS-treated donors results in significant liver damage (see section 2). To investigate whether the damaging action of thrombin is due to a direct toxicity to liver parenchymal cells, we examined the effect of thrombin administration to hepatocytes. Incubation of untreated primary hepatocyte cultures resulted in a small increase in ALT activity in the medium over time. At no time did the addition of thrombin (0.04 to 40 nM) to the culture medium increase the release of ALT from hepatocytes obtained from untreated rat donors (Figure 3.1).

Prior exposure to LPS is required for the toxic action of thrombin in the isolated liver (see Chapter 2). Accordingly it was determined if exposure of parenchymal cells to LPS was sufficient to induce sensitivity to thrombin. Rat hepatocytes were exposed to various concentrations of LPS for 2 hours, and this was followed by the addition of 4 or 40 nM thrombin. As shown in Figure 3.2, LPS addition to the culture medium did not increase ALT activity significantly. Moreover, thrombin was not toxic to hepatocytes in the presence or absence of LPS.

Figure 3.1. Effect of Thrombin on Isolated Rat Hepatocytes. Isolated hepatocytes (2.5 X 10^5/ml) were incubated in serum-free Williams' medium E containing α-thrombin at the indicated concentrations. The activity of ALT was determined in the cell-free medium and cell lysates 3, 6, and 16 hours later. The percentage of total ALT released was calculated as described in Methods. Values represent mean ± SEM, N=4 donors.

tocytes. Isolated

Villiams' medium

The activity of

tes 3, 6, and 16

s calculated as

donors.

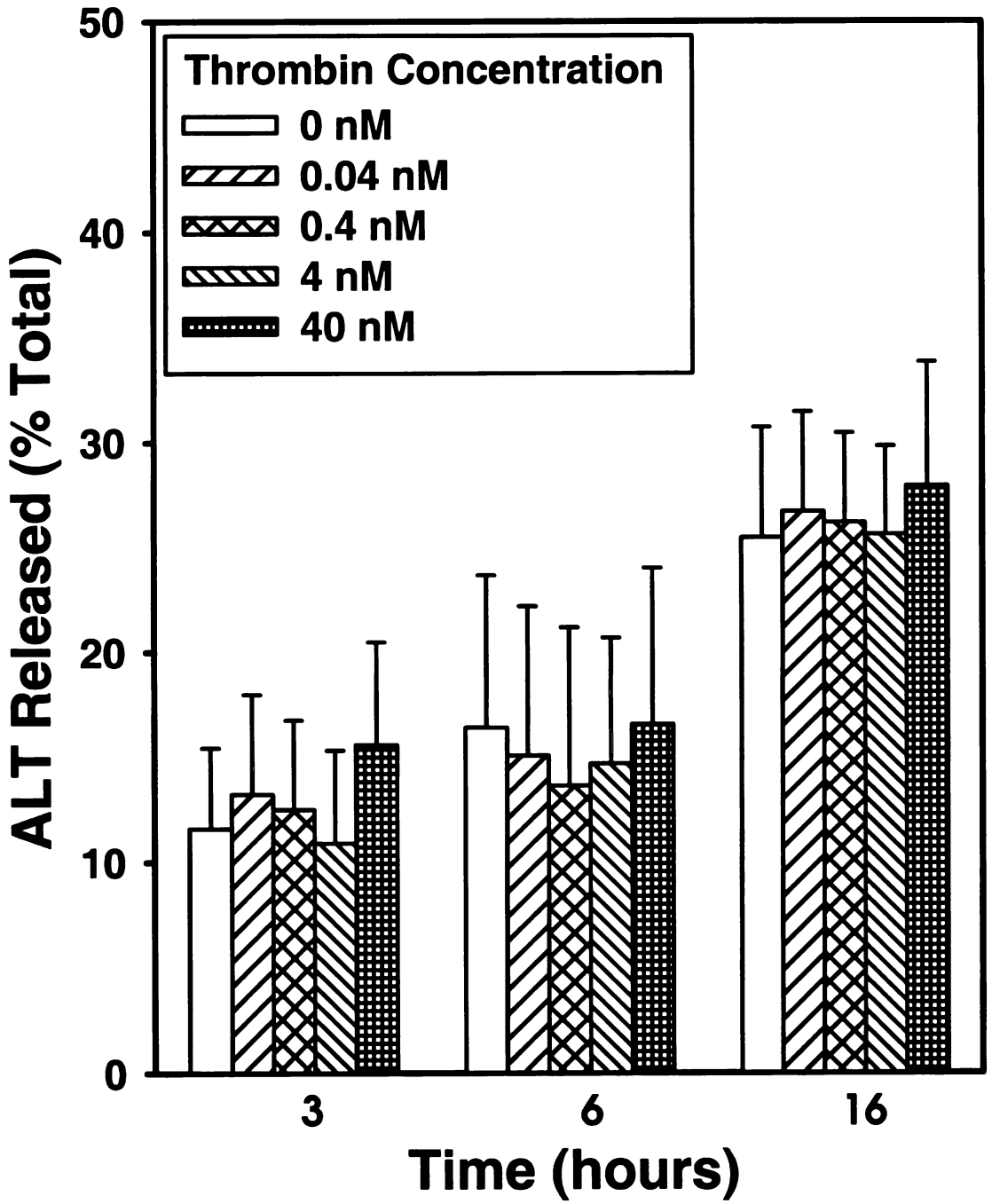


Figure 3.1

Figure 3.2. Effect of Thrombin on Isolated, Rat Hepatocytes in the Presence of LPS. Isolated hepatocytes (2.5 X 10^5/ml) were incubated in serum-free Williams' medium E containing α-thrombin and LPS at the indicated concentrations. The activity of ALT was determined in the cell-free medium and cell lysates 16 hours later. ALT released was calculated as described in Methods. Values represent mean ± SEM, N=4 donors.

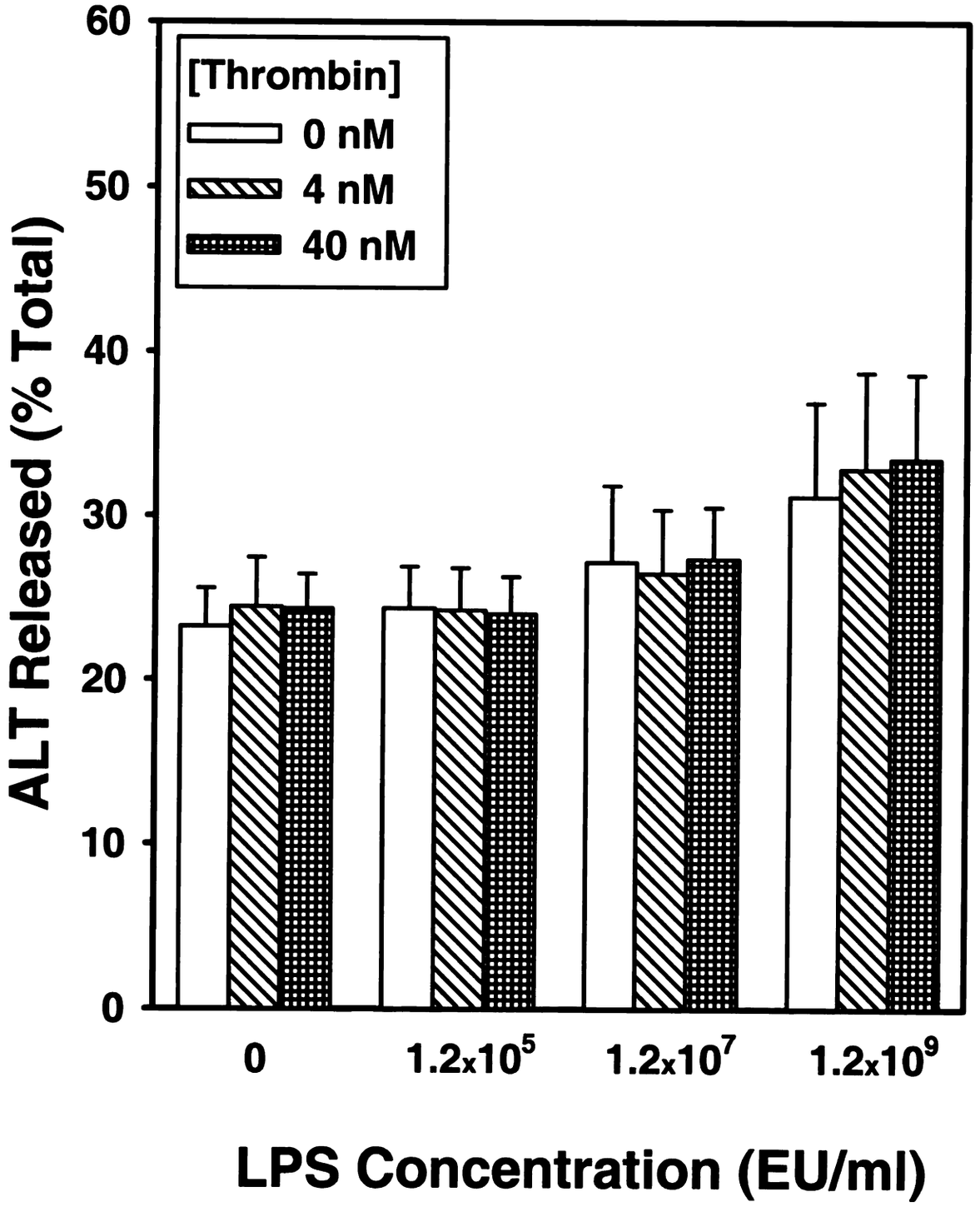

60
50
40
30
20
10
0
[Thrombin]
0 nM
4 nM
40 nM
ALT Released (% Total)
0
1.2x10^5
1.2x10^7
1.2x10^9
LPS Concentration (EU/ml)

Figure 3.2

Similar results were obtained using hepatocytes isolated from donors treated with LPS ($96x10^6$ EU/kg, i.v.) two hours before isolation. As shown in Figure 3.3, prior exposure to LPS *in vivo* neither compromised viability of hepatocytes nor rendered them sensitive to the action of thrombin.

Dose-dependent injury from thrombin in the perfused liver. To determine the concentration-dependence of liver damage in response to thrombin, we perfused isolated livers from LPS-treated rats with various concentrations of the enzyme. Thrombin caused dose-dependent release of ALT from isolated livers of endotoxic rats, with an EC_{50} of approximately 0.4 nM (Figure 3.4). The maximum effect of thrombin occured at 4 nM. None of the thrombin concentrations significantly altered either oxygen consumption or perfusion pressure (data not shown). Histopathologic evaluation at the conclusion of perfusion revealed no striking morphologic changes due to thrombin administration, and no fibrin clots were observed within vessel or sinusoidal lumens.

Effect of TRAP in the perfused liver. We used TRAP (SFFLRN) (Connolly *et al.*, 1994) corresponding to the N-terminal sequence of the cleaved rat PAR-1 to investigate whether or not the effects of thrombin in the isolated liver were linked to activation of this receptor.

Figure 3.3. Effect of Thrombin on Hepatocytes from LPS-Treated Animals. Rats were treated with LPS (96 X 10^6 EU/kg, iv). Two hours later, hepatocytes were isolated from the LPS-treated rats. Isolated hepatocytes (2.5 X 10^5/ml) were incubated in serum-free Williams' medium E containing human α-thrombin at the indicated concentrations. Values represent mean ± SEM, N=4 donors.

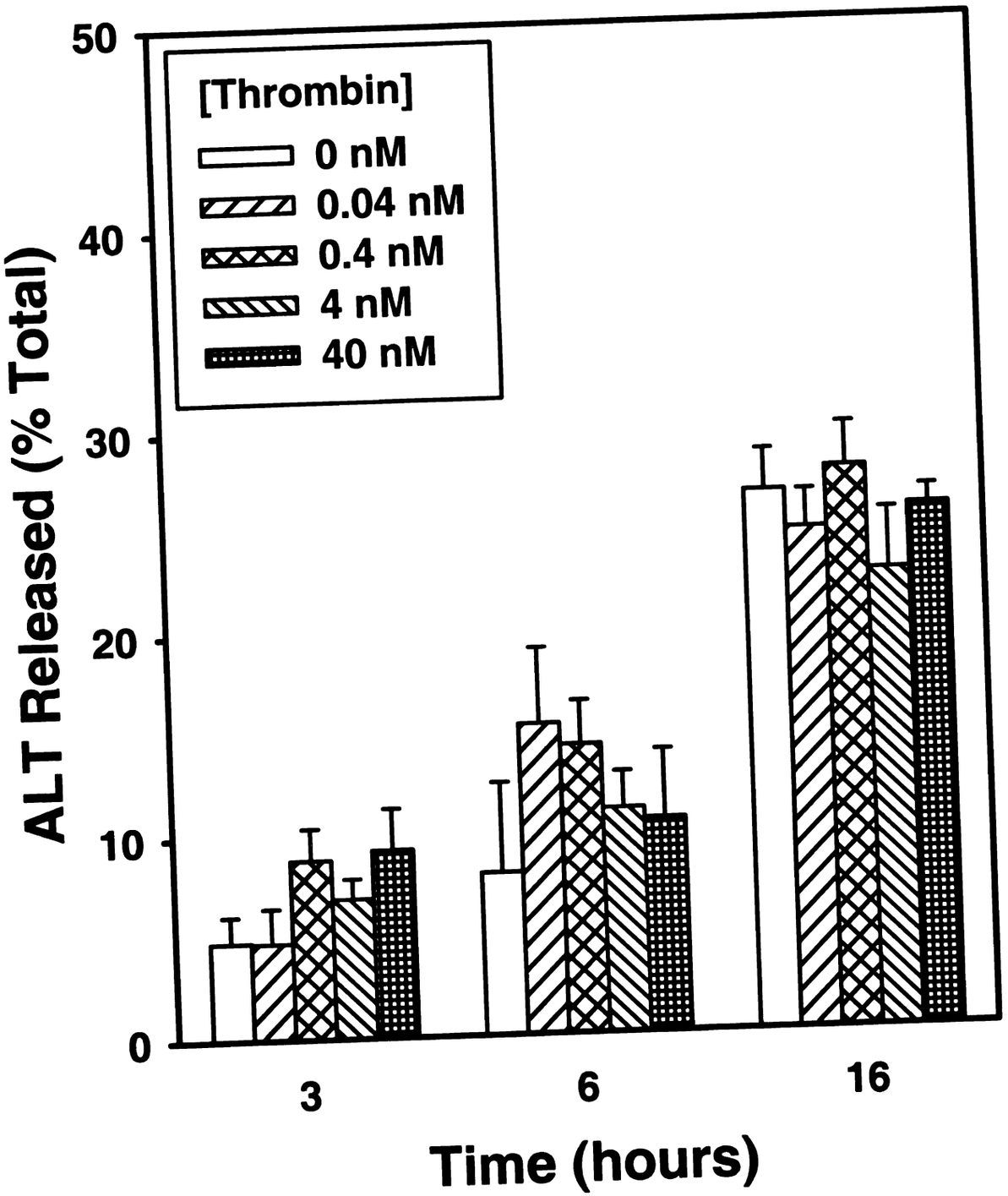

[Thrombin]
0 nM
0.04 nM
0.4 nM
4 nM
40 nM
ALT Released (% Total)
50
40
30
20
10
0
3
6
16
Time (hours)

Figure 3.3

Figure 3.4. Concentration-Dependence of Thrombin-Induced Injury to the Perfused Liver. Liver donors received LPS (96 X 10^6 EU/kg) intravenously 2 hours before perfusion. Perfusion medium comprised Krebs-Henseleit buffer with 2% BSA and various concentrations of thrombin (0 to 40 nM). ALT activity was measured in the recirculating medium at the end of perfusion. Values represent mean ± SEM, N = 9.

* Significantly different from livers perfused with thrombin-free medium.

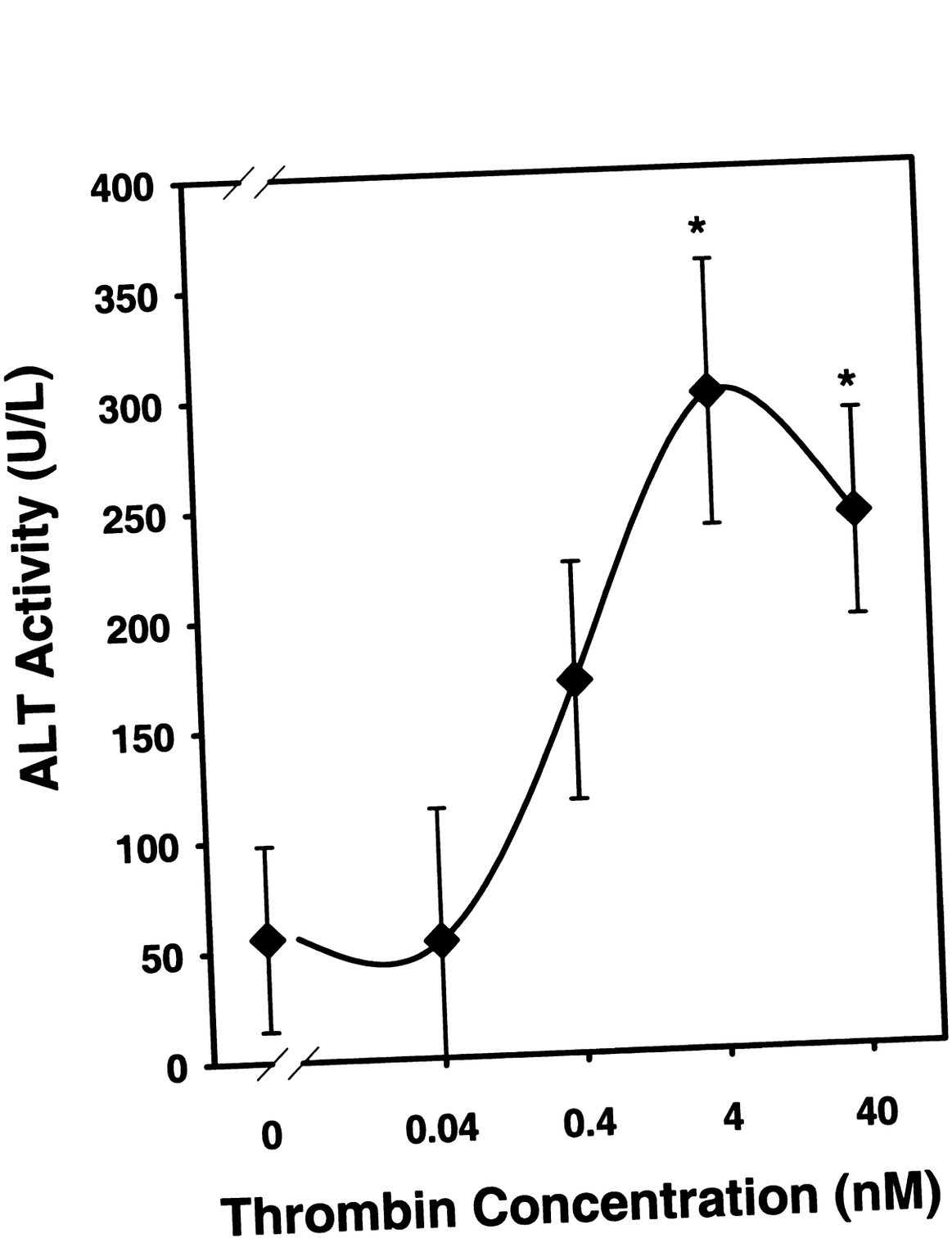

400
350
300
250
200
150
100
50
0
ALT Activity (U/L)
*
*
0
0.04
0.4
4
40
Thrombin Concentration (nM)

Figure 3.4

Isolated livers from LPS-treated rats were perfused with Krebs-Henseleit buffer to which was added either rat TRAP (10 μM) or the reverse sequence peptide (10 μM). A pronounced time-dependent increase in ALT activity occurred in media from livers perfused with TRAP compared to those perfused with the control peptide (Figure 3.5). The amount of ALT released was comparable to that obtained by perfusing livers with 4 nM thrombin (see Figure 3.4).

Effect of platelet depletion on thrombin-induced damage to the isolated liver. Previous studies have shown that platelets accumulate in the liver within minutes after LPS administration and are required for LPS-induced liver injury (Pearson *et al.*, 1995). Thrombin is a potent stimulator of platelets (Hanson and Harker, 1988; Eidt *et al.*, 1989; Badimon *et al.*, 1991), and in humans its action occurs through activation of PAR-1 (Lau *et al.*, 1994). To investigate further the relationship between platelets and thrombin in LPS-induced liver injury, circulating platelet numbers were reduced in rat liver donors with anti-platelet serum (APS), and this was followed by injection of LPS. The APS treatment depleted platelets to a degree that is associated with complete protection from LPS-induced liver injury *in vivo* (Pearson *et al.*, 1995) (Figure 3.6). White blood cell concentration in the circulation was unaffected by the treatment.

Figure 3.5. Release of ALT from Isolated Livers Perfused with TRAP. Liver donor rats received LPS (96 X 10^6 EU/kg, iv) two hours before liver isolation. The livers were then perfused with buffer containing either rat TRAP (SFFLRN, 10 μM) or the inactive, reverse-sequence peptide (NRLFFS, 10 μM). ALT activity was measured in the perfusion effluent as described in Methods. Results are expressed as mean ± S.E.M. N=6 per group.

* Significantly different from reverse sequence peptide.

with TRAP. Liver

hours before liver

ntaining either rat

sequence peptide

rfusion effluent as

± S.E.M. N=6 per

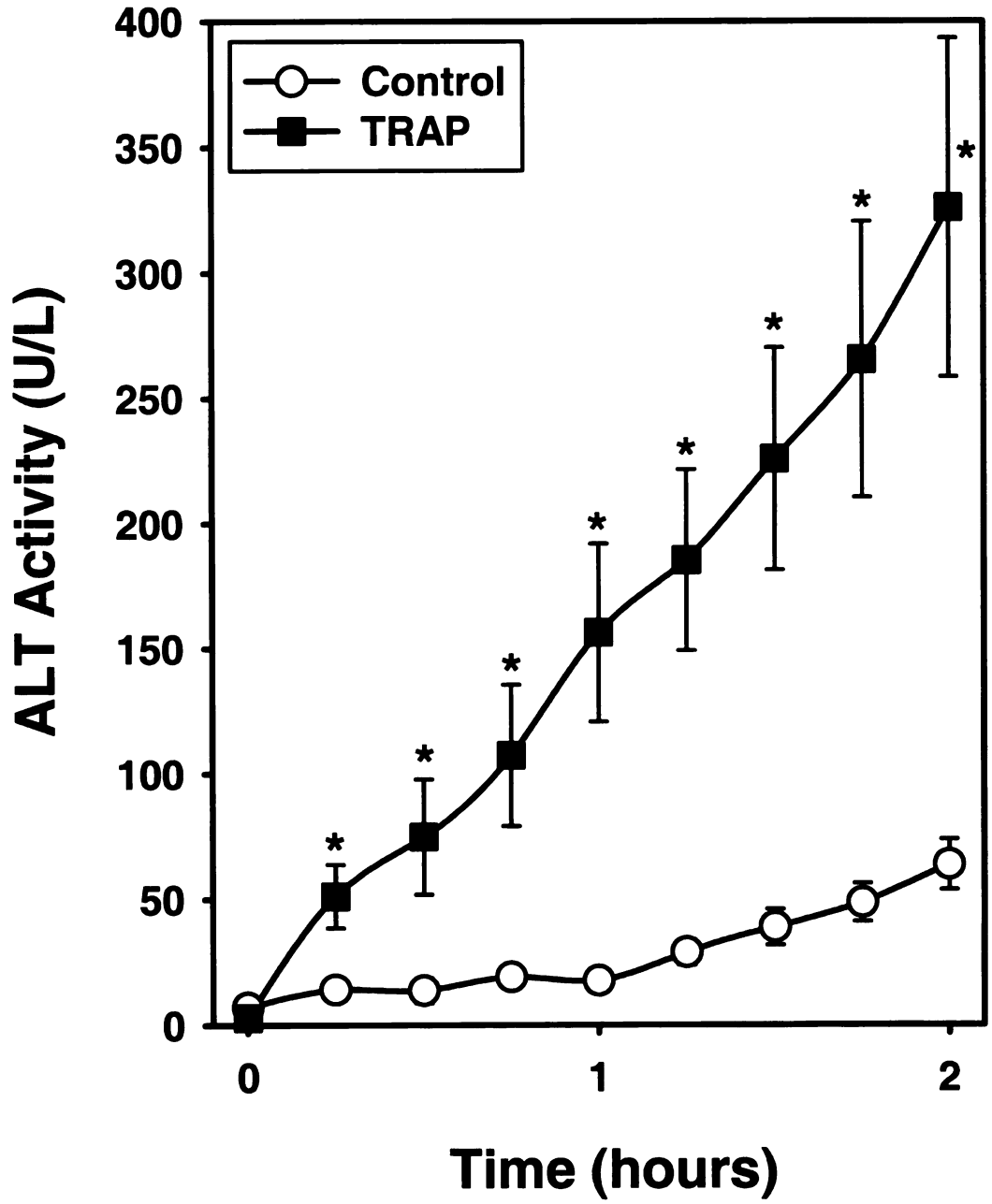


Figure 3.5

Figure 3.6. Effect of Platelet Antiserum on Blood Platelet Concentration. Rats were pretreated with either control serum (CS) or anti-platelet serum (APS) 22 hours before i.v. administration of LPS (96 X 10^6 EU/kg) or saline vehicle. Two hours after LPS administration, blood samples were collected from the inferior vena cava just prior to liver perfusion, and platelets were enumerated as described in Methods. Results are expressed as mean ± SEM, N=5.

* significantly different from CS.

significantly different from vehicle treatment.

et Concentration.

nti-platelet serum

^{6} EU/kg) or saline

es were collected

nd platelets were

essed as mean ±

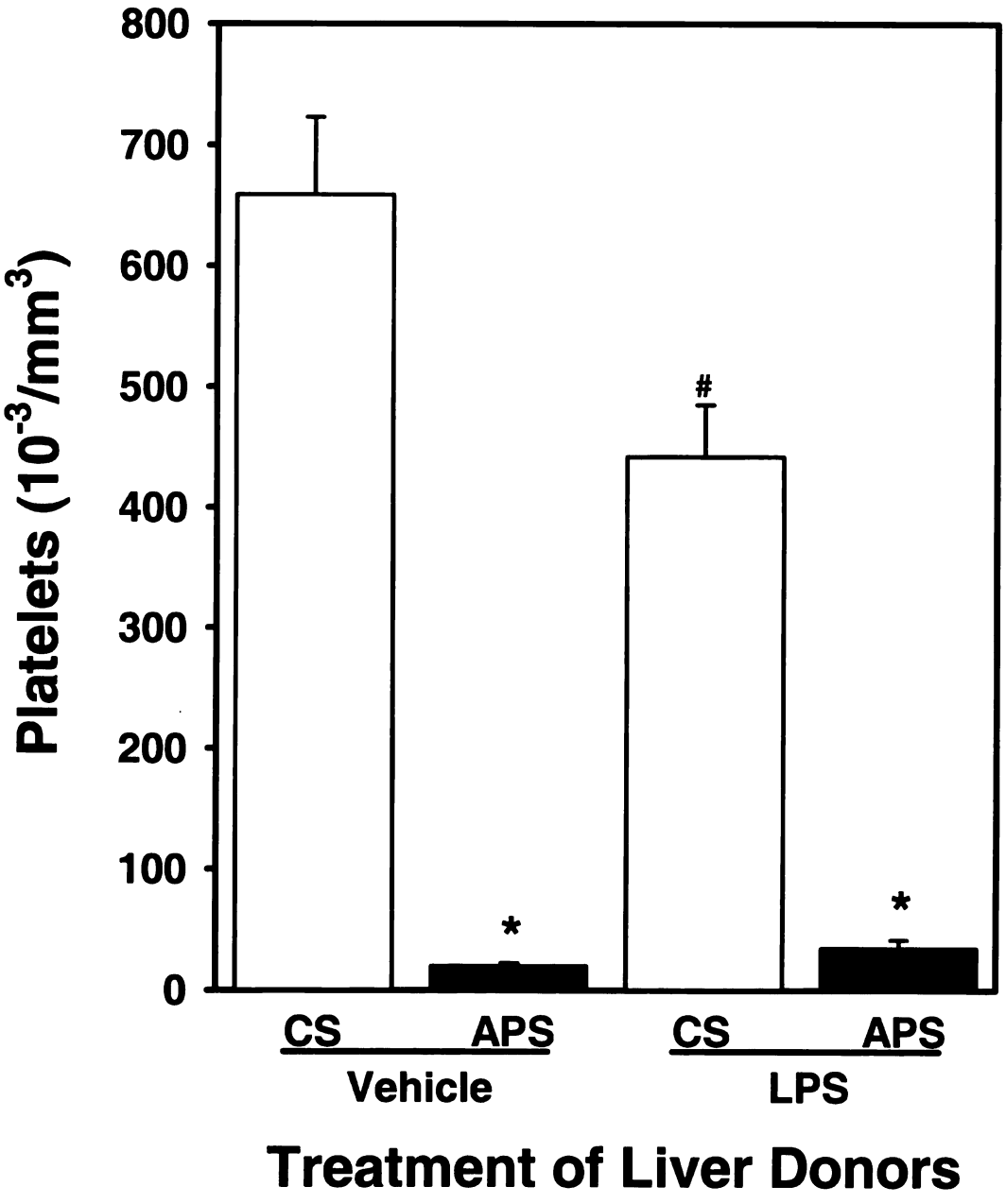


Figure 3.6

Two hours after LPS administration, livers were removed and perfused with buffer containing thrombin. As before, perfusion of livers from LPS-treated animals with thrombin resulted in significant ALT release into the perfusion medium. This injury occurred in livers from rats treated with APS as well as those from rats treated with control serum (Figure 3.7).

Figure 3.7. Thrombin-Induced Injury in Isolated Livers from Platelet-depleted Rats. Liver donors received anti-platelet serum (APS) or control serum (CS) 22 hours before LPS administration (96 X 10^6 EU/kg, iv). Two hours later, livers were removed and perfused with buffer containing thrombin (10 nM). ALT activity was measured in the recirculating medium. Control represents CS-treated liver donors perfused with buffer without thrombin. Results are expressed as mean ± SEM, N = 5.

* Significantly different from livers perfused with thrombin-free medium.

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>S) or control

J/kg, iv). Two

er containing

ıting medium.

>uffer without

medium.

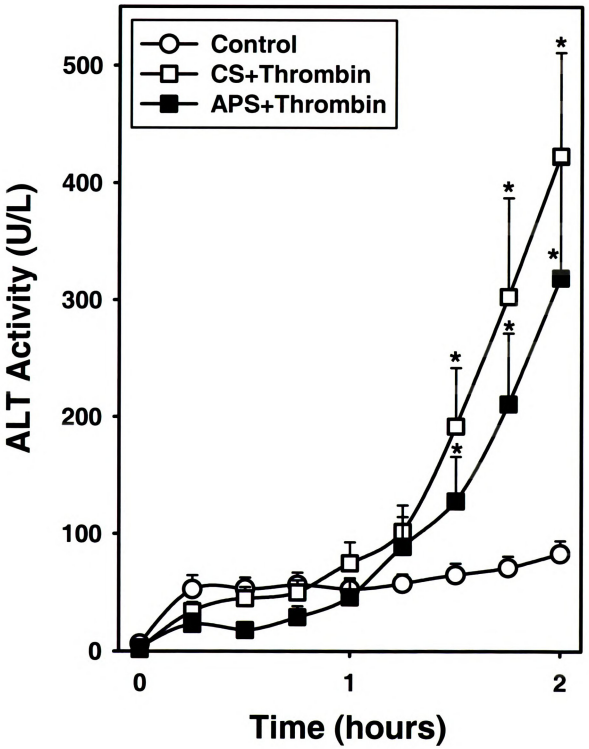

Figure 3.7

Discussion

Disorders of coagulation are frequent in patients with gram-negative bacterial sepsis (Penner, 1998; Vervloet *et al.*, 1998; Gando *et al.*, 1996) and occur in several animal models of endotoxemia (Kruithof *et al.*, 1997; Uchiyama *et al.*, 1992; Ito *et al.*, 1990). Evidence for activation of the coagulation system following exposure to LPS includes a decrease in circulating concentrations of clotting factors (Prager *et al.*, 1979) and an increase in the prothrombin and partial thromboplastin times (Gurewich *et al.*, 1976; Yoshikawa *et al.*, 1981). A decrease in the concentration of plasma fibrinogen within 3 hours after intravenous injection of LPS in rats is followed by an increase in plasma levels of fibrin split products, indicating transformation of prothrombin into thrombin. Previous studies in rats have shown that activation of the coagulation cascade, with subsequent formation of thrombin, is important for LPS-induced liver injury. Anticoagulants, such as warfarin, heparin, and hirudin, block hepatic parenchymal cell damage and prevent endotoxin-induced lethality (Margaretten *et al.*, 1967; Nowak and Markwardt, 1980; Pearson *et al.*, 1996). Although these studies point to thrombin as a critical factor in the pathogenesis of LPS-induced liver injury in the rat, its exact role has not been elucidated.

Because thrombin is a serine protease, one possibility we tested was that the enzyme might damage hepatocytes directly. Other serine

proteases, i.e. cathepsin G and elastase, can induce hepatocellular killing (Ho *et al.*, 1996; Ganey *et al.*, 1994) *in vitro*. Indeed, thrombin itself is capable of damaging other cell types in culture (Cunningham and Donovan, 1997). In studies presented here, concentrations of thrombin which led to significant hepatic injury in isolated livers did not damage cultured hepatocytes (Figure 3.1). This observation was consistent with results from isolated liver studies in which perfusion of livers from naive animals with thrombin alone did not produce liver damage. However, prior exposure of cultured hepatic parenchymal cells to LPS itself (Figure 3.2) or to LPS and the mediators released within two hours of its administration *in vivo* (Figure 3.3) did not induce sensitivity of the hepatocytes to thrombin. Accordingly, thrombin is not directly toxic to liver parenchymal cells in culture.

In cell cultures, thrombin activates cellular responses through receptor-mediated mechanisms at concentrations between 0.5-50 nM (De Caterina and Sicari, 1993). In the isolated, perfused liver the maximum effect was reached at 4 nM (Figure 3.4). This suggested the possibility of a receptor-mediated mechanism of toxicity. Inasmuch as thrombin produces effects on several cell types through activation of PAR-1, we investigated further the role of PAR-1 in LPS-induced liver injury.

TRAP is a peptide with an amino acid sequence identical to the active portion of the tethered ligand for PAR-1 but devoid of any proteolytic

activity (Gerszten *et al.*, 1994; Chao *et al.*, 1992; Hui *et al.*, 1992). In various cell culture systems, TRAP can reproduce the PAR-1-mediated actions of thrombin. It usually is about 1000 fold less potent than native thrombin in producing cellular effects (Lasne *et al.*, 1995; Chen *et al.*, 1994; Hui *et al.*, 1992). From the observation that the concentration of thrombin leading to maximal effect in the isolated liver was 4 nM, we chose to infuse TRAP at a concentration of 10 μM. Isolated livers from LPS-treated rats released ALT into the perfusion medium when perfused with TRAP (Figure 3.5). ALT release was not observed from livers perfused with a control, reverse-sequence peptide that does not activate the thrombin receptor. The ability of TRAP to replace thrombin during endotoxin-induced liver injury suggests that thrombin promotes toxicity through activation of the PAR-1 receptor, since the other member of the PAR family which is activated by thrombin (PAR-3) is not activated by TRAP (Hou *et al.*, 1998).

The exact mechanism by which activation of PAR-1 by thrombin leads to hepatic parenchymal cell death remains subject to conjecture. Since hepatocytes were not directly damaged by thrombin *in vitro*, the injury is likely indirectly mediated through another cell type. PAR-1 has been identified on the surface of human platelets, endothelial cells, neutrophils and Ito cells. It can modify gene expression and protein synthesis in endothelial cells (de Groot *et al.*, 1987; DeMichele and

Minnear, 1992). This leads to an increased production of inflammatory mediators, modification of the interactions between endothelial cells and either the underlying matrix or other endothelial cells and expression of adhesion glycoproteins on the cell surface. Thrombin stimulates proliferation of the liver fat-storing cells and expression of monocyte chemotactic protein-1, and this effect is reproduced by administration of TRAP (Marra *et al.*, 1995). In addition, thrombin was recognized early for its ability to stimulate platelets and cause them to spread, aggregate, adhere to the vascular wall and release components of their alpha and dense granules (Holmsen and Day, 1970; Venturini and Kaplan, 1992; Lasne *et al.*, 1995; Molino *et al.*, 1995).

Platelets accumulate in the liver shortly after LPS administration *in vivo* and are a critical component of the mechanism of liver injury. Accordingly, it seemed conceivable that thrombin might play a role in LPS-induced liver injury through an interaction with platelets. If so, thrombin could either promote the sequestration of platelets in the liver and/or activate platelets. However, results from previous studies suggest that thrombin does not influence the sequestration of platelets in the liver: hepatic platelet accumulation begins within 15 minutes after intravenous injection of LPS into rats (Pearson *et al.*, 1995), and activation of the coagulation system does not occur until 2-5 hours. Moreover hirudin, a selective inhibitor of thrombin, failed to affect the accumulation of platelets

in the liver (Pearson *et al.*, 1996). The possibility still remained that thrombin acts through activation of platelets already in the liver. However, platelet depletion of rat liver donors by a method that afforded protection from LPS-induced liver injury *in vivo* failed to alter the damaging effect of thrombin in the isolated liver (Figure 3.7). These results indicate that thrombin does not act through platelet activation to injure hepatocytes. A possibility consistent with the results *in vivo* and in the isolated liver is that platelets promote formation of thrombin in the sinusoids, perhaps by providing a surface for assembly of the prothrombinase complex (Ofosu *et al.*, 1996; Billy *et al.*, 1997; Bevers *et al.*, 1991).

The exact mechanism by which thrombin causes tissue damage after exposure to LPS remains unknown but most likely requires other inflammatory cells. For example neutrophil depletion protects against LPS-induced liver injury *in vivo*, and in the presence of platelets thrombin enhances neutrophil activation *in vitro* (Moon *et al.*, 1990). Activated neutrophils are required for LPS-induced liver injury (Hewett *et al.*, 1992), and thrombin is a potent chemoattractant for these cells (Bizios *et al.*, 1986). Accordingly, it is possible that neutrophils interact in some way with thrombin to promote parenchymal cell death. Since TRAP and thrombin were able to induce hepatic injury in the isolated liver, we have to consider the likelihood of another cell type responding to both agonists via activation

of PAR-1 and in turn stimulating neutrophils to kill the hepatocytes. This possibility will be explored in Chapter 4.

In summary, these studies demonstrate that thrombin damages liver previously exposed to LPS in a dose-dependent manner. In doing so, the enzyme does not induce direct hepatic parenchymal cell death, but rather requires the intervention of secondary mediators of toxicity. The cell type on which thrombin acts after LPS exposure remains unknown; however the interaction between thrombin and these mediators can be reproduced by TRAP, and therefore most likely proceeds through activation of PAR-1. Finally platelets, which play a critical role in the mechanism of toxicity *in vivo*, appear to act upstream from thrombin's action and most likely contribute by increasing local thrombin concentration and/or activity. To our knowledge, this is the first report of thrombin involvement via a receptor-mediated mechanism in an uncontrolled inflammatory process producing injury in an intact tissue.

CHAPTER 4

INTERDEPENDENCE OF THROMBIN AND PMNs IN LIVER INJURY DURING LPS EXPOSURE

Introduction

Lipopolysaccharide is a component of the cell walls of gram-negative bacteria. It is released in the blood during bacterial lysis (Hewett and Roth, 1993). Many of the adverse effects of LPS are dependent on the activation of cellular and soluble inflammatory mediators, among which are platelets, polymorphonuclear leukocytes (PMNs) (Hewett *et al.*, 1992; Jaeschke *et al.*, 1991), Kupffer cells (Iimuro *et al.*, 1994), various cytokines (Hewett *et al.*, 1991), and components of the coagulation cascade (Hewett and Roth, 1993). The liver is exposed to each one of these factors at some point during the cascade of events that follows administration of LPS *in vivo* (see Figure 1.6).

PMNs are phagocytic cells that play an essential role in the defense against microorganisms. Upon activation, these cells undergo a respiratory burst generating free radicals and release into the extracellular space the contents of their cytosolic granules. Although PMNs are undoubtedly critical components of the host immune defense, their ability to become activated and release cell-damaging intermediates has been implicated in host tissue injuries (Smith, 1994). Several models of neutrophil-dependent injury have been described, including endotoxin-induced lung (Brigham and Meyrick, 1986) and liver damage (Jaeschke *et al.*, 1991) and ischemia/reperfusion injury in the heart (Romson *et al.*, 1983) and liver (Jaeschke and Smith, 1997b).

Increasing evidence suggests that there may be a link between PMNs and coagulation components in the pathogenesis of LPS-induced liver injury. For example thrombin can stimulate degranulation (Baranes *et al.*, 1986) and the release of tissue kallikrein (Cohen *et al.*, 1991) and thromboxane B2 (Del Vecchio *et al.*, 1987) from PMNs. Thrombin also induces chemotaxis of PMNs (Bizios *et al.*, 1986), an important step in neutrophil-induced hepatocellular killing *in vivo* (Jaeschke *et al.*, 1996).

Both PMNs and thrombin are required *in vivo* for LPS-induced liver injury (Hewett *et al.*, 1992; Pearson *et al.*, 1996), but their interactions with the numerous other extrahepatic mediators released in response to LPS, as well as their specific mechanism of toxicity remain poorly understood. Experiments in the isolated liver model have shown that exposure to thrombin of livers from LPS-treated donors but not from naïve donors resulted in hepatic injury (Figure 2.7). This result demonstrates that the some of the events occuring after LPS exposure *in vivo* are required for thrombin to induce hepatic injury. Accordingly, in this study we verified the critical role of PMNs in LPS-induced injury in the isolated liver. To determine if PMNs, thrombin and LPS are sufficient to produce hepatic damage, we perfused livers from naive donors with various combinations of these three factors. Finally, to clarify some of the interactions between the PMNs and thrombin, we evaluated the influence of this enzyme on PMN functions involved in cellular killing.

Materials and Methods

Materials

Lipopolysaccharide (*Escherichia coli*, serotype 0128:B12, $24x10^6$ EU/mg), bovine serum albumin, Glycogen (Type II from Oyster), Kit 59 for determination of ALT activity, Kit 826-UV for determination of lactate concentration and Kit 510-A for the determination of glucose concentration were purchased from Sigma Chemical Company (St. Louis, MO). The specific activity of the LPS was confirmed in our laboratory using a kinetic chromogenic modification of the *limulus* amebocyte lysate assay from BioWhittaker (Walkersville, MA). Human α-thrombin (3048 NIH U/mg) was purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Collagenase type B was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Williams' medium E and gentamicin were purchased from Gibco (Grand Island, NY) and fetal calf serum from Intergen (Purchase, NY). Triton X-100 was purchased from Research Products International (Mount Prospect, IL). Tissues were fixed using neutral buffered formalin (Surgipath Medical Industries, Inc. , Richmond, IL).

Animals

Sprague-Dawley, male rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 250-350g were used as hepatocyte and liver

donors. Male, retired breeders were used for PMN collection. The animals were maintained on a 12 hr light/dark cycle under controlled temperature (18-21^0 C) and humidity (55 ± 5%). Food (Rat chow, Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out according to the guidelines of the American Association of Laboratory Animal Sciences and the University Laboratory Animal Research Unit at Michigan State University.

Isolation of rat PMN

Retired breeders received 35 ml of a 1% glycogen solution in 0.9% sterile saline solution intraperitoneally. Four hours later, animals were anesthetized with diethyl ether and sacrificed by exsanguination. 30 ml of heparinized (1 U/ml), phosphate-buffered saline solution (PBS, pH 7.4) were injected into the peritoneum. The abdominal wall was incised, and the content of the abdominal cavity was poured through 3 layers of gauze into a centrifugation tube. The final volume in the tube was then brought to 50 ml with PBS, and it was spun in a centrifuge at 500g for 7 minutes. The supernatant was discarded, and the pellet was resuspended in 15 ml of NH_4Cl (0.15 M) to lyse red blood cells. After 2 minutes, 35 ml of PBS was added and the combined contents spun for 7 minutes at 320g. The supernatant was discarded, the pellet resuspended into 50 ml PBS and spun again for 7 minutes at 320g. The supernatant was discarded again

and the pellet resuspended into 10 ml PBS. The number of PMNs per ml was determined using an hemacytometer and adjusted to a final concentration of $3x10^6$ PMNs/ml.

Isolation and perfusion of rat livers

The recirculating perfusion system used in these experiments was described in detail previously (see Chapter 2). Under pentobarbital anesthesia (50 mg/kg, i.p.), the abdominal cavity of the rat was opened, and the portal vein was cannulated. Perfusion was started immediately so that the period of ischemia was less than 15 seconds. The perfusion medium was composed of Krebs-Henseleit bicarbonate buffer containing 2% bovine serum albumin and saturated with 95% O_2 and 5% CO_2 gas. Flow was constant at 35 ml/min. An outflow cannula was inserted into the thoracic portion of the inferior vena cava, and the liver was transferred into a temperature-controlled perfusion cabinet maintained at 37ºC. Livers were allowed to stabilize for 15 min under conditions of single-pass perfusion. At the end of the stabilization period, a sample of the outflow was taken and analyzed for ALT. Livers for which this initial sample contained more than 20 U/L ALT were considered damaged during the surgical procedure and discarded. For the remaining livers, a sample of the perfusion solution was taken from the reservoir before passage through the liver (time 0), and the system was switched to recirculating perfusion. The total volume of

solution in the recirculating system was 50 ml. The temperature of the perfusion solution was maintained at 38.5ºC, and the pH was monitored (7.4) by an in-line electrode connected to a pH controller (Chemtrix Inc., Hillsboro, OR). Experiments were performed using two identical systems, allowing simultaneous perfusion of treated and control livers. In all studies, liver donors were randomly assigned to the various treatment groups and the two perfusion systems.

Preparation of immunoglobulins (Igs)

Rat PMNs were elicited from the peritoneum of male retired breeders as described above. Rabbits were then immunized against rat PMNs by injecting the cells (10^6 cells/ml) in suspension in Freund's adjuvant (Sigma Chemical Company, St. Louis, MO) in three subsequent administrations at two week intervals (Hewett *et al.*, 1992). To prepare anti-PMN immunoglobulin (AN-Ig), blood was collected from the central ear artery one week after the last PMN booster injection, and the total Ig fraction was precipitated from serum using ammonium sulfate. The precipitate was dialyzed against saline to remove contaminating ammonium sulfate, and flocculent material was removed by centrifugation. Control immunoglobulin (C-Ig) was prepared by the same procedure from the blood of non-immunized rabbits. The supernatant fluids were tested for endotoxin content by the LAL assay and stored at -20°C until used.

Perfusion with thrombin of livers from PMN-depleted rats

Liver donors received immunoglobulins isolated from rabbits immunized with rat PMN (AN-Ig) or from untreated rabbits (C-Ig) as two separate injections (0.5 ml) in the tail vein at 18 and 6 hours before LPS ($96x10^6$ EU/kg, i.v.) administration. This dose of AN-Ig has been shown previously to reduce the number of circulating PMNs to less than 5% of control (Hewett *et al.*, 1992). A sample of blood was collected into 0.38% sodium citrate at the time of sacrifice. Platelets and white blood cells were counted using a system 9000 cell counter (Serono Baker Diagnostics, Allentown, PA) and a differential count made on blood smears to verify neutrophil depletion. Livers were isolated for perfusion 2 hours after LPS administration. Perfusion medium comprised Krebs-Henseleit buffer with 2% BSA and 10 nM thrombin. This concentration of thrombin was shown in earlier studies to be above the dose producing maximal injury to perfused livers from LPS-treated animals (Figure 3.4). Samples (350 μL) of the recirculating solution were taken every 15 min, and ALT activity was measured in the recirculating medium over time.

Liver perfusion with LPS, PMNs and thrombin

Five hours before liver isolation, PMN donors were injected with glycogen. PMNs were isolated as described above and resuspended at a concentration of $3x10^6$ PMN/ml in Krebs-Henseleit perfusion buffer

containing 2% BSA. Liver donor rats were anesthetized, and the liver was removed and perfused for 10-15 minutes in a single pass manner as described above. The livers were then perfused for four hours in a recirculating manner with or without PMNs ($2x10^6$ PMNs/ml final concentration). LPS ($96x10^6$ EU/kg of donor rat weight) or its saline vehicle was introduced into the perfusion buffer 10 min after the start of recirculation. Perfusate samples (350 µL) were taken every 30 minutes for the first 2 hours. After two hours of recirculating perfusion, either thrombin (10 nM final concentration) or an equal volume of saline vehicle was introduced into the perfusion buffer, and samples were collected every 15 minutes. All samples were used for determination of ALT activity and glucose and lactate concentrations. The rates of production of glucose and lactate (µmol/g/h) were calculated from changes in concentration in the perfusate, flow rate and liver weight. ALT activity in the outflow was used as a marker of hepatic parenchymal cell injury since PMNs contain negligible amounts of this enzyme (Guigui *et al.*, 1988).

Preparation and culture of isolated hepatocytes

Hepatocytes were isolated from male rats according to the method of Seglen (Seglen, 1973) as modified by Klaunig (Klaunig *et al.*, 1981). Rats were treated with LPS ($96X10^6$ EU/kg) or its saline vehicle two hours prior to isolation. Rats were anesthetized with sodium pentobarbital

(50 mg/kg, i.p.), and a cannula was inserted into the portal vein. The liver was then perfused with approximately 150 ml of Mg^{2+}-free, Ca^{2+}-free Hanks' balanced salt solution followed by 250 ml of collagenase type B (0.5 mg/ml), and the liver digest was collected and filtered through gauze. The digestion product was subsequently centrifuged at 50xg for two minutes. Cells from the pellet fraction were resuspended in Williams' medium E containing 10% fetal calf serum and 1% gentamicin and plated in six-well plates at a density of $5x10^5$ cells per well. After a three hour attachment period, the medium with unattached cells was removed, and fresh medium without fetal calf serum was added. Using this isolation procedure, 98% of the cells in the final preparation were hepatic parenchymal cells and the viability of the hepatocytes was routinely greater than 90%.

Hepatocyte/Neutrophil Cocultures

PMNs ($5X10^6$/well) were added to adherent cultures of hepatocytes ($5X10^5$/well). Cells were incubated as described above in the absence of fetal calf serum. PMNs were allowed to attach for 30 minutes, after which thrombin at concentrations of 0.04, 0.4, 4, or 40 nM was added. Following a 3, 6, or 16 hour incubation period, the medium was collected. The cells remaining on the plate were lysed with 1% Triton X-100 and sonication. Both the medium and the lysates from the plates were spun in a centrifuge

at 600*g* for 10 minutes. The activity of ALT in the cell-free supernatant fluids was determined. The ALT activity in the medium was expressed as a percentage of the total activity (activity in the medium plus activity in cell lysates). It was shown previously that at a PMN/hepatocyte ratio of 10:1, the total activity of ALT in the PMNs is less than 10% of the total activity in hepatocytes (Ganey *et al.*, 1994). Thus, ALT activity released into the medium was taken as an index of hepatocellular injury in these experiments.

In subsequent experiments, the addition of PMNs to cultures of hepatocytes was followed by the addition of 40 nM thrombin in the presence or absence of cytocholasin B (5 µg/ml) and fMLP (100 nM). These concentrations of cytocholasin B and fMLP activate PMNs to damage hepatocytes in PMN/hepatocyte cocultures (Ganey *et al.*, 1994). After a 16 hour incubation ALT released into the medium was analyzed as described above.

Measurement of Myeloperoxidase Release from PMN

Rat PMNs ($5X10^6$ PMN/ml) in serum-free Williams' medium E were incubated with 0, 0.4, or 4 nM thrombin in the presence or absence of 1, 10, or 100 nM fMLP with or without 5 µg/ml cytocholasin B. The cells were incubated in a shaking water bath at 37°C. After a 1 hour incubation, the PMNs were removed from the samples by centrifugation at 400*g* for 7

minutes. Myeloperoxidase (MPO) release into the medium was then measured according to the method of Henson (Henson *et al.*, 1978). An additional sample of PMNs ($5X10^6$ PMN/ml) was lysed by sonication. This sample was used to measure total MPO in the PMN samples. The percent of total MPO released into the supernatant was calculated by dividing the value obtained for the supernatant by the value obtained for the PMN lysate and multiplying by 100.

Statistical Analysis.

Results are presented as the mean ± standard error of the mean (SEM). For all studies, N represents the number of repetitions of the experiment, each experiment using cells or liver from a different rat. In cell culture experiments, data expressed as a percentage of total enzyme release were subjected to angular transformation and analyzed by Student's *t*-test. In the isolated liver studies, time-dependent changes in ALT activity were analyzed using repeated measurements analysis of variance (ANOVA). Multiple comparisons were performed using the Games/Howell test. Data sets that did not meet the criterion of homogeneity of variance or normality were transformed before analysis. For all studies, the criterion for significance was $p<0.05$.

Results

Effect of PMN depletion on thrombin-induced damage to the isolated liver. PMNs accumulate in the liver within minutes after LPS administration and are required for LPS-induced liver injury *in vivo* (Hewett *et al.*, 1992). Thrombin damages perfused livers isolated from rats treated with LPS two hours earlier but not livers from naive rats (see Chapter 2). To investigate whether PMNs are required in isolated livers from LPS-treated rats for thrombin-mediated hepatic injury, blood neutrophil numbers were reduced in rat liver donors using AN-Ig treatment, and this was followed by injection of LPS. The immunoglobulin treatment depleted blood PMNs to less than 5% of control values, a degree that almost completely eliminates LPS-induced liver injury *in vivo* (Hewett *et al.*, 1992). Two hours after LPS administration, livers were isolated and perfused with buffer containing thrombin. As previously reported (Chapter 2 and 3), perfusion of livers from LPS-treated animals with thrombin resulted in pronounced ALT release over time into the perfusion medium when the donors had received the C-Ig (Figure 4.1). This injury was markedly reduced by pretreatment with AN-Ig, bringing the time-dependent ALT release of these livers down to the level which was observed in livers from LPS-treated donors perfused without thrombin (Figure 2.7).

Figure 4.1. Thrombin-Induced Injury in Isolated Livers from PMN-Depleted Rats treated with LPS. Liver donors received anti-neutrophil immunoglobulin or control immunoglobulin 18 and 6 hours before LPS administration ($96x10^6$ EU/kg, iv). Two hours later, livers were removed and perfused with Krebs-Henseleit buffer containing thrombin (10 nM). ALT activity was measured in the recirculating medium. Results are expressed as mean ± SEM, N = 5.

* Significantly different from livers from control immunoglobulin-treated rats perfused with thrombin.

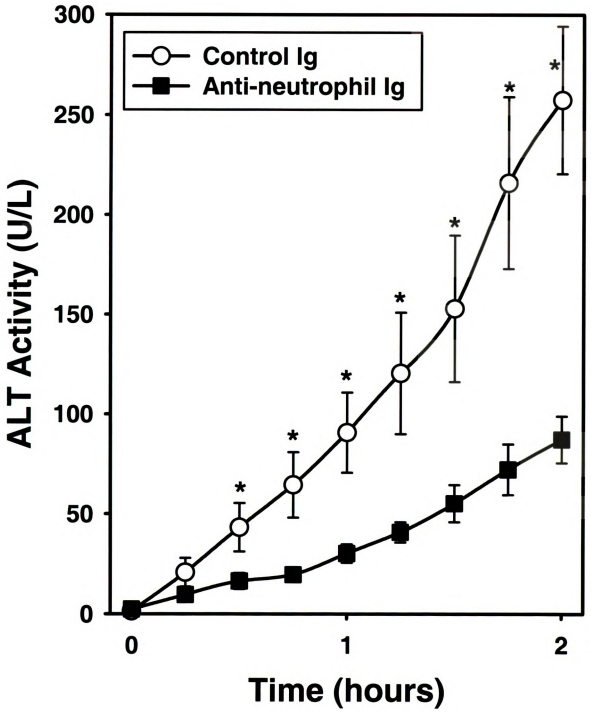
Control Ig
Anti-neutrophil Ig
ALT Activity (U/L)
Time (hours)
300
250
200
150
100
50
0
0
1
2
*

Figure 4.1

Requirement for PMNs, LPS and thrombin for injury to livers from naive donors. The addition of thrombin to livers from rat donors treated two hours before perfusion with LPS results in a marked release of ALT into the medium over time. Within this time period, liver cells as well as PMNs accumulated in the sinusoids, have been exposed to various soluble inflammatory mediators released during systemic LPS exposure. It is known from previous studies that the presence of PMNs, thrombin activity and the exposure to LPS are three necessary conditions for the expression of endotoxic liver injury in the rat. However, other extrahepatic factors may be important determinants of the hepatocellular damage *in vivo* or in the isolated, perfused liver. To determine if the presence of LPS, neutrophils and thrombin is sufficient to produce hepatocellular injury after LPS exposure, livers isolated from naive donors were perfused with various combinations of these three factors.

Perfusion of livers from naive donors with buffer resulted in a small increase in ALT activity in the recirculating medium which resembled what we had seen previously in control livers. At two hours, the ALT activity in the perfusion buffer of controls was 27 ± 4 U/L. During the first two hours, the addition of PMNs alone to the perfusion buffer did not significantly change the release of ALT (Figure 4.2-A). Similarly, perfusion with LPS alone did not elicit release of ALT.

Figure 4.2-A. Release of ALT from Isolated Livers During the First Two Hours of Perfusion with LPS and PMN. Livers from untreated donor rats were perfused with Krebs-Henseleit buffer containing 2% bovine serum albumin. Rat PMNs (final concentration $2x10^6$ PMN/ml) were added to the perfusion buffer at time 0 in the indicated treatment groups. After 10 minutes, LPS ($96x10^6$ EU/kg of donor rat weight) or its saline vehicle was added to the perfusion buffer. Samples of the recirculating medium were collected every 30 minutes for two hours. ALT activity was determined in all samples as described in Methods. Results are expressed as mean ± SEM, N=16 per group.

* significantly different from respective group without LPS.

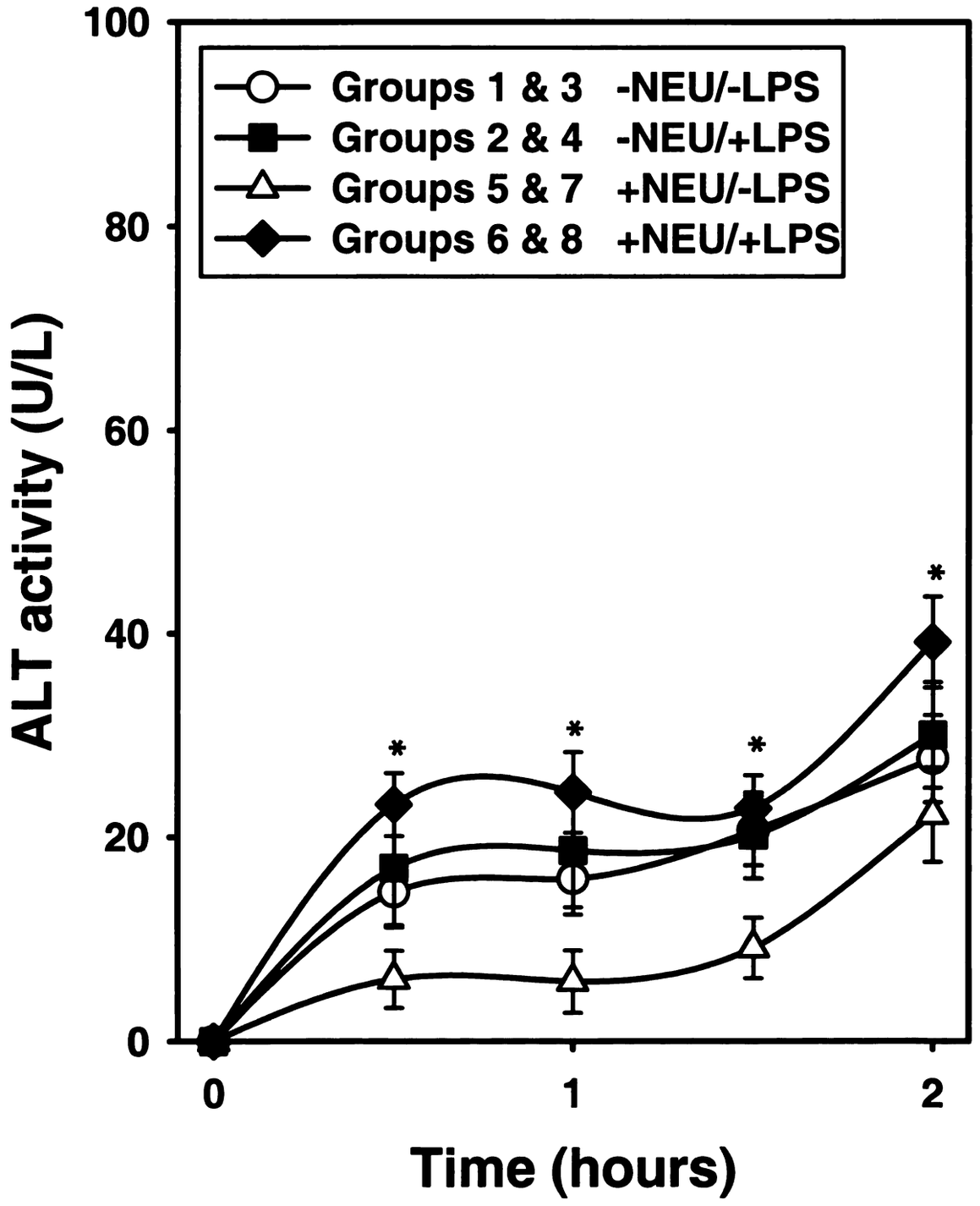
Groups 1 & 3 -NEU/-LPS
Groups 2 & 4 -NEU/+LPS
Groups 5 & 7 +NEU/-LPS
Groups 6 & 8 +NEU/+LPS
ALT activity (U/L)
100
80
60
40
20
0
0
1
2
Time (hours)
*
*
*
*

Figure 4.2-A

The addition of LPS to the perfusion buffer containing PMNs caused a modest yet statistically significant increase in ALT activity during the first two hours. From two to four hours, none of the factors individually had any effect on the increase of ALT activity with time. However, the addition of thrombin to livers containing either LPS or PMNs (group 4 or 7) significantly increased the time-dependent release of ALT. The combination of all three factors (group 8) resulted in a significantly greater increase in ALT activity with time compared to the combination of thrombin with either LPS or PMNs. In this group, ALT activity at the end of the experiment was more than 5 times greater than the value for the group receiving no treatment (group 1)(Figure 4.2-B). No increase in ALT release was observed with the combination of LPS and PMNs in the absence of thrombin ($P>0.97$, ANOVA).

The rate of release of lactate over time was not altered by any of the treatments. LPS addition significantly increased the rate of glucose released into the perfusion buffer (Figure 4.3). However, no significant interaction of LPS with either PMNs or thrombin was detected, and the LPS effect on glucose output became statistically significant only after two hours of perfusion.

Figure 4.2-B. Release of ALT from Isolated Livers During the Last Two Hours of Perfusion with LPS and PMN in the Presence of Thrombin. Livers from untreated donor rats were perfused with Krebs-Henseleit buffer containing 2% bovine serum albumin. Rat PMNs (final concentration $2x10^6$ PMN/ml) were added to the perfusion buffer at time 0 in the indicated treatment groups. After 10 minutes, LPS ($96x10^6$ EU/kg of donor rat weight) or its saline vehicle was added to the perfusion buffer. After two hours, human α-thrombin (10 nM) or its vehicle was added to the perfusion solution, and livers were perfused for another two hours. Samples were taken every 15 minutes from 2 to 4 hour. ALT activity was determined in all samples as described in Methods. Results are expressed as mean of ALT activity without SEM for clarity, N=8 per group.

significantly different from all other groups.

* Significantly different from groups 1,2,3,5,6 and 8.

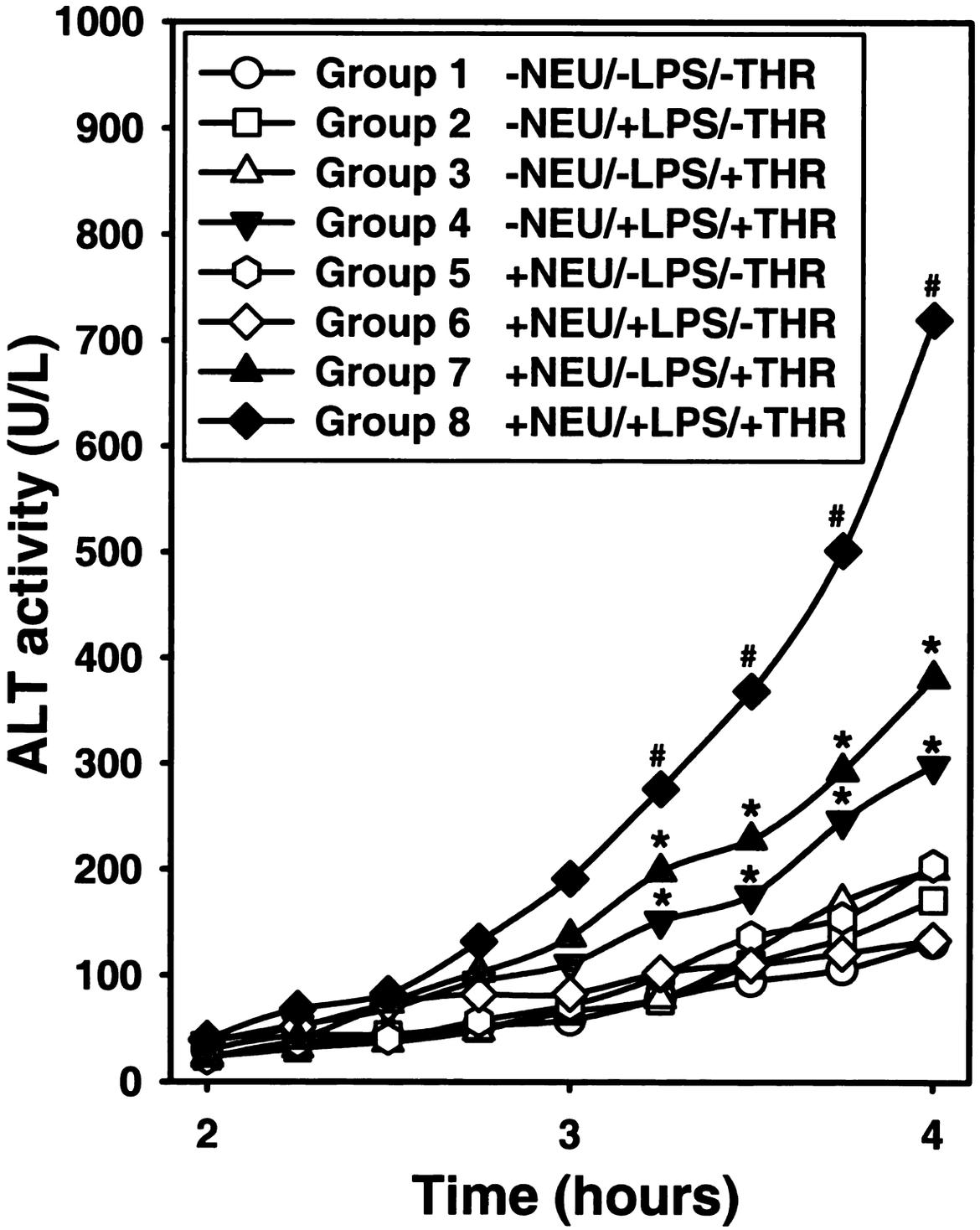
Group 1 -NEU/-LPS/-THR
Group 2 -NEU/+LPS/-THR
Group 3 -NEU/-LPS/+THR
Group 4 -NEU/+LPS/+THR
Group 5 +NEU/-LPS/-THR
Group 6 +NEU/+LPS/-THR
Group 7 +NEU/-LPS/+THR
Group 8 +NEU/+LPS/+THR
ALT activity (U/L)
1000
900
800
700
600
500
400
300
200
100
0
2
3
4
Time (hours)
#
*

Figure 4.2-B

Figure 4.3. Rate of Release of Glucose from Isolated Livers Perfused with LPS, PMN and Thrombin. Livers from untreated donor rats were perfused with Krebs-Henseleit buffer containing 2% bovine serum albumin. Rat PMNs (final concentration $2x10^6$ PMNs/ml) were added to the perfusion buffer at time 0 in the indicated treatment groups. After 10 minutes, LPS ($96x10^6$ EU/kg of donor rat weight) or its saline vehicle was added to the perfusion buffer. After two hours, human α-thrombin (10 nM) or its vehicle was added into the circulating solution, and livers were perfused for another two hours. Samples of medium were collected every 30 minutes for four hours, and glucose concentrations were determined in all samples. Net rates of release (μmol/g/h) were calculated from changes in concentration in the perfusate, flow rate and liver weight. Results are expressed as mean, N=8 per group.

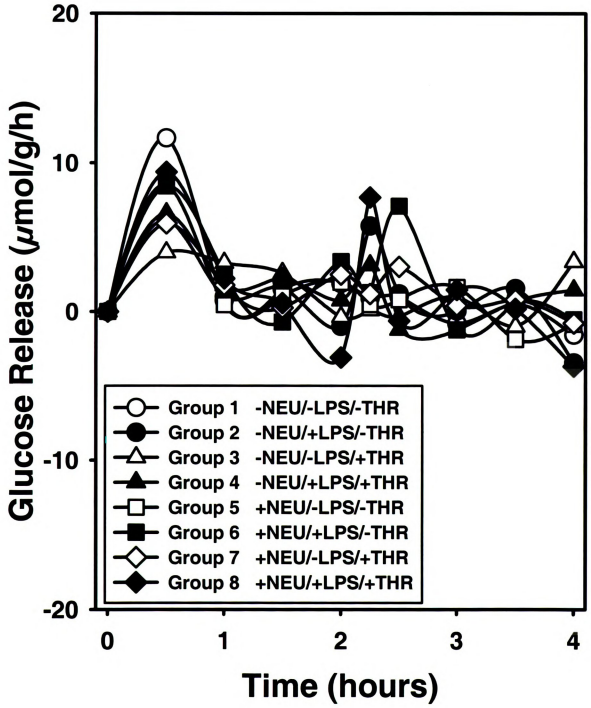
Glucose Release (μmol/g/h)
20
10
0
-10
-20
0
1
2
3
4
Time (hours)
Group 1 -NEU/-LPS/-THR
Group 2 -NEU/+LPS/-THR
Group 3 -NEU/-LPS/+THR
Group 4 -NEU/+LPS/+THR
Group 5 +NEU/-LPS/-THR
Group 6 +NEU/+LPS/-THR
Group 7 +NEU/-LPS/+THR
Group 8 +NEU/+LPS/+THR

Figure 4.3

Effect of thrombin administration on PMN degranulation in culture. PMN-dependent hepatocellular killing *in vitro* is mediated by proteases released during degranulation of activated PMNs (Ganey *et al.*, 1994). To examine whether thrombin could increase the degranulation of these cells, we measured the activity of MPO in the medium of PMNs in culture as an indicator of lysosomal contents release. fMLP is a stimulus for degranulation of lysosomes, and its action is greatly potentiated by the activity of cytochalasin B on the PMN actin filament system (Bengtsson *et al.*, 1991). In the absence of fMLP and cytochalasin B thrombin, at concentrations up to 40 nM, did not increase the release of MPO from PMNs (Figure 4.4). Consistent with previous reports (Bengtsson *et al.*, 1991), in the absence of cytochalasin B, fMLP did not increase MPO release from PMN (Figure 4.4). Since thrombin also has effects on the actin filament network of endothelial cells (Lum and Malik, 1996) and platelets (Fox, 1994), we investigated whether thrombin could replace cytochalasin B in promoting PMN degranlation. However, MPO release was not increased by the combination of fMLP and thrombin (Figure 4.4). Finally, we examined whether thrombin could increase the sensitivity of PMNs to the combination of fMLP and cytochalasin B. As previously reported (Bengtsson *et al.*, 1991), in the presence of cytochalasin B fMLP caused a dose-dependent release of MPO.

Figure 4.4. Effect of Thrombin on fMLP-Stimulated Release of Myeloperoxidase in the Absence of Cytochalasin B. Rat PMNs were suspended ($5x10^6$ PMN/ml) in serum-free Williams' medium E. The cells were incubated with 0, 4 or 40 nM human α-thrombin and fMLP (0, 1, 10 and 100 nM) at 37ºC for one hour. Myeloperoxidase (MPO) activity was measured in the cell-free medium and expressed as a percentage of total MPO content. Values represent mean ± SEM, N=5 donors.

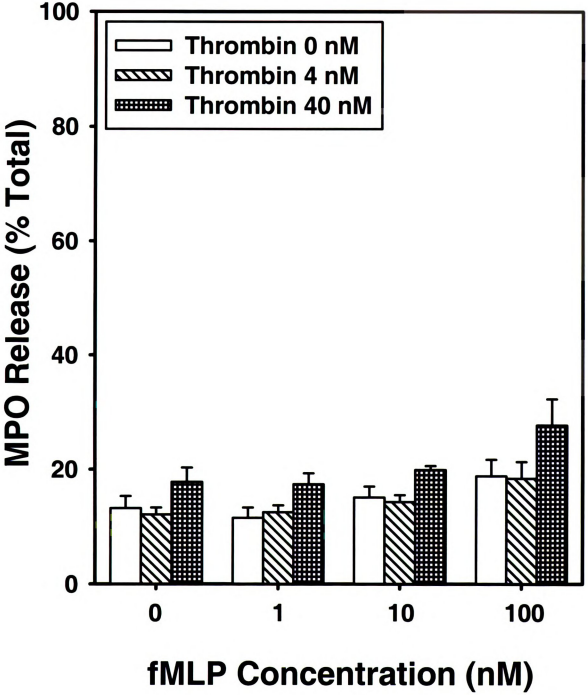
Thrombin 0 nM
Thrombin 4 nM
Thrombin 40 nM
MPO Release (% Total)
100
80
60
40
20
0
0
1
10
100
fMLP Concentration (nM)

Figure 4.4

At any concentration, thrombin did not augment the MPO release due to the combination of fMLP and cytochalasin B (Figure 4.5).

Effect of thrombin administration to hepatocyte/PMN cocultures. In coculture with hepatocytes, activated PMNs can damage hepatocytes through the release of toxic proteases (Ganey *et al.*, 1994). Since several studies suggest that thrombin can directly stimulate PMNs (Baranes *et al.*, 1986) and the administration of thrombin and PMNs to the isolated liver results in liver injury in the absence of LPS (Figure 4.2B), we investigated in a coculture system whether the damaging action of thrombin could be due to an ability to directly activate PMNs to kill hepatocytes. As reported previously (Ganey *et al.*, 1994), coculture of hepatocytes with unstimulated PMNs lead to a moderate release of ALT over 16 hours of incubation, and thrombin at concentrations up to 40 nM did not stimulate PMNs to kill hepatocytes isolated from donor rats treated with LPS (Figure 4.6).

We investigated the ability of thrombin to enhance hepatic parenchymal cell killing by PMNs activated with a primary stimulant. Administration to hepatocyte/PMN cocultures of 100 nM fMLP in the presence of cytochalasin B resulted in a significant increase in ALT released into the medium (Figure 4.5). However, thrombin at 40 nM did not enhance the hepatic parenchymal cell killing.

Figure 4.5. Effect of Thrombin on fMLP-Stimulated Release of MPO from PMN in the Presence of Cytochalasin B. Rat PMNs suspended ($5x10^6$ PMNs/ml) in serum-free Williams' medium E were incubated with human α-thrombin (0, 4 or 40 nM), fMLP (0, 1, 10 and 100 nM) and cytochalasin B (5 µg/ml) at 37ºC for one hour. MPO activity was measured in the cell-free medium after centrifugation and expressed as a percentage of total MPO activity. Values represent mean ± SEM, N=5 donors.

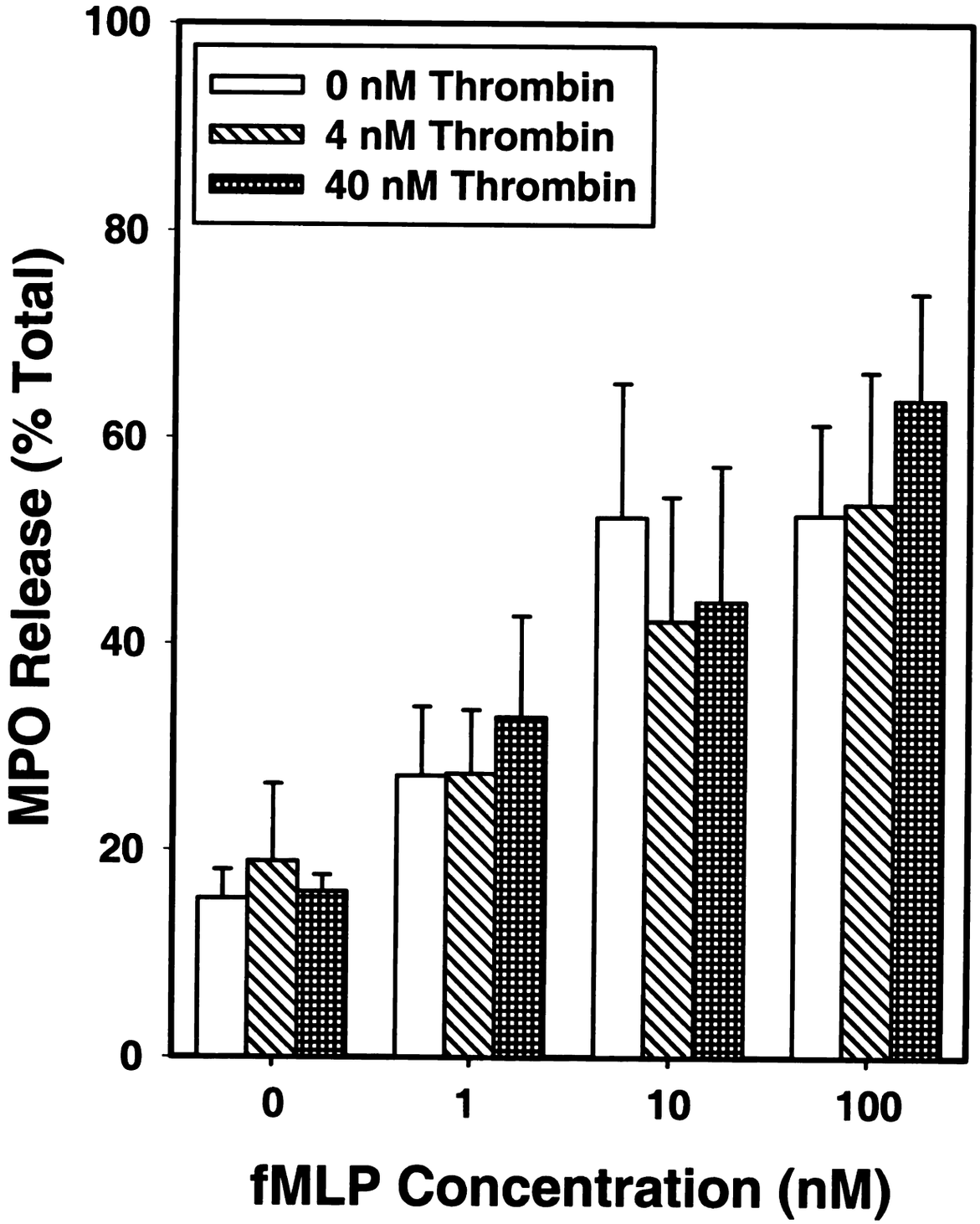
100
80
60
40
20
0
MPO Release (% Total)
0 nM Thrombin
4 nM Thrombin
40 nM Thrombin
0
1
10
100
fMLP Concentration (nM)

Figure 4.5

Figure 4.6 Effect of Thrombin on the Viability of Rat Hepatocytes from LPS-Treated Donors in Culture with PMNs. Hepatocyte donor rats received LPS (96 x 10^6 EU/kg, i.v.). Two hours later, hepatic parenchymal cells were isolated and plated (5x10^5/well) as described in Methods. The medium was replaced after three hours, and rat PMNs (5 x 10^6/well) were added. PMNs were allowed to attach for 30 minutes, and human α-thrombin was added at concentrations of 0.04, 0.4, 4 or 40 nM. The activity of ALT was determined in the cell-free medium and the cell lysate 3, 6 or 16 hours after thrombin addition. The percentage of total ALT released was calculated. Values represent mean ± SEM, N=4 donors.

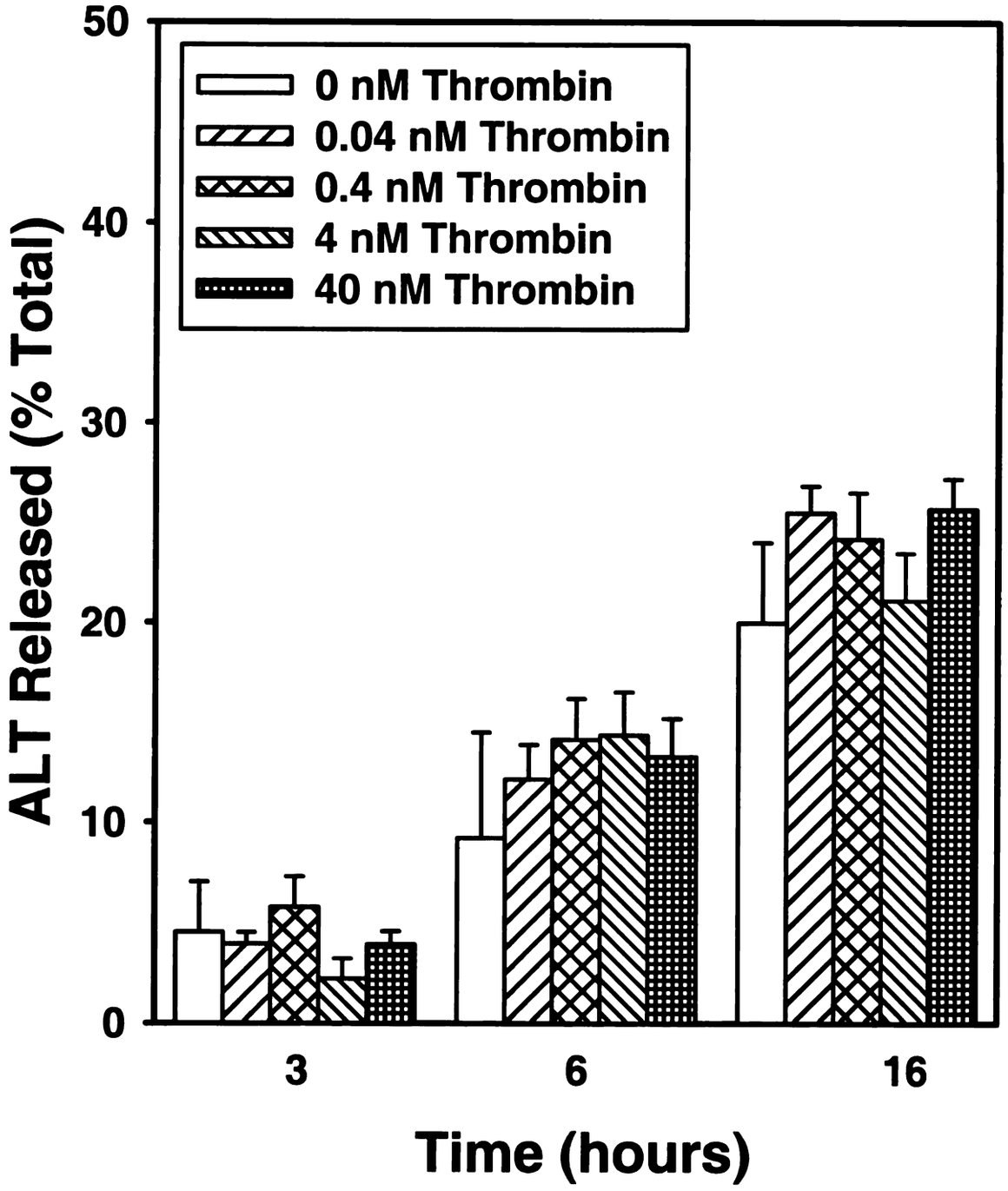
50
40
30
20
10
0
ALT Released (% Total)
0 nM Thrombin
0.04 nM Thrombin
0.4 nM Thrombin
4 nM Thrombin
40 nM Thrombin
3
6
16
Time (hours)

Figure 4.6

Figure 4.7. Effect of Thrombin on the Release of ALT from Hepatocytes in the Presence of fMLP-Stimulated PMNs. Isolated hepatocytes ($5x10^5$/well) were plated as described in Methods, and after three hours the medium was changed and PMN were added at a ratio of 10 PMNs/hepatocyte. After 30 minutes, human α-thrombin (40 nM) or its vehicle and cytochalasin B (5 μg/ml) were added to each well, followed 5 minutes later by either fMLP(100 nM) or its vehicle. The activity of ALT was determined in the cell-free medium and the cell lysates 16 hours later, and the percentage of total ALT released was calculated. Values represent mean ± SEM, N=4 donors.

* Significantly different from the respective group without fMLP.

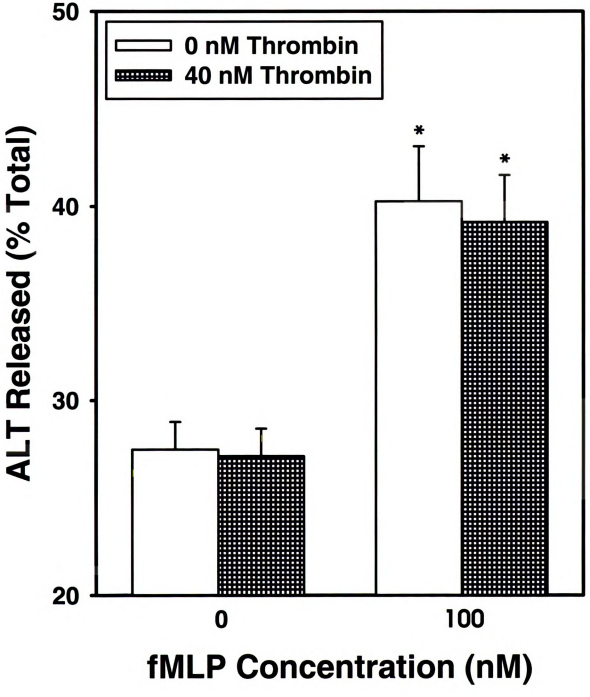
0 nM Thrombin
40 nM Thrombin
ALT Released (% Total)
50
40
30
20
*
*
0
100
fMLP Concentration (nM)

Figure 4.7

Discussion

Accumulating evidence indicates that thrombin plays an important role in liver injury and repair during various inflammatory processes (Vercellotti, 1998; Stadnicki *et al.*, 1997; Gando *et al.*, 1997). Thrombin formation occurs during liver diseases, and an accumulation of fibrin deposits in necrotic areas has been described in several models of acute liver injury. In addition, neutrophils are able to bind FXa and convert prothrombin to thrombin (Altieri and Edgington, 1990). Since areas of liver necrosis are often infiltrated with PMNs and platelets, it is likely that the procoagulant abilities of these cells are activated during liver damage. These observations indicate that thrombin is generated during hepatic injury and repair. However, recent studies suggest that thrombin formation could be more than the result of liver damage. The observations that thrombin inactivation completely blocked hepatic necrosis *in vivo* after LPS exposure (Margaretten *et al.*, 1967; Pearson *et al.*, 1996) and that thrombin was able to injure isolated perfused livers from LPS-treated donors (see Chapters 2 and 3) point toward thrombin as one of the key mediators of liver injury in response to LPS.

PMN depletion protects against LPS-induced hepatic necrosis (Hewett *et al.*, 1992), demonstrating the importance of these cells in the mechanism of tissue damage *in vivo*. Depletion of PMNs from liver donors prior to LPS injection also provided protection from thrombin-induced

hepatic injury in the isolated, perfused liver (Figure 4.1), reducing the release of ALT in the perfusion medium to the level of livers never exposed to thrombin. The protection afforded by PMN depletion or thrombin inhibition suggests that (1) each of these two factors is critical to the pathogenesis and (2) they are interdependent.

The inflammatory response to LPS is complex, and interference with any one of the critical mediators presented in Figure 1.6 markedly reduces or prevents liver injury. Interactions among these mediators are likely. Increasing evidence suggests that coagulation and inflammation pathways are intricately linked (Gillis *et al.*, 1997), yet our understanding of these interactions is still imperfect. Indeed, two hours after LPS administration, PMNs as well as hepatic cells have been exposed to a multitude of soluble mediators, including several cytokines, components of the complement system and products of platelet activation (Hewett and Roth, 1993).The present studies were undertaken for the purpose of uncovering potential interactions between thrombin and PMNs in the mechanism of LPS-induced liver damage, and to identify possible additional extrahepatic mediators necessary for liver injury.

In order to identify the minimum extrahepatic elements required for LPS-induced liver injury, naive livers were perfused with buffer containing LPS, PMN and/or thrombin. In the absence of *in vivo* exposure to endotoxin, livers were damaged by the combination of LPS, PMNs and

thrombin (Figure 4.2-B). This result suggests that any additional cellular or soluble mediator required for hepatic injury is either already present in the liver or can be synthesized by liver cells. The combination of all three elements produced the greatest amount of injury (Figure 4.2-B), supporting the idea of an interaction between thrombin and PMNs in response to LPS that maximizes hepatocellular killing.

Even in the absence of LPS, the combination of PMNs and thrombin was sufficient to result in significant liver damage (Figure 4.2-B). Accordingly, we hypothesized that the enzyme could interact with PMNs to cause injury in the absence of an inflammatory cytokines. Human PMNs can be activated directly by thrombin to degranulate (Baranes *et al.*, 1986). To investigate this possibility, we first looked at the effect of thrombin on rat PMN degranulation. Activation of PMNs initiates the fusion of alpha and dense granules with the cell membrane, resulting in the discharge of the lysosomal contents (Ricevuti, 1997; Weiss, 1989). The released products contain various proteases, such as cathepsin G and elastase, which contribute to hepatocellular killing by neutrophils *in vitro* (Ho *et al.*, 1996).

Cytoskeletal changes are important for PMN degranulation in response to an inflammatory stimulus (Bengtsson *et al.*, 1991). For example, cytochalasin B enhances degranulation in response to fMLP through its effects on the actin filament system (Honeycutt and Niedel, 1986). Since thrombin has been shown to affect the cytoskeleton of

endothelial cells (Lum and Malik, 1996) and platelets (Fox, 1994), we hypothetized that thrombin might either replace or enhance the effects of cytochalasin B in fMLP-stimulated cells. However, thrombin neither stimulated the degranulation of rat PMNs (Figure 4.6) nor enhanced the degranulation of fMLP-stimulated PMN in the presence (Figure 4.7) or absence (Figure 4.6) of cytochalasin B. These observations suggest that thrombin has no direct effect on the release of lysosomal contents from rat PMNs.

In an attempt to reproduce in a cell culture system the results from the isolated liver experiments, we investigated the ability of thrombin to modulate neutrophil-induced hepatocyte killing. In the coculture system, doses of thrombin up to 4 times those which produced injury in the isolated liver had no effect on the viability of hepatocytes isolated from either control (data not shown) or LPS-treated liver donors (Figure 4.4), confirming that thrombin was neither directly toxic to hepatocytes nor able to stimulate PMNs to kill these cells. Furthermore, although addition of fMLP to a PMN/hepatocyte coculture system yielded significant hepatic parenchymal cell death, thrombin was unable to enhance the cytotoxic effect of PMNs activated by fMLP (Figure 4.5). Thus, none of the conditions explored in cell cultures were consistent with the thrombin/PMN interdependence in hepatocyte killing suggested by experiments *in vivo* and in the isolated, perfused liver.

Surprisingly, ANOVA revealed no direct interaction between PMNs and LPS in our study. The mechanism by which neutrophils accumulate and become activated in the liver in response to LPS is not entirely clear, but it appears related to the the expression of adhesion molecules promoted by LPS (Jaeschke *et al.*, 1996; Jaeschke and Smith, 1997a). However, this effect is dependent on the presence of LPS-binding protein or soluble CD14 receptor (Hailman *et al.*, 1996), two elements that were not added to the perfusion buffer. The mere process of perfusing PMNs through the sinusoids of an isolated liver causes neutrophil adhesion, independently of the addition of LPS (Dahm *et al.*, 1991). Accordingly, it is possible that endothelial cell surface modification due strictly to the experimental procedure of liver isolation and perfusion supplanted the need for LPS interaction with PMNs that is required *in vivo*.

The observation that thrombin and LPS in combination (ie, without PMNs) led to significant liver damage (Figure 4.2-B) suggests that either thrombin has the ability to compromise the viability of hepatocytes directly after they are exposed to LPS, or that a cell type constitutively present in the liver can become activated by thrombin to damage hepatocytes during LPS exposure. As noted above, thrombin is not directly toxic to cultured hepatocytes either in the presence of LPS (Figure 3.2) or after a 2 hour exposure to LPS in vivo (Figure 3.3). Therefore, it appears likely that a cell type other than the PMN can be activated by thrombin to injure

hepatocytes after exposure to LPS. This cell type must be a component of the nonparenchymal cell population since naive, buffer-perfused livers are virtually devoid of blood cells. The response of this cell to thrombin requires prior LPS exposure, since administration of thrombin alone (Figure 4.3-B) did not result in liver injury. Finally, this cell type must possess the machinery to kill hepatic parenchymal cells. The observation that livers treated with the combination of all three factors experienced more damage than the ones treated either with thrombin and PMNs or with thrombin and LPS also suggests the possibility of an interaction between PMNs and this cell type that results in increased killing ability.

One candidate that fits these criteria is the Kupffer cell. Kupffer cells express the CD14 receptor and are activated by the binding of LPS to this receptor. They have been implicated in the mechanism of hepatic parenchymal cell death in various liver diseases (Ayala *et al.*, 1991; Deaciuc and Spitzer, 1996; Nolan *et al.*, 1980; Przybocki *et al.*, 1992; Sarphie *et al.*, 1996) and can enhance the activation of neutrophils through the release of various pro-inflammatory cytokines.

As previously reported (Filkins and Buchanan, 1977), endotoxin stimulated glucose output from the liver, probably as a result of enhanced glycogenolysis. Activation of glycogenolysis in hepatic parenchymal cells *in vitro* has been shown to depend on the release of prostaglandin D_2 from Kupffer cells activated by LPS (Casteleijn *et al.*, 1988). Therefore, the

stimulation of glucose output by LPS in isolated livers confirms the viability and responsiveness of the liver's resident macrophages.

In summary, we have shown in the isolated liver as *in vivo* that PMN depletion prevents liver damage caused by intravenous injection of endotoxin. This confirms the critical role of PMNs in the pathogenesis and supports the usefulness of the model in the exploration of LPS-induced injury. It was possible to reproduce in naive livers the thrombin-mediated injury observed during perfusion of livers isolated from LPS-treated rats by adding LPS and PMNs to the perfusion buffer. This demonstrates that the only critical, extra-hepatic mediators involved in LPS-induced liver damage are thrombin and PMNs. Moreover, maximal injury occured in the presence of PMNs, LPS and thrombin, and statistical analysis indicated significant interaction among these three factors. Thrombin did not stimulate or enhance degranulation of rat PMNs, and it was not directly toxic to isolated rat hepatocytes in the presence PMNs *in vitro* even after LPS exposure. Accordingly, hepatocyte killing by the PMN/thrombin combination appears to require the intervention of an additional cellular and/or soluble mediator(s).

CHAPTER 5

SUMMARY AND CONCLUSIONS

Sepsis and septic shock represent the leading cause of death in noncoronary intensive care units, and sepsis kills an estimated 150,000 patients each year. The observation that intensive antibiotic therapy could paradoxically worsen the clinical manifestation of gram-negative infections focused attention on a component of the bacterial cell wall: LPS. Mortality rate was found to be well correlated with blood concentration of LPS in patients suffering from gram-negative infection, and further studies provided support for the role of LPS in the development of the multiple organ failure syndrome

Numerous studies have documented the activation of the coagulation system either in septic patients or in animal models, and a wealth of evidence suggests that the coagulation cascade is important in clinical endotoxemia and multiple organ failure. Its importance in the pathogenesis of tissue injury was further emphasized by the ability of anticoagulants to abolish hepatotoxicity in septic patients and animal models of endotoxemia. In MODS, the lack of effective supportive therapy for hepatic functions makes the onset liver failure a sign of very poor prognosis during the late phase of sepsis.

Experiments *in vivo* suggest that among the components of coagulation, thrombin plays a critical role in the mechanism of liver damage following exposure to LPS in rats. In view of these observations, the overall aim of this dissertation was to examine the mechanisms by

which thrombin participates in LPS-induced liver injury. In particuliar, we confirmed that thrombin is a critical mediator of injury and that its action does not occur through the formation of fibrin clots in the vasculature. We showed that thrombin activation shortly precedes liver damage, and that its action requires stimulation of a specific receptor. Finally, we examined the interdependence between thrombin and cellular or soluble mediators released after LPS exposure.

Thrombin is a critical mediator of LPS-induced liver injury

Pretreatment with heparin 1 hr before LPS protected rats against liver injury, confirming previous reports. Heparin inhibits thrombin but also has several other actions that could influence LPS hepatotoxicity. Accordingly, a similar study was conducted with hirudin, a highly selective inhibitor of thrombin's catalytic activity. As with heparin, treatment with hirudin markedly reduced liver injury. We then perfused livers from LPS-treated donors with blood taken from ancrod- or heparin-treated rats. Ancrod prevents coagulation by depleting blood of fibrinogen but does not inhibit thrombin or prevent its formation. ALT activity in the perfusion medium from livers perfused with heparinized blood was modest and unchanged during the two-hour perfusion. By contrast, perfusion of livers with ancrod-treated blood resulted in a marked increase in ALT activity over time. Finally, we perfused livers from LPS-treated rats with thrombin

in buffer and observed a similar increase in ALT activity in the perfusate, confirming that thrombin is, indeed, a critical mediator of liver damage following LPS exposure.

Thrombin is a distal mediator of liver injury and acts independently of clot formation.

To test the hypothesis that the critical action of thrombin is a late event in the genesis of LPS-induced liver injury, heparin or hirudin was administered either 1.5 or 2.5 hrs after the administration of LPS, and liver injury was evaluated. Both treatments prevented the increase in plasma ALT activity. This result shows that activation of the coagulation cascade shortly precedes the onset of tissue damage that appears between 3 and 4 hours in our model. The observation that injury results from the addition of thrombin to buffer used to perfuse livers from LPS-treated rats demonstrates that other components of the coagulation system are not required for this effect.

Thrombin action requires activation of a specific receptor

To determine the concentration-dependence of liver damage in response to thrombin, we perfused isolated livers from LPS-treated rats with various concentrations of the enzyme. Thrombin caused dose-dependent injury in isolated livers from endotoxic rats, with an EC_{50} of

approximately 0.4 nM. The maximum effect of thrombin occured at 4 nM. These concentrations are similar to those effective in receptor-mediated responses to thrombin. We used the rat thrombin-receptor activating peptide (TRAP) corresponding to the N-terminal sequence of the cleaved PAR-1 to investigate whether or not the effects of thrombin were linked to activation of this receptor. A pronounced, time-dependent increase in ALT activity occurred in media from livers perfused with TRAP compared to those perfused with a control peptide, demonstrating that the effect of thrombin in the isolated liver is linked to the activation of PAR-1.

Thrombin requires interaction with other mediators

Thrombin is not directly toxic to hepatocytes

Addition of thrombin to the culture medium of primary hepatic parenchymal cells obtained from LPS-treated or naive rat donors did not result in increased release of ALT. Similarly, addition of LPS to the culture medium did not render thrombin toxic, demonstrating that thrombin does not injure hepatocytes in the presence or absence of LPS.

Platelets are not necessary for LPS-induced hepatic damage

Previous studies have shown that platelets accumulate in the liver within minutes after LPS administration and are critical for LPS-induced liver injury. For example, prior platelet depletion protects against LPS-induced

liver damage *in vivo*. To investigate further the relationship between these cells and thrombin, circulating platelet numbers were reduced in liver donors with anti-platelet serum (APS). The APS treatment failed to abolish the appearance of liver damage following addition of thrombin. This result is in contrast with the protective effect of platelet depletion *in vivo* and suggests that platelets act upstream from thrombin's action. It seems likely that platelets contribute to local formation of thrombin in the liver sinusoids.

Neutrophils are required for injury

PMNs accumulate in the liver within minutes after LPS administration and are required for LPS-induced liver injury *in vivo.* To investigate whether PMNs are required in isolated livers from LPS-treated rats for thrombin-mediated hepatic injury, blood neutrophil numbers were reduced in rat liver donors using anti-neutrophil immunoglobulin (AN-Ig). This was followed by injection of LPS, isolation of the livers and perfusion with thrombin. The injury observed in response to thrombin was markedly reduced by pretreatment with AN-Ig, confirming the critical role of neutrophils in the mechanism of LPS-induced liver damage and suggesting an interdependence between the PMNs and thrombin.

The mechanism may require intervention of another cell type

The perfused liver studies mentioned above employed livers from LPS-treated animals, which had experienced early inflammatory events (eg, cytokine release, platelet and PMN accumulation) critical to LPS-induced liver injury. In naive livers, it was possible to reproduce the thrombin-mediated injury by adding LPS and PMNs to the perfusion buffer. However, thrombin did not activate rat PMNs *in vitro*, and did not enhance hepatocyte killing in the presence of PMNs *in vitro,* even after LPS exposure. Thus, liver damage by the PMN/thrombin combination appears to demand the intervention of an additional cellular and/or soluble mediator present in the liver.

Hypothetical mechanism

The cascade of events underlying liver damage in endotoxemia is complex and still incompletely understood. It is likely that LPS action on endothelial cells and Kupffer cells contribute to the early influx of PMNs and platelets into the liver, as well as to the release of various cytokines in the general circulation. The mechanism by which thrombin suddenly appears in the circulation after two to three hours is unclear. It is possible that the response of the endothelium to LPS, either through retraction of the cells or apoptotic death exposes a sufficient surface of the basal membrane to initiate the formation of the prothrombinase complex. The

interactions between thrombin and PMNs are not completely explained, but one might speculate that thrombin is involved in the migration of these cells from the sinusoids into the liver parenchyma. At the same time, hepatocytes could change their surface proteins (ie, express new adhesion molecules) in response to the cleavage of PAR-1, and the contact between the PMN surface integrins and those newly expressed proteins could increase adhesion of PMNs to the hepatocytes and initiate PMN activation. The products of PMN degranulation and the free radicals generated near the surface of the hepatic parenchymal cells may cause disturbances in the plasma membrane and eventually lyse the cell. The release of cytosolic contents is a potent chemotaxin and activator of PMNs, resulting in more inflammatory cells called into the liver tissue and more activation. At the same time, expression of tissue factor on the membranes of dead cells and assembly of the prothrombinase complex on the activated PMNs would lead to more thrombin formation, more PAR-1 cleavage and more hepatocytes expressing targeting signals. The overall result would be the installment of a vicious cycle where tissue damage would result in more coagulation and inflammation, leading to more tissue damage until complete destruction of the liver occurs (Figure 5.1).

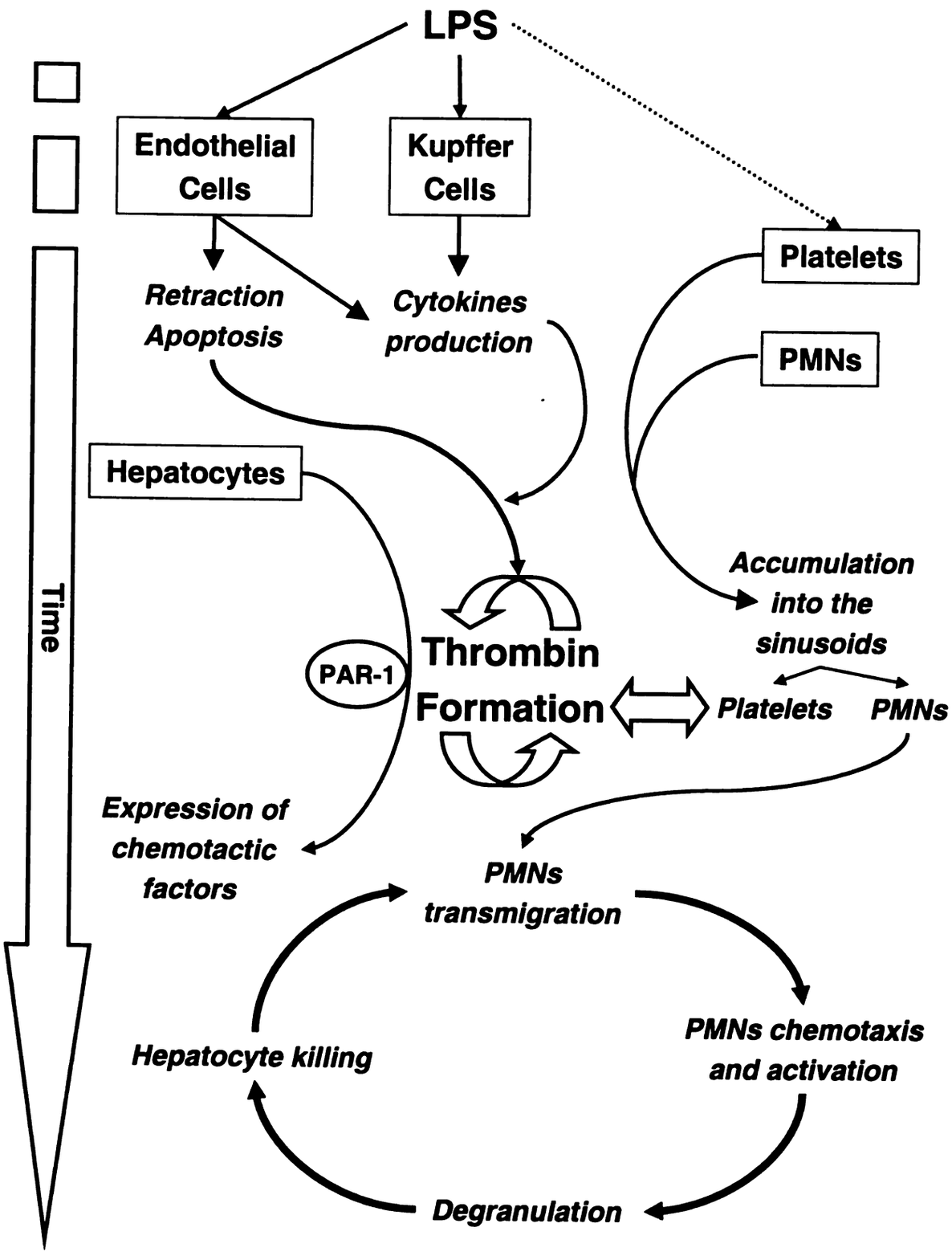

Figure 5.1 Hypothetical Mechanism of Thrombin Action in LPS-Induced Liver Injury

Importance of our research

Despite the lack of a complete description of the mechanism underlying liver injury after exposure to endotoxin, two major elements can be gathered from this work that may have direct impact on future strategies for the treatment of sepsis.

First, thrombin is indeed a critical mediator, and blocking thrombin should block the onset of liver failure. This result is not actually new in amimal models, and various antithrombotic therapies have been or still are in clinical trial today. However, anticoagulant therapies are not highly regarded by physicians in the treatment of critically ill patients because of the high risks of fatal hemorrhage that are invariably attached to the blockage of fibrin clot formation. Thrombin's action in LPS-induced liver injury appears linked to the activation of a receptor and is independent of fibrin clot formation. Therefore it should be possible to target not thrombin itself, but its receptor, and to block its cellular actions without dramatically interfering with the blood coagulation cascade. It has been shown that the magnitude of response to thrombin is determined by the number of receptors cleaved and the time over which cleavage occurs, a full response requiring the cleavage of most of the receptors in a short period of time. Even partial inhibition of the receptor cleavage might dramatically reduce the pathogenic response to thrombin.

Second, thrombin is a distal mediator of injury. The evolution of our understanding of the interactions implicated in the multiple organ failure syndrome resulted in numerous attempts to block various steps of the process. Anti-endotoxin antibodies or anti-cytokines have demonstrated efficacy when administered preventively in animal models, but not in clinical trials. One of the reasons currently invoked to explain the lack of efficacy in the clinic of techniques that were successful in the laboratory is the delay between the actual exposure to LPS and the time of treatment. From a medical viewpoint, the first exposure to LPS and the rise in TNF-α or IL-1 in the patient's circulation are not in close enough temporal apposition to the appearance of the first clinical symptoms to be useful targets for therapeutic intervention. In essence, when TNF-α peaks, around 90 minutes after exposure to a critical dose of LPS, the patient is still experiencing flu-like symptoms that usually do not warrant consultation of a physician or transport to an intensive care unit. Only after several hours does the onset of sepsis become clearly visible, and at that time most of the early events, including the presence of LPS in the circulation, the rise in cytokine concentrations and the influx of inflammatory cells are either finished or well on their way. However, because the activation of thrombin shortly preceeds organ damage it should be possible to administer drugs blocking its action before the appearance of MODS and DIC, but after the appearance of characteristic sepsis symptoms.

Finally, thrombin involvement in the pathogenesis of respiratory distress following endotoxin has also been reported, and the enzyme is now believed to mediate some of the symptoms of SIRS. Anti-thrombin agents prevent the lethal consequences of Gram-negative bacterial infections in several animal models and improve the outcome of sepsis in clinical trials. Thus, despite the focus of this thesis to the study of the liver, it is reasonable to envision a broader implication of thrombin in the mechanism of endotoxin-induced, multiple organ dysfunction syndrome.

In conclusion, we have advanced the understanding of the cascade of events resulting in liver damage after exposure to LPS. However, the interactions between thrombin and PMNs and the exact mechanisms by which liver parenchymal cells are damaged remain unexplained. Such mechanisms and interactions are difficult to study even in the isolated liver, and a coculture system may be more likely to provide answers.

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