MECHANISMS OF ENHANCED ALLYL ALCOHOL HEPATOTOXICITY BY ENDOTOXIN

By

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ABSTRACT

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Lipopolysaccharide (LPS) causes enhancement of toxicity from a number of xenobiotics including allyl alcohol. The mechanism by which LPS enhances the hepatotoxicity of allyl alcohol was investigated. Kupffer cells are critical for the enhancement of allyl alcohol hepatotoxicity by LPS. Stimulation of Kupffer cells with LPS in either Kupffer cell-hepatocyte cocultures or isolated, perfused livers from naïve rats did not induce the enhancement of allyl alcohol hepatotoxicity. These data indicate that Kupffer cells require the participation of other factors not present in the medium-perfused liver. Extrahepatic factors critical to large dose LPS-induced liver injury include neutrophils and thrombin. A role for each of these factors in the potentiation of allyl alcohol-induced liver injury by LPS was investigated. Neutrophils were previously known to play a critical role in this potentiation model. Allyl alcohol is well known to rapidly deplete glutathione stores in hepatocytes. Therefore, it was hypothesized that neutrophils enhance the toxicity of allyl alcohol through the release of reactive oxygen species onto hepatocytes depleted

of their glutathione. While neutrophil-derived reactive oxygen species caused a slight enhancement of allyl alcohol-induced cytotoxicity *in vitro*, *in vivo* administration of the antioxidants superoxide dismutase and catalase or apocynin did not afford protection from the potentiation of allyl alcohol toxicity by LPS. These data indicate that neutrophil-derived reactive oxygen species do not play a critical role in this potentiation model. To begin to investigate the role of thrombin rats were pretreated with the anticoagulants heparin or warfarin. Both of these anticoagulants protected against LPS enhancement of allyl alcohol-induced liver injury. These results suggested that thrombin might play a critical role in this potentiation model. To determine whether thrombin was acting through a receptor mediated mechanism to enhance the toxicity of allyl alcohol, isolated, perfused livers from LPS-treated rats were perfused with medium containing allyl alcohol in the absence or presence of thrombin. In these experiments thrombin did not enhance the toxicity of allyl alcohol indicating that the role of thrombin in this potentiation model may be dependent on other coagulation factors or other components of the blood. In conclusion, LPS enhances the hepatotoxicity of allyl alcohol through a complex mechanism in which the roles of Kupffer cells, neutrophils and thrombin are interdependent.

Dedications

I dedicate this accomplishment to those I love the most. My parents Jan and Martin Kinser, my brother Eric, and of course my girl Mary.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a	activated factor
AA	allyl alcohol
AALAS	American Association of Laboratory Animal Sciences
ADP	adenosine diphosphate
ALT	alanine aminotransferase
ANOVA	analysis of variance
AP-1	adaptor protein-1
AST	asparate aminotransferase
ATP	adenosine triphosphate
BPI	bactericidal/permeability-increasing protein
C	Celsius
Ca^{++}	calcium
CETP	cholesterol ester transfer protein
CINC-1	cytokine-induced neutrophil chemoattractant
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
DAG	diacylglycerol
DD	death domain
ECSIT	Evolutionarily-Conserved Signaling Intermediate in Toll
ERK	extracellular regulated kinase
Fe^{3+}	iron
fMLP	n-formyl-methionyl-leucyl-phenylalanine
g	gravity
Gal	D-galactose
GBS	Gey's balanced salt solution
$GdCl_3$	gadolinium chloride
Glc	D-glucose
GlcNAc	N-acetyl-D-glucosamine
GSH	glutathione (reduced form)
GSSG	glutathine (oxidized form)
HBSS	Hanks' balanced salt solution
HCs	hepatocytes
Hep	Heptose
IKK	Iκ-B kinase
IL-1R	interleukin-1 receptor
ILs	interleukins
ip	intraperitoneal
IRAK	IL-1 receptor associating kinase
iv	intravenous
IX	plasma thromboplastin component (Christmas factor)
JNK	C-Jun NH_2-Terminal kinase

KCs	Kupffer cells
Kdo	2-keto-3-deoxyoctonic acid
kg	kilogram
L	liter
LBP	LPS binding protein
LPS	lipopolysaccharide
M	molar
Man	D-mannose
MAP	mitogen-activated protein
MAP3K	mitogen-activated protein kinase kinase kinase
mCD14	membrane-bound CD14
MEKK-1	mitogen-activated protein kinase/ERK kinase kinase
μg	microgram
mg	milligram
μM	micromolar
mM	millimolar
min	minute
MKK	mitogen-activated protein kinase kinase
N	number
NADH	nicotine adenine dinucleotide, reduced form
NADPH	nicotine adenine dinucleotide phosphate, reduced form
NF-κB	nuclear transcription factor kappa B
ng	nanogram
NIK	NF-κB-inducing kinase
O_2	molecular oxygen
$O_2^{\cdot}$	superoxide anion
PAF	platelet activating factor
PAR	protease-activated receptor
PC-PLC	phosphatidylcholine-specific phospholipase C
PGE_2	prostaglandin E_2
PGI_2	prostacyclin
PI3	phosphatidylinositol-3
PL	phospholipid surface
PLA_2	phospholipase A_2
PLTP	phospholipid transfer protein
PMA	phorbol myristate acetate
PMN	neutrophil
Rha	rhamnose
ROS	reactive oxygen species
RT-PCR	reverse transcriptase-polymerase chain replication
sCD14	soluble CD14
S.E.M.	standard error of the mean
SOD	superoxide dismutase

TAK1	transforming growth factor-β -activated kinase
TGFα	transforming growth factor α
TIR	TolI/IL-1R
TLR4	TolI-like receptor 4
TLRs	TolI-like receptors
TNF-α	tumor necrosis factor α
TRAF6	TNF receptor-associated factor
VII	proconvertin
X	Stuart-Prower factor
XI	plasma thromboplastin
XII	Hageman factor

CHAPTER 1

General Introduction

INTRODUCTION

1.1 Endotoxin

1.1.1 Structure

Endotoxin is a component of gram-negative bacteria that elicits a strong inflammatory response in mammals. The term endotoxin was first coined by Richard Pfeiffer in 1893 to describe the toxic component found in the lysates of heat-inactivated *Vibrio cholerae*. This toxic component was different from other known exotoxins in that it was heat stable and its toxicity was only found in lysed cells, indicating that it may be attached to the cell body. Because the first endotoxin components identified were polysaccharide and lipid, the endotoxins were also called lipopolysaccharides (LPS). This molecule is found in the outer portion of cell wall of gram-negative bacteria. One bacterial cell contains approximately 3.5×10^6 LPS molecules that occupy an area of about 4.9 μm^2. Because the surface of the cell is about 6.7 μm^2, LPS comprises approximately three-quarters of the bacterial surface (Nikaido *et al*, 1987).

A mutation in the early steps of LPS synthesis is fatal to the bacterium, indicating that LPS is an essential component of the outer membrane. LPS represents the main surface antigen (O-antigen) of

gram-negative bacteria. It is essential for the specific recognition and elimination of gram-negative bacteria from the host. It may be that, because gram-negative bacteria display this essential component on their surface, the host defense mechanisms were able to evolve an ability to recognize this component at very low concentrations in the plasma. LPS isolated from different species of gram-negative bacteria elicit different pathological and physiological activities. Depending on the exact structure, LPS may also help prevent activation of complement and phagocytosis by macrophages, thereby protecting the pathogen from cellular host defenses.

Over the past several decades scientists have investigated the structural components of LPS to understand better the ability of host cells to recognize gram-negative bacteria. LPS structure can be broken down into 4 basic segments: the O-specific chain, outer core, inner core, and lipid A. Each segment is different in structure and biosynthesis and elicits different functions and biological activities. A brief understanding of each of these segments will be described. For additional information the reader is encouraged to read any of the following reviews: Morrison et al, 1992; Decker, 1997; Haeffner-Cavaillon, 1999; Darveau, 1998; Raetz, 1988; Rietschel, 1994; Wang, 1996; Fenton, 1998; Tobias, 1999; Ulevitch, 1999; Cunningham, 2000; Antal-Salma's, 2000; Ulevitch, 2000; Doun, 2000; Medzhitov, 2000; Beutler, 2000; Means, 2000.

The O-specific region is a carbohydrate polymer consisting of up to 50 oligosaccharides (Figure 1.1). This oligosaccharide chain is made up of many repeated units, each unit consisting of up to seven monosaccharides. The structure of the O-specific region is highly variable among species and even within a single bacterium. Variation can occur in the ring form, linkage type, monosaccharide, monosaccharide sequence within a repeating unit, number of repeating units, and extent of branching. The diversity of sugar constituents and the variability of linkage sites allows for a vast number of possible O-specific chain structures (Rietschel *et al*, 1982).

O-specific chains are either synthesized on the surface of the bacteria or synthesized within the bacterium and transported to the surface (Mulford *et al*, 1983). *S. typhimurium* O-specific chain biosynthesis occurs by first making oligosaccharide repeating units and then connecting the units using a polymerase found on the periplasmic surface of the inner membrane. Elongation of the chain occurs at the reducing end. In other bacteria (i.e. *Samonella*) elongation occurs at the nonreducing end (Makela *et al*, 1984).

A cluster of genes termed *rfb* encodes proteins critical for the synthesis of the O-specific chain. Mutants with a defect in the *rfb* gene synthesize LPS that lacks the O-specific region. LPS lacking the O-specific region is called R-form LPS because it produces bacteria that

look rough on their surface. These mutant bacteria are able to grow in vitro, indicating that the O-specific chain is not required for viability. However, when these mutants are put into tissue or body fluids they do not survive, suggesting a protective role for the O-specific chain (Jann *et al*, 1984).

The O-specific chain plays a role in many of the biological properties of LPS. For example, these chains determine the serological O-specificity of LPS and can act as receptors for bacteriophages (Luderity *et al*, 1982; Lindberg *et al*, 1983). Some bacteria utilize their O-specific chain to help bind to their leguminous host (Wolpert *et al*, 1976) or inhibit attachment of the C5b-9 membrane attack complex (Makela *et al*, 1990), which makes it important for bacterial virulence. The O-specific chain of other bacteria is able to activate the alternative complement pathway (i.e. phagocytosis via C3b) leading to removal from the host (Grossman *et al*, 1984). Due to the large variability in the structure of this region of LPS, hosts may not have been able to evolve to recognize this region with specificity. Thus, the O-specific region is not critical for host recognition and often serves to protect the bacteria from recognition and can even down-regulate the endotoxic activity of LPS (Morrison *et al*, 1985; Luderitz *et al*, 1989).

The core region of LPS is a branched heteropolysaccharide without repeating glycosyl structures. Its biosynthesis starts at the lipid

A-bound 2-keto-3-deoxyoctonic acid (Kdo). The chain then elongates by the addition of activated glycosyl residues to the nonreducing end of the chain (Jann et al, 1984). This biosynthesis occurs independently of and with separate pathways from O-specific chain synthesis. The core oligosaccharide can be broken into two regions based on the type of sugars incorporated; the outer core and the inner core.

Most species variation in core structure is found in the outer core. The outer core is also called the hexose region due to its content of various hexoses such as D-glucose, D-galactose, and N-acetyl-D-glucosamine (Figure 1.1). Not much is known about the biological activities that are elicited by this region. However, it can recognize an Ra-specific serum factor (Ihara *et al*, 1982) and may be involved in binding certain bacterial mutants to lymphocytes (Lehmann *et al*, 1980; Jirillo *et al*, 1990).

The inner core connects the outer core and O-specific region to the lipid A region (Figure 1.1). It is made up of heptose sugars such as L-glycero-D-manno-heptose. The structure of the inner core varies across species but all gram-negative bacterial LPS contain at least one α-bound pyranosidic or furanosidic Kdo residue. This Kdo group is always found in a lipid A-proximal location and serves to anchor the polysaccharide to the lipid portion of LPS. The inner core structure has a slightly negative

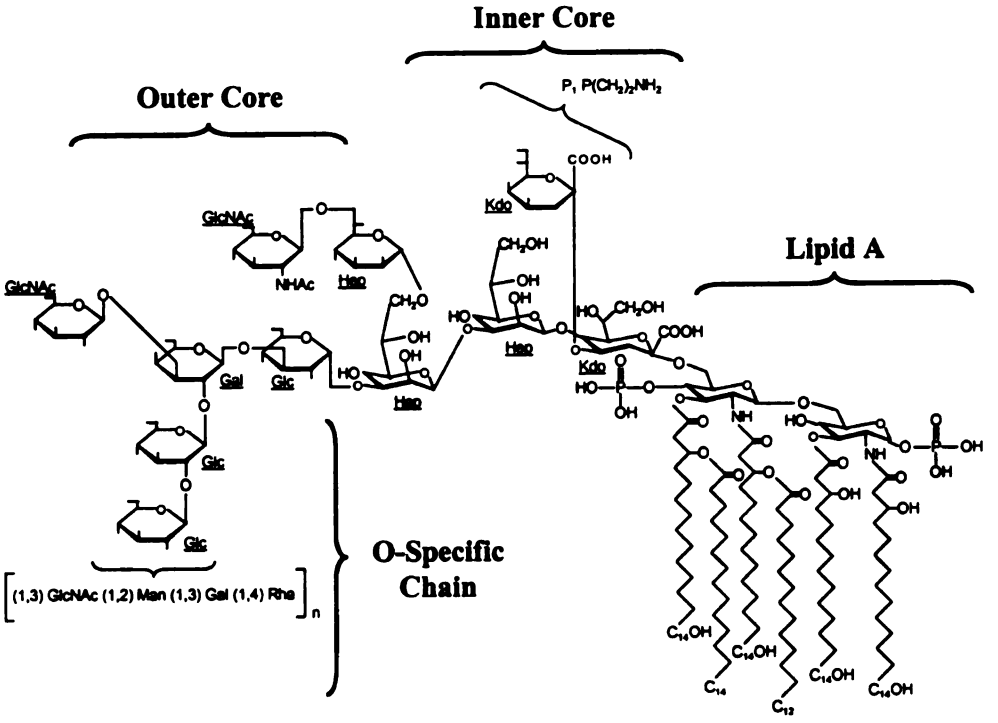
charge which is responsible for the negative charge of LPS. This charged region of LPS aids in membrane stability.

The inner core region is a rather conserved region of LPS. This may have enabled the host to evolve the mechanisms that recognize this region of LPS. In fact this region is required for some of the biological activities of lipid A and for bacterial viability (Haeffner-Cavaillar *et al*, 1989; Vaara *et al*, 1984; Osborn MJ, 1979).

The lipid A region of LPS represents the covalently bound lipid component (Figure 1.1). Though its structure varies among species and within a given LPS preparation, much of the structural format remains similar. Nearly all lipid A contains gluco- configured and pyranosidic- D-hexosamine residues which are usually present as a β(1-6)-linked disaccharide. This disaccharide makes up the backbone of the lipid. Hydroxy fatty acids are linked to the disaccharide via amine or ester bonds. Typically the hydroxy fatty acids are 10 to 28 carbons long and are often acylated at their 3-hydroxyl group. The amount and position of fatty acid acylation and phosphorylation play key roles in host-cell responses to LPS (Takada *et al*, 1992). Some structural prerequisites for bioactivity have been elucidated by analyzing the biological activities of synthetic lipid A molecules. In general, a biologically active lipid A will contain two hexosamine residues, two phosphoryl groups, and six fatty acids in a defined location as it is presented in *E. coli*.

Figure 1.1. Structure of LPS molecule.

Structure depicting the four major segments of LPS the O-specific chain, outer core, inner core, and lipid A. Abbreviations: Glc: D-glucose; Gal: D-galactose; GlcNAc: N-acetyl-D-glucosamine; Hep:heptose; Kdo: 2-keto-3-deoxyoctonic acid; Man: D-mannose; Rha: rhamnose; n: the number of repeating units may vary from 0 to approximately 50. (adapted from Haeffner-Cavaillon et al, 1999)


Inner Core
Outer Core
Lipid A
O-Specific Chain
P, P(CH2)2NH2
COOH
Kdo
GlcNAc
NHAc
Hep
CH2OH
OH
HO
Gal
Glc
COOH
NH
(1,3) GlcNAc (1,2) Man (1,3) Gal (1,4) Rha
n
C14OH
C14
C12

Lipid A represents the least variable region of LPS. This may be the reason that Lipid A contains the endotoxic principle of LPS. Lipid A has been shown to induce the various classical effects of endotoxin such as fever, local Schwartzman reactivity (a standard *in vivo* test of LPS tissue toxicity), and lethal toxicity. In addition to all of the physiological effects elicited by LPS, the lipid A region is essential for bacterial viability (Osborn MJ, 1979).

1.1.2 Cell Signaling

The ability of cells to recognize LPS has been an area of intense study for many years. The cellular receptor for LPS has only recently been discovered and is thought to be Toll-like receptor 4 (TLR4). The ability of cells to detect levels of LPS in the picogram per milliliter range is due to the LPS carrier proteins LPS binding protein (LBP) and CD14 which effectively deliver LPS to its receptor. A brief review of the mechanism by which these proteins interact to elicit LPS detection by cells follows as reviewed by: Tobias, 1999;Ulevitch, 1999; Cunningham, 2000; Antal-Szalma's, 2000; Ulevitch, 2000; Daun, 2000; Medzhitov, 2000; Beutler, 2000; Means, 2000.

LBP is a 61-65 kDa glycoprotein, acute-phase reactant that is found in the blood at concentrations of about 2-20 μg/ml (Gallay *et al*, 1993, Myc *et al*, 1997, Calvano *et al*, 1994). The levels of LBP can

increase 50-100 fold during inflammation by its formation mainly in the liver and to a lesser extent in the lungs and other tissues (Martin *et al*, 1997). LBP is structurally very similar to three other proteins: bactericidal/permeability-increasing protein (BPI), cholesterol ester transfer protein (CETP), and phospholipid transfer protein (PLTP) (Day *et al*, 1994; Tobias *et al*, 1988; Gray *et al*, 1989). Because no x-ray crystallography has been done on LBP, much of the knowledge of its structure is based on the x-ray crystallography of the very similar BPI protein. Like LBP, BPI will also bind LPS molecules, however it does not transfer them to CD14. The BPI protein will deposit LPS molecules back onto LPS aggregates, effectively neutralizing LPS (Tobias *et al*, 1997). According to its crystallographic structure, BPI contains two apolar pockets that interact with the acyl chains of phosphatidylcholine (Beamer *et al*, 1997). These apolar pockets could be the sites of interaction with LPS for both BPI and LBP. These proteins appear to associate with the fatty acid portion of LPS. Although LBP binds to LPS from different bacterial strains with differing affinities (Cunningham *et al*, 1996), this is not thought to be the biological mechanism by which the host can differentiate among LPS molecules.

In general, LBP binds LPS entering the blood in very small concentrations and helps present it to cells expressing CD14. LPS binds to CD14 as a monomer, however LPS is found as aggregates in aqueous

environments such as tissue culture medium or blood. These aggregates spontaneously release LPS monomers at a very slow rate. The addition of LBP dramatically accelerates the binding of LPS monomers from LPS aggregates to CD14 (Hailman *et al*, 1994), and in this capacity LBP increases the sensitivity of cells to LPS (Wright *et al*, 1991). Though LBP can transfer LPS to membrane-bound CD14 and form an LBP-LPS-CD14 complex, LBP does not form a complex with the soluble CD14 (sCD14). LBP can also transfer LPS to lipoproteins, which functionally neutralizes LPS (Wurfel *et al*, 1994; Munford *et al*, 1982), and in these instances LBP decreases the sensitivity of cells to LPS.

The importance of LBP is demonstrated by its ability to increase the sensitivity of cells to LPS. For example, the threshold concentration of LPS required to induce tissue factor release from a monocyte cell line was reduced 20-fold by purified LBP (Steinemann *et al*, 1994). In addition, antibodies to LBP prevented LPS-induced activation of cells and lethality from LPS administration (Gallay *et al*, 1994). Blood from LBP knock-out mice was less sensitive to LPS-stimulated release of TNF-α, but LBP knock-out mice responded similarly to wild type mice during LPS exposure *in vivo* (Wurfel *et al*, 1997). These contrasting results may be explained by local production *in vivo* of the sCD14 or some alternate protein that can act as an LPS carrier. However, galactosamine-sensitized LBP knock-out mice were less sensitive to LPS than

heterozygous controls, indicating the importance of LBP. In addition, LBP knock out mice were more sensitive to *Salmonella*-induced death, indicating that LBP plays a critical role in host defense (Fenton *et al*, 1998). Despite the inconsistency of these results, it is clear that LBP plays an important role in the recognition of LPS.

CD14 is a membrane glycoprotein of 356 amino acids with a 19 amino acid N-terminal leader peptide. Its gene is found on chromosome 5 in the region q23-21. It has two exons and encodes a single 1.4 kb mRNA transcript (Ferrero *et al*, 1988). After translation, 28-30 amino acids of the C-terminal end are replaced with a glycosyl-phosphatidyl inositol group which serves to anchor CD14 to the membrane but does not facilitate a transmembrane signal (Haziot *et al*, 1988). Although only one mRNA transcript has been reported for CD14, different subtypes have been proposed based on differences in antigenicity, LPS binding affinity, and sensitivity to enzymatic cleavage (Pedron *et al*, 1995). Cells known to express CD14 on their surface include monocytes, macrophages, and neutrophils (Goyert *et al*, 1986).

The number of CD14 molecules on a human neutrophil has been estimated to be about 3000 (Antal-Szalmas *et al*, 1997) and on monocytes between 6000 and 190,000 (Marchant *et al*, 1992, Couturier *et al*, 1991). The number of membrane bound CD14 receptors varies between monocytes of different tissues. Peritoneal, pleural, and

perivascular brain macrophages express large numbers, whereas microgliae, alveolar macrophages, Kupffer cells, and macrophages of the intestinal mucosa express smaller copy numbers of CD14 (Antal-Szalmas et al, 2000).

The expression of CD14 can be altered by various stimuli. Tumor necrosis factor-alpha, granulocyte-colony stimulating factor, n-formyl-met-leu-phe (fMLP), and LPS can upregulate CD14 about two fold in neutrophils. In monocytes, interleukin-4 and interleukin-13 decrease CD14 mRNA, whereas interferon-α, interferon-γ, interleukin-2, and transforming growth factor beta induce a rapid increase in CD14. LPS also alters the monocytic-expression of CD14; however, the response appears to be dependent on dose and time. At early time points (30-180 min.) LPS causes a rapid increase in CD14 expression (50-100%) followed by a decrease (50-75%) at 3-6 hours. Finally, CD14 expression increases dramatically (200-300%) after 1-6 days.

sCD14 exists in two different forms and can be found in the plasma at concentrations of 3-5 μg/ml (Kruger *et al*, 1991). One form is produced from the shedding of membrane bound CD14 (mCD14) as a result of various stimuli including PMA, IFN-γ, Ca^{2+}-ionophore A23187, or LPS. This stimulus-initiated shedding is thought to involve a membrane-associated serine protease and results in a protein with a molecular mass of 48-49 kDa. The second form consists of CD14

molecules that escape glycosyl-phosphatidyl inositol anchor attachment, retaining their C-terminal leader which results in a protein with a molecular mass of 55-56 kDa (Haziot *et al*, 1988). This form may be stored intracellularly. In neutrophils sCD14 molecules appear to be stored in secretory vesicles and azurophilic granules. Their localization in monocytes is more diffuse and in the perinuclear region (Detmers *et al*, 1995). There as yet does not appear to be any biologically functional difference between the two forms of sCD14.

CD14 binds and shuttles a wide variety of microbial ligands. The mechanism by which CD14 binds to LPS is not entirely known. There are no crystallographic data available for CD14; based on what is known about the sequence for CD14, it is thought to bind the polar portion of LPS. There are many regions within the amino terminal portion of CD14 that are required for LPS binding (Shapiro *et al*, 1997). Only the first 152 amino acids of the 356 amino acids in CD14 are important for functionality (Viriyakosol *et al*, 1996; Juan *et al*, 1995). Charge reversal mutations are the most effective at reducing LPS binding affinity (Shapiro *et al*, 1997). It is thought that many of the critical regions are on the same side of the molecule forming a charged pocket that effectively works as a selective molecular flypaper, similar to macrophage scavenger protein (Cunningham *et al*, 2000).

There is a vast body of data supporting a central role for CD14 in LPS responses. CD14 has a high affinity for and binds stoichiometrically to LPS (Hailman *et al*, 1994). Specific monoclonal antibodies against CD14 inhibit LPS-enhanced phagocytosis (Wright *et al*, 1990; Lynn *et al*, 1991; Wright *et al*, 1991; Kitchens *et al*, 1992). sCD14 is found in the blood and it allows for the activation of cells that do not express CD14 (Bazil *et al*, 1989; Frey *et al*, 1992; Arditi *et al*, 1993; Haziot *et al*, 1993). Transfection of CD14 into cell lines enhances their responsiveness to LPS more than 1000-fold (Lee *et al*, 1992; Golenbock *et al*, 1993; Viryakosol *et al*, 1995). CD14-deficient knock-out mice are 10,000-fold less sensitive to LPS than wild-type mice (Haziot *et al*, 1996; Haziot *et al*, 1995). Whereas small concentrations of sCD14 increase the sensitivity of cells to LPS, high concentrations of sCD14 can act to neutralize LPS activity (Troelstra *et al*, 1997).

CD14 was originally thought to be the receptor for LPS, however, it was proven to assist merely in the recognition of LPS by the actual LPS receptor (Kitchens *et al*, 1995). Until recently, the LPS receptor remained unknown. It is now known to be TLR4.

The discovery of the LPS receptor occurred rather serendipitously. Initial studies in *Drosophilla melanogaster* showed that a protein family known as Toll played a critical role in innate immune responses to microbial pathogens. This finding prompted the search for a human

homologue of the Toll receptor family. Human homologs were discovered and named Toll-like receptors (TLRs) (Medzhitov *et al*, 1997; Chaudhary *et al*, 1998). The members of the TLR family, TLR2 and TLR4, have been found to respond to microbial pathogens (Kirschning *et al*, 1998; Yang *et al*, 1998; Chow *et al*, 1999).

Structurally the TLR group contains transmembrane regions with leucine-rich repeats in the extracellular region, and a hydrophobic transmembrane domain (Rock *et al*, 1998). The cytoplasmic tail is similar to the interleukin-1 receptor (IL-1R) and is termed the Toll/IL-1R (TIR) domain. This region shows 45% sequence identity with IL-1R (Gay *et al*, 1991). It is estimated that there are 1000 or fewer molecules of TLR4 on a cell. Currently the amount of TLR4 expressed on the surface of cells is thought to regulate the limit of LPS sensitivity. Little is known about the regulation of the TLR4 gene.

Shortly after the discovery of human homologs to the Toll receptor, their potential contributions toward the immune system were investigated. Two groups independently showed that TLR2 supports a CD14-dependent LPS signal that activates nuclear factor kappa B (NF-κB) using a pathway similar to IL-1R (Yang *et al*, 1998; Kirschning *et al*, 1998). Another group found that hamster cells carrying a null allele for TLR2 are capable of responding to LPS (Heine *et al*, 1999). These findings suggested that while TLR2 is sufficient for an LPS response, it is

not required. It was later found that activation of TLR2, in the presence of LPS, may have actually been stimulated by bioactive contaminants found in commercial preparations of LPS. Deletion of TLR2 in mice resulted in loss of peptidoglycan recognition and responsiveness with no inhibition of LPS response. TLR2 recognized a wide variety of other microbial products *in vitro*, including components of gram-positive bacterial cell walls, zymosan, bacterial lipoproteins, as well as mycobacteria and glycolipids derived from mycobacterial cell walls (Underhill *et al* 1999; Schwandner *et al*, 1999; Aliprants *et al*, 1999; Brightbill *et al*, 1999; Flo *et al*, 2000). While it appears that TLR2 can recognize many different molecules, it must be remembered that neither *in vitro* transfection nor targeted deletion of individual TLRs can tell the specificity of a single TLR type. It has been hypothesized that some TLRs may form heterodimers which would influence ligand specificity. In general, TLR2 is thought to recognize predominately gram-positive bacterial cell wall components. Thus there had to be some other LPS-responsive receptor.

Beutler and coworkers suggested the role for TLR4 in LPS signaling (Poltorak *et al*, 1998). There is a large amount of data supporting a role for TLR4 in LPS signaling; the most convincing data come from LPS-resistant mouse strains. The defect in the *Lps* gene that caused LPS resistance in the mouse strains C3H/HeJ and

C57BL/10ScCr was found to encode the TLR4 protein. In the C3H/HeJ strain there is a proline-histidine change at position 712 and in the C57B1/10ScCr strain there is a null mutation in the TLR4 gene (Poltorak et al, 1998). In summary, mutation of TLR4 in mice resulted in a complete loss of LPS responsiveness.

TLR4 intracellular signaling has been the most extensively studied LPS signaling pathway and reviewed by Ulevitch, 2000; Medzhitov, 2000; Means, 2000 (Figure 1.2). LPS is well known to stimulate the phosphorylation of p38, extracellular regulated kinase (ERK) 1 and 2, C-Jun NH_2-Terminal kinase (JNK), adaptor protein-1 (AP-1), and NFκ-B. Most of what is known about this signaling pathway involves the proteins associated with the activation of NF-κB. A protein called MD-2 associates with TLR4 to aid in LPS stimulation. MD-2 lacks a transmembrane domain but can form a complex on the extracellular domain of TLR4 (Akashi *et al*, 2000; Shimazu *et al*, 1999). Upon stimulation the protein MyD88 associates with TLR4 via an interaction at the TIR region (Muzio *et al*, 1998; Medzhitov *et al*, 1998). MyD88, originally isolated as a myeloid differentiation primary response gene, contains both an N-terminal death domain and a C-terminal TIR domain (Daun JM, 2000). MyD88-deficent mice are resistant to the lethal effect of LPS, and macrophages from these mice do not produce IL-6, TNF-α, or NO in response to LPS (Kawai *et al*, 1999). However, LPS is capable

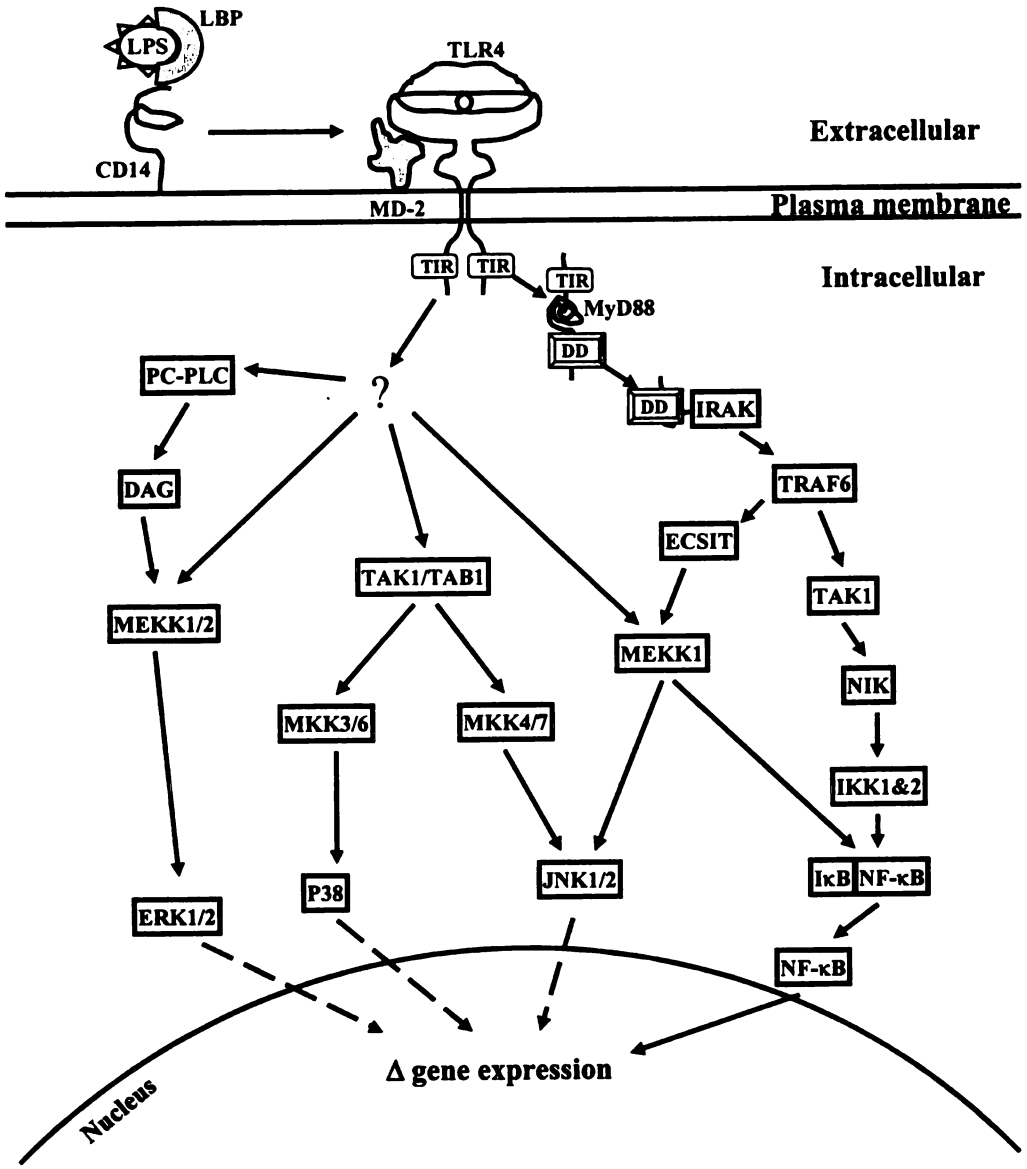
of signaling through both MyD88-dependent and -independent pathways (Daun JM, 2000). MyD88 recruits IL-1 receptor associating kinase (IRAK) to the TLR4 receptor. IRAK is a serine/threonine protein kinase that contains an N-terminal death domain. It is the homophilic interaction between MyD88 and IRAK death domains that leads to association and autophosphorylation of IRAK (Wesche *et al*, 1997). This stimulates the activation of the downstream kinase TNF receptor-associated factor (TRAF6), which leads to the stimulation of the transforming growth factor-β-activated kinase (TAK1) (Cao *et al*, 1996; Ninomiya-Tsuji *et al*, 1996). TAK1 activation leads to induction of NF-κB-inducing kinase (NIK), which leads to the activation of the Iκ-B kinases IKK1 and IKK2 (Ninomiya-Tsuji *et al*, 1996). Activated IKKs phosphorylate Iκ-B and stimulate its degradation through the proteasome pathway leading to the release of NFκ-B (Ninomiya-Tsuji *et al*, 1996). Once NFκ-B is activated and translocates to the nucleus many of the genes involved in the inflammatory response are activated.

Other portions of LPS-stimulated signal transduction have been discovered, however, much more work must be done to get a clear picture of the intracellular pathways activated by LPS. Some of the other portions of LPS-stimulated pathways include TRAF6 association with Evolutionarily-Conserved Signaling Intermediate in Toll pathways (ECSIT). ECSIT appears to bridge TRAF6 to the mitogen activated

protein kinase kinase kinase (MAP3K), MEKK-1 via a direct interaction (Kopp *et al*, 1999). MEKK-1 also can activate JNK (Means TK, 2000). Alternatively JNK may be activated by TAK-1/TAB1, which are thought to activate the mitogen activated protein kinase kinases MKK3/6 and MKK4 that in turn activate p38 and JNK respectively (Ninomiya-Tsuji *et al*, 1999; Muzio *et al*, 1998). MAPKs can be activated by LPS independently of MyD88 (Means TK, 2000). Along other pathways LPS stimulates the activation of phosphatidylinositol-3 (PI3) kinase. PI3 kinase may associate with the NIK/IKK complex leading to Iκ-B phosphorylation and degradation (Figure 1.2). LPS stimulation also results in the breakdown of phosphatidylcholine to the second messengers diacylglycerol and ceramide, which leads to mitogen activated protein (MAP) kinase activation (Daun JM, 2000). A phosphatidylcholine-specific phospholipase C (PC-PLC) is important for ERK activation with LPS-stimulation (Daun JM, 2000). It is easy to see that LPS stimulates many different intracellular pathways. This causes the stimulated cells to produce many different acute phase proteins and other mediators that can alter the physiology of the cells and the organs surrounding them.

Figure 1.2. Signaling cascade from LPS-stimulated TLR4 receptor.

Schematic diagram representing the signaling cascade of the TLR4 receptor initiated by LPS as described in the text. Abbreviations: LPS, lipopolysaccaride; LBP, lipopolysaccaride binding protein; TLR4, toll-like receptor-4; TIR, toll/interleukins-1 receptor; DD, death domain; IRAK, interleukins-1 receptor associating kinase; TRAF6, tumor necrosis factor receptor-associated factor-6; TAK1, transformaing growth factor β-activated kinase; NIK, NFκ-B inducing kinase; IKK, I-κB kinases; ECSIT, evolutionarily-conserved signaling intermediate in toll pathways; MKK, mitogen activated protein kinase kinase; MEKK-1, mitogen activated protein kinase/ERK kinase kinase; ERK, extracellular regulated kinase; JNK, C-Jun NH_2-terminal kinase; DAG, diacylglycerol; PC-PLC, phosphatidylcholine-specific phospholipase C.


LBP
LPS
TLR4
CD14
Extracellular
MD-2
Plasma membrane
TIR
TIR
TIR
MyD88
Intracellular
DD
PC-PLC
?
DD
IRAK
TRAF6
DAG
ECSIT
TAK1/TAB1
TAK1
MEKK1/2
MEKK1
NIK
MKK3/6
MKK4/7
IKK1&2
P38
JNK1/2
IκB
NF-κB
ERK1/2
NF-κB
Δ gene expression
Nucleus

1.1.3 Critical Factors for LPS-Induced Responses in Liver

LPS stimulates cells through a receptor-mediated process to release a large array of cellular mediators. The physiological effects induced by LPS are many. Only the effects of LPS on the liver will be discussed here. In the liver LPS can induce injury at sufficient doses. Liver injury induced by LPS is critically dependent on the activity of several factors in the liver including Kupffer cells, neutrophils, thrombin, and hepatocytes (Fujita *et al*, 1995; Iimuro *et al*, 1994; Hewett *et al*, 1992; Sato *et al*, 1993; Pearson *et al*, 1996; Moulin *et al*, 1996). Each of these factors will be discussed further below.

1.1.3.1 Hepatic Response

Although the exact mechanisms are unknown, LPS stimulates the release of many cellular mediators including complement factors, clotting factors, oxygen radicals, lysosomal enzymes, nitric oxide, arachidonic acid metabolites, platelet activating factor (PAF), tumor necrosis factor-α (TNF-α), interleukins (ILs), and endothelin. The number of physiological effects elicited by such mediators is enormous.

After LPS administration, various receptors are down-regulated including hepatic vasopressin, α1-adrenergic, prostaglandin F2α, and glucocorticoid receptors. Other receptors become sensitized such as

receptors for glucagons, insulin, and TNF-α. Cells exposed to LPS exhibit increased sensitivity to cyclic AMP-dependent hormones, which may be due to LPS-induced increase in membrane-bound adenylate cyclase activity.

Upon LPS administration *in vivo*, inflammatory cells release mediators that stimulate the production of acute phase proteins. Medium from LPS treated monocytes is capable of inducing hepatocytes to produce acute phase proteins, supporting the contention that stimulation of hepatocytes *in vivo* is mediated through the release of cytokines from monocytes. Three main cytokines released from Kupffer and other cells that are responsible for induction of acute phase proteins are IL-1, IL-6, and TNF-α. Hepatocytes are stimulated to increase the synthesis of many acute phase proteins and decrease the synthesis of negative acute phase proteins, such as albumin. Synthesizing results from many labs it is apparent that the net effect on total protein synthesis is dependent on the dose of LPS administered and the time proteins are measured. At early time points there is an increase in total protein synthesis.

Glycogen depletion is an early event after LPS administration that causes hyperglycemia. This is stimulated by catecholamines and prostaglandins at an early phase and by glucagons at a later phase of endotoxemia. Additionally, gluconeogenesis is increased in LPS-treated

animals. Cyclooxygenase products are thought to play a role in the LPS-stimulated glycogenolytic burst.

It also affects lipid metabolism in hepatocytes. LPS causes a switch in fatty acid synthesis from oxidation to esterification. It also causes a marked increase in triacylglycerolemia, which is partly due to a decrease in lipoprotein lipase activity and an increase in fatty acid synthetase activity.

1.1.3.2 Kupffer Cells

Kupffer cells originate from promyloblast stem cells in the bone marrow. Once the cells differentiate into monocytes they are released into the circulation. The monocytes may circulate for several days before settling in various organs where they differentiate into tissue macrophages. The macrophages that settle in the liver are called Kupffer cells. These represent 80% of all resident macrophages, and they represent about 10 to 12 percent of the liver cell population.

In addition to LPS, Kupffer cells play an important role in the development of liver injury from many different xenobiotics. For example, activating Kupffer cells with retinol enhances carbon tetrachloride and vinylidene chloride hepatotoxicity (Badger *et al*, 1996; Wueweera *et al*, 1996). Furthermore, inhibition of Kupffer cells with gadolinium chloride, dextran sulfate, or glycine-containing diet protects

against hepatotoxicity induced by carbon tetrachloride, vinylidene chloride, acetaminophen, D-galactosamine, 1,2-dichlorobenzene, LPS, ethanol, diethyldithiocarbamate, and ischemia reperfusion (Badger *et al*, 1996; Wueweera *et al*, 1996; Michael *et al*, 1999; Stachlewitz *et al*, 1999; Hoglen *et al*, 1998; Sarphie *et al*, 1996; Fujita *et al*, 1995; Iimuro *et al*, 1994; Bautista *et al*, 1999; Hisama *et al*, 1996; Ishiyama *et al*, 1995; Adachi *et al*, 1994).

The mechanism by which Kupffer cells contribute to liver injury is not fully understood. I have found that the most common mechanisms reported are the generation of oxidative stress and the release of TNF-α. Kupffer cell-derived oxidative stress is important for the toxicity of many compounds including acetaminophen, 1,2-dichlorobenzene, LPS, and diethyldithiocarbamate (Michael *et al*, 1999; Hoglen *et al*, 1998; Iimuro *et al*, 1994; Ishiyama *et al*, 1995). TNF-α release is important for the toxicity of other compounds such as D-galactosamine (Stachlewitz *et al*, 1999).

Though Kupffer cells can increase the toxicity of many xenobiotics, they can also protect against liver injury from xenobiotics. Based on a review of the literature it appears that the ability of the Kupffer cell to protect the liver as opposed to contributing to injury is dependent upon the time at which the Kupffer cells are activated relative to chemical exposure. Activation of Kupffer cells with Corynebacterium parvum five

days before treatment protected against carbon tetrachloride and acetaminophen toxicity. This protective effect may be due to inhibition of microsomal oxidizing enzymes and an increased production of glutathione (Pereira *et al*, 1997).

Kupffer cells are the major cell type responsible for LPS clearance. After an i.v. injection of LPS, 100 times more LPS is found in Kupffer cells than in endothelial cells and 1000 times more than in hepatocytes (Fox, 1990). Most of the effects of LPS administration have been attributed at least in part to signal molecules generated by resident macrophages and by blood monocytes (Decker, 1997). Upon stimulation with LPS, Kupffer cells change from a quiescent state to an inflammatory or active state. While in the quiescent state, Kupffer cells release only low levels of apolipoprotein E and prostanoids. They also respond very weakly to external stimuli. Upon stimulation with LPS they become swollen and contain an increased number of cytoplasmic lysosomal granules and phagocytic vacuoles. The endoplasmic reticulum appears dilated and damage to the plasma and nuclear membranes is also apparent at large doses of LPS. In the active state Kupffer cells increase their secretion of mediators and are much more sensitive to external stimuli. Kupffer cells are the major suppliers of LPS-stimulated TNF-α in the liver. Upon LPS-stimulation they also release IL-1β, IL-6, IL-8, IL-10, Interferon-α/β, TGF-α, TGF-β, PGE_2,

PGD_2, $PGF_{2\alpha}$, PGI_2, thromboxane A_2, platelet activating factor, superoxide, and nitric oxide (Decker, 1997).

Though no one single cytokine can be responsible for all of the pathological effects of LPS, TNF-α is widely considered the most relevant and typical cytokine mediating the effects of LPS. Plasma TNF-α levels reach their peak at about 90 minutes after LPS administration and administration of TNF-α elicits many effects similar to those of LPS. Furthermore, the effects of LPS administration can be blocked by inhibition of TNF-α (Decker, 1997; Stachlewitz *et al*, 1999; Hewett *et al*, 1993).

The effects of IL-1β are very similar to those of TNF-α. IL-1β plays a role in antigen presentation by increasing adhesion molecules on antigen presenting cells. Glucose homeostasis can also be modulated by IL-1. Blockage of IL-1β inhibits the effects of LPS (Decker, 1997). IL-1 from Kupffer cells stimulates hepatocytes to release the chemokine cytokine-induced neutrophil chemoattractant-1 CINC-1 (Mawet *et al*, 1996).

IL-6 plays a role in enhancing the synthesis of acute phase proteins. The amount of IL-6 produced by Kupffer cells is only a fraction of what blood monocytes produce; however, Kupffer cells are spatially much closer to hepatocytes and are thus thought to play a critical role in

the regulation of acute-phase protein synthesis in hepatocytes (Decker, 1997).

Platelet activating factor released from LPS-stimulated Kupffer cells stimulates the Kupffer cells to form oxygen free radicals and peripheral blood monocytes to produce TNF-α (Lo *et al*, 1997). Platelet activating factor plays a role in the early stages of neutrophil infiltration of the liver (Coughlan *et al*, 1994; Zimmerman *et al*, 1994; Lorant *et al*, 1991). Platelet activating factor primes neutrophils to respond to secondary immunological stimuli more robustly (Kulikov *et al*, 1998). It is thought to play a role in the early stages of endotoxemia (Balsa *et al*, 1997; Terashita *et al*, 1992, Qi *et al*; 1990).

Prostaglandins are released from Kupffer cells upon LPS-stimulation of phospholipase A_2, which provides arachidonic acid by cleavage of membrane-attached phospholipids. The major prostaglandins released from LPS-stimulated Kupffer cells are PGE_2 and PGD_2. These prostaglandins are involved in vasodilation and are also thought to play a role in the regulation of inflammation (Decker, 1997).

Kupffer cells can release nitric oxide upon stimulation by LPS. Nitric oxide leads to the relaxation of contracted hepatic stellate cells. It can also be converted to the highly toxic molecule peroxynitrite in the presence of superoxide. Peroxynitrite plays a role in the development of tissue injury in many models of toxicity (Decker, 1997).

LPS alone does not stimulate superoxide production by Kupffer cells. LPS enhances phorbol ester- or phagocytotic stimuli-activated production of superoxide catalyzed by membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This is likely due to the ability of LPS to enhance the formation of glucose-6-phosphate dehydrogenase, which is an enzyme that limits the rate of NADPH regeneration needed for oxygen reduction (Decker, 1997).

1.1.3.3 Neutrophils

Neutrophils originate in the bone marrow from promyelocytic stem cells. The majority of their maturity occurs in the bone marrow before release into the systemic circulation. Neutrophils play a key role in the host's defense against bacterial infection. This is illustrated by the fact that animals depleted of their circulating neutrophils have a greater susceptibility to bacterial infection (Matsumoto *et al*, 1991).

Accumulation at the site of infection is the first step in the neutrophil response to a bacterial infection. This involves a complex series of events that can be subdivided into chemotaxis, adherence, and diapedesis. Chemotaxis refers to the process by which neutrophils follow a concentration gradient of soluble chemotactic factors that directs them to the site of infection. Adherence is used to describe the ability of the neutrophil to adhere to the vascular endothelium.

Diapedesis is the process by which neutrophils migrate from the vascular lumen into the interstitium.

Once the neutrophils arrive at the site of infection they can kill the bacteria by either phagocytosis or the release of cytotoxic factors into the surrounding area. Bacteria that are phagocytosed are subsequently killed and degraded by cytotoxic factors released into the phagocytic vacuole. The same cytotoxic factors are also released into the extracellular milieu. These include reactive oxygen metabolites and proteases.

The production of reactive oxygen metabolites is dependent upon the activation of the enzyme NADPH oxidase. This enzyme is assembled on the plasma membrane upon LPS-stimulation and catalyzes the transfer of one electron from NADPH to molecular oxygen to produce superoxide. Though superoxide is itself cytotoxic it is also converted into more toxic oxidants such as hydrogen peroxide, hydroxyl radical, and peroxynitrites. With the additional release of myeloperoxidase the major oxidant hypochlorous acid can be formed. Oxygen metabolites can damage cells by initiation of lipid peroxidation and alterations in proteins and DNA.

LPS stimulates the expression of neutrophil adhesion molecules on the surface of vascular endothelial cells and the production of chemokines, which stimulate the migration of neutrophils into the liver.

Molecules that act as chemokines include IL-8 and C5a as well as other complement products.

Neutrophils contribute to LPS-induced liver injury. This is demonstrated by the fact that depletion of circulating neutrophils protects against LPS-induced liver injury (Hewett *et al*, 1992; Sato *et al*, 1993). The mechanism by which neutrophils impart LPS-induced toxicity has been debated. There are many reports that describe an increased release of reactive oxygen during endotoxemia (Bautista *et al*, 1990; Spitzer *et al*, 1993). Administration of antioxidants improve survival of endotoxemic animals (Rahman *et al*, 1999; Villa *et al*, 1995). Superoxide dismutase (SOD) protects against liver injury from Corynebacterium parvum and endotoxin treatment (Aurthur *et al*, 1985). In addition, phorbol myristate acetate (PMA)-stimulated neutrophils injure the isolated, perfused liver (Dahm *et al*, 1991). However, in cocultures of hepatocytes and neutrophils, superoxide does not contribute to hepatotoxicity (Guigui *et al*, 1988; Ganey *et al*, 1994). Rather, proteases released from stimulated neutrophils are responsible for hepatocellular death (Mavier *et al*, 1988; Harbrecht *et al*, 1993; Ganey *et al*, 1994). Cathepsin G and elastase are the cytotoxic proteases released from stimulated neutrophils (Ho *et al*, 1996). *In vivo*, reactive oxygen species may increase the hepatotoxicity of proteases by inhibiting the activity of antiproteases found in the plasma (Jaeschke *et*

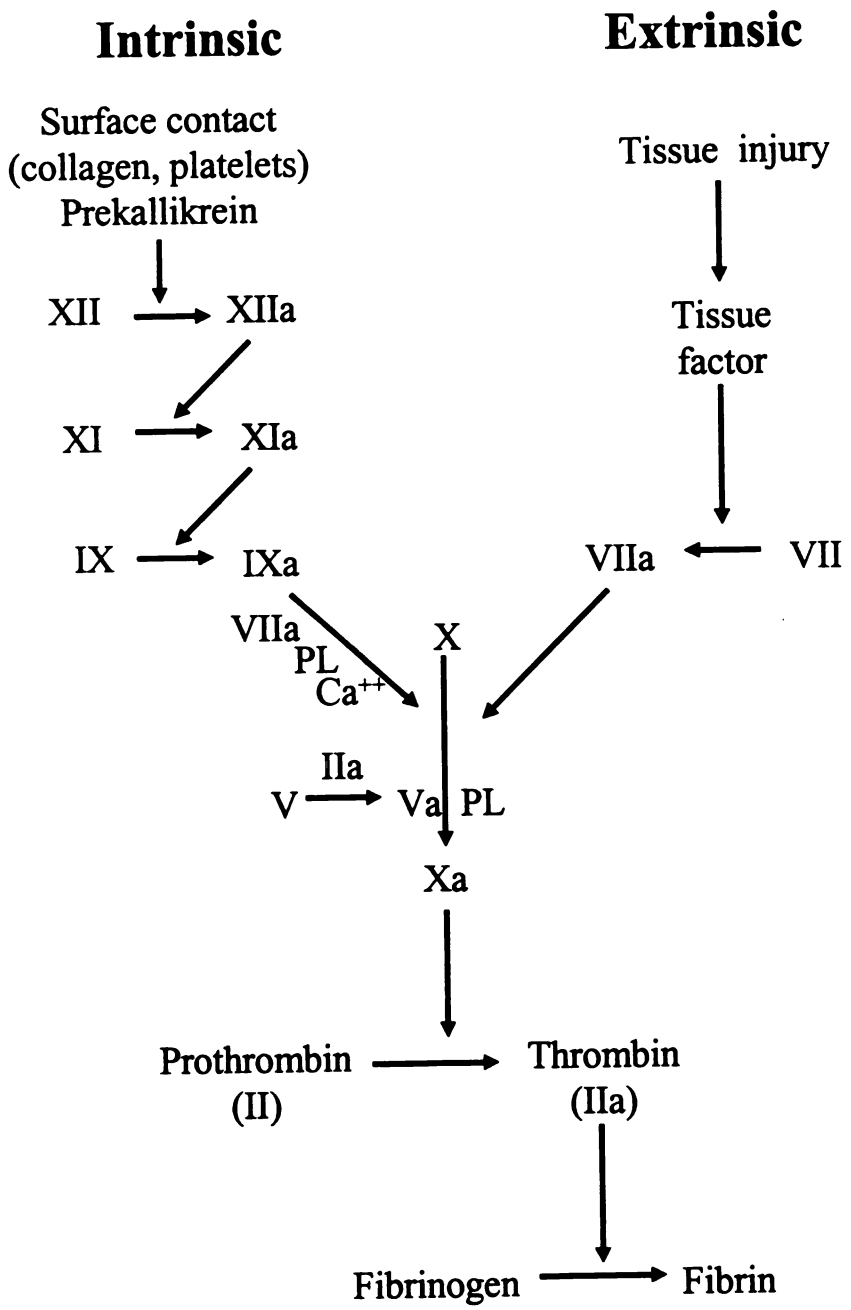
al, 1997). Additionally, reactive oxygen species are known to activate NF-κB pathways that also may contribute to hepatocellular injury by enhancing the inflammatory process (Schreck *et al*, 1991; Canty *et al*, 1999; Feng *et al*, 1995). Thus, considering all of the results, one can conclude that neutrophils may contribute to LPS-induced liver injury through a protease-dependent mechanism that is enhanced by reactive oxygen species.

1.1.3.4 Thrombin

Initiation of coagulation can occur either intrinsically by surface-mediated reactions or extrinsically through a tissue-derived factor pathway. In hemostasis, first platelets adhere to macromolecules in subendothelial regions of the injured blood vessel. This aggregate forms the primary hemostatic plug. The platelets then stimulate local activation of plasma coagulation factors. At each step of the coagulation cascade a previously activated enzyme activates the next enzyme by cleavage of one or more peptide bonds in the proenzyme. This activity requires a cofactor, usually calcium, and an organizing surface, usually platelets. At the end of the cascade prothrombin is converted into its active form, thrombin, by factor Xa, which cleaves two bonds on prothrombin. Thrombin can then convert fibrinogen into the insoluble fibrous protein fibrin (Figure 1.3).

Figure 1.3. The coagulation cascade.

Schematic diagram of the coagulation cascade representing both the intrinsic and extrinsic pathways. Abbreviations: Ca^{++}, calcium; PL, phospholipid surface; a, activated factor; XII, Hageman factor; XI, plasma thromboplastin antecedent; IX, plasma thromboplastin component (Christmas factor); VII, proconvertin; X, Stuart-Prower factor.


Intrinsic
Extrinsic
Surface contact
(collagen, platelets)
Prekallikrein
XII
XIIa
XI
XIa
IX
IXa
Tissue injury
Tissue
factor
VIIa
VII
VIIa
PL
Ca++
X
IIa
V
Va PL
Xa
Prothrombin
(II)
Thrombin
(IIa)
Fibrinogen
Fibrin

The proenzymes involved in the coagulation cascade include factors II (prothrombin), VII, IX, X, XI, XII, and prekallikrein. Each of these proenzyme proteases contain a trypsin-like domain containing about 200 amino acids of the carboxyl end. In addition, 9 to 12 glutamate residues near the amino-terminal ends of factors II, VII, IX, and X are converted to γ-carboxyglutamate residues during biosynthesis in the liver. These γ-carboxyglutamate residues are important for calcium binding and are required for coagulation.

Factors V and VII act as cofactors once they are activated by thrombin cleavage into Va and VIIa. Va and VIIa have no enzymatic activity themselves but will increase the proteolytic efficiency of the factors Xa and IXa respectively. Tissue factor is also a nonenzymatic cofactor that greatly increases the proteolytic efficiency of VIIa. It is found on the surface of cells not normally exposed to blood. In addition, monocytes and endothelial cells also may express tissue factor when exposed to a variety of stimuli, including LPS, TNF-α, and IL-1.

LPS-stimulated enhancement of tissue factor gene activity plays an important role in the initiation of LPS-induced coagulation (Franco *et al*, 2000). This is supported by the ability of tissue factor inhibition to block LPS-activated coagulation (de Jonge *et al*, 2000; Levi *et al*, 1994). In particular, neutrophils release a large amount of tissue factor in

response to LPS, and it is their contribution of tissue factor that seems to be important (Todoroki *et al*, 2000).

Thrombin has been found to elicit many effects beyond its "classical" role in the coagulation cascade. For instance, human α-thrombin stimulates migration of human peripheral blood monocytes in a dose-dependent manner. Thrombin stimulates endothelial cells to produce and release many cellular mediators including PAF, prostacyclin (PGI_2), nitric oxide, adenine nucleotides, plasminogen activator, plasminogen activator inhibitor, von Willebrand factor, fibronectin, superoxide, and platelet-derived growth factor (Carney *et al*, 1992; Bar-Shavit *et al*, 1992; Ritchie *et al*, 1995; De Meyer *et al*, 1995; Holland *et al*, 1998). Thrombin enhances the release of TNF-α and IL-1 from monocytes (Hoffman *et al*, 1995) and stimulates platelet activating factor release from stellate cells (Pinzani *et al*, 1994). In isolated, perfused livers thrombin stimulates glycogenolysis (Yamanaka *et al*, 1992). In platelets, thrombin stimulates the release of neutrophil-activating peptide 2 variants and adherence to endothelial cells (Piccardoni *et al*, 1996; Carney *et al*, 1992). In neutrophils, thrombin may enhance generation of superoxide, adherence to endothelial cells and release of thromboxane (Chan *et al*, 1988; Carney *et al*, 1992; Bizios *et al*, 1987). Thrombin also acts as a growth factor for smooth muscle cells (Bar-Shavit *et al*, 1992).

Some of the effects of thrombin are dependent on its proteolytic activity whereas others are dependent on different sites on thrombin called exosites or subsites. These sites provide recognition centers for receptors, of which thrombin has several. One class of receptors that thrombin has been found to activate is called the protease-activated receptor (PAR). Our knowledge of this family of G-protein-coupled receptors is growing. There are currently four known receptors in this family (PARs 1-4). These receptors are activated by proteolytic cleavage of the N-terminal sequence, which then acts as a tethered receptor ligand. It is well known that PAR-1 plays a key role in hemostasis. Activation of PAR-1 contributes to proinflammatory effects by stimulating vasodilatation, increased vascular permeability to plasma proteins, and chemotaxis. Thrombin, via activation of PAR-1, stimulates various inflammatory cells to release mediators such as histamine, eicosanoids, and cytokines, and to induce leukocyte rolling and adherence on post-capillary mesenteric venules (reviewed in Vergnolle *et al*, 2001). Thrombin induced IL-6 production by endothelial cells is potentiated by LPS and TNFα, indicating that thrombin and inflammatory mediators may cooperate to amplify vascular inflammation (Chi *et al*, 2001).

With the many cellular and physiological effects stimulated by thrombin, it should be no surprise that thrombin plays a role in LPS-

induced liver injury. Within three hours of a lethal dose of LPS to rats, circulating fibrinogen concentrations decrease by more than 90% (Margaretten *et al*, 1967; Prager *et al*, 1979). At the same time fibrin clots appear in the microcirculation of the liver. Infusion of thrombin into the portal vein leads to morphological changes in the liver that are similar to portal venous infusion of LPS. These changes include fibrin deposition, neutrophil accumulation, and hepatic injury (Shibayama, 1987; Hewett *et al*, 1993). The anticoagulants, heparin and warfarin, and the thrombin specific inhibitor, hirudin, protect against LPS-induced liver injury (Pernerstorfer *et al*, 1999; Moulin *et al*, 1996; Pearson *et al*, 1996), underlining the critical role played by thrombin. However, ancrod, which depletes circulating fibrinogen and thereby prevents formation of fibrin clots, does not protect against LPS-induced liver injury (Hewett *et al*, 1995; Moulin *et al*, 1996). This result suggests that thrombin contributes to injury by a mechanism independent of clot formation. This interpretation was supported by the observation that perfusion of livers isolated from LPS-pretreated rats with medium containing thrombin produced injury whereas no injury was observed in the absence of thrombin (Moulin *et al*, 1996).

Though thrombin induces neutrophil chemotaxis and aggregation, this does not appear to be the mechanism by which thrombin contributes to LPS-induced liver injury (Bizios *et al*, 1986). Many

investigators have found that thrombin is not critical for LPS-induced neutrophil accumulation into the liver (Hoffmann *et al*, 2000; Woodman *et al*, 2000; Pearson *et al*, 1996). However, the role of thrombin in neutrophil extravasation has not yet been fully investigated. The ability of the neutrophil to extravasate may be a critical step for neutrophils to damage hepatocytes (Jaeschke, 1997).

1.1.3.5 Summary

From the text above it is clear that hepatocyte injury induced by LPS is critically dependent on the roles of Kupffer cells, neutrophils, and thrombin. The role of each of these factors is intertwined with that of other factors. Though the exact mechanism of LPS-induced liver injury has yet to elucidated, it is clear that endotoxin stimulates the inflammatory system, and when the inflammatory system is stimulated to some threshold, cellular and/or soluble mediators cause hepatocytes to die. The point at which inflammatory cells reach this threshold, lose their anti-microbial specificity for bacteria and begin to injure host cells is not known. Equally unknown is the mechanism that allows an underlying inflammatory state to enhance the toxicity of a xenobiotic agent.

1.2 Endotoxin Enhancement of Xenobiotic Toxicity

LPS administration can enhance the toxicity of many compounds including ozone, aflatoxin B_1, ethanol, benzyl alcohol, verotoxin 2, halothane, carbon tetrachloride, and galactosamine (Peavy *et al*, 1987; Barton *et al*, 2000; Thurman, 1998; Cebula *et al*, 1984; Sugatani *et al*, 2000; Lind *et al*, 1984; Formal *et al*, 1960; Galanos *et al*, 1979). The biological relevance of this enhancement was demonstrated through the reduction of xenobiotic toxicity by eliminating LPS from animals. Animals pretreated with LPS binding compounds such as polymyxin B, lactulose, or LPS-neutralizing antibody were protected against the toxicity of carbon tetrachloride, halothane, and galactosamine (Nolan *et al*, 1978; Lind *et al*, 1984; Czaja *et al*, 1994). D-galactosamine-induced acute liver injury was attenuated by removal of normal gut flora (Camara *et al*, 1983). Induction of endotoxin tolerance by pretreatment with LPS protected against the toxicity of ozone, carbon tetrachloride, and acetaminophen (Peavy *et al*, 1987; Liu *et al*, 2000). A dose equivalent to 1% of the lethal dose of LPS negated the protective effects of colectomy on murine hepatitis induced by frog virus 3 (Kirn *et al*, 1982).

The culmination of these data and others not described, clearly is that LPS is capable of altering the toxic threshold of a wide variety of chemicals. Synthesizing the literature it becomes evident that the timing of exposure to LPS relative to exposure to the chemical is

important in determining the nature of the response. In general, pretreatment with LPS 16 or more hours before a toxic insult decreases an animal's toxic threshold. The mechanism for this is thought to be dependent on either decreased cytochrome P450 levels (Liu *et al*, 2000) or an increased amount of antioxidants (Peavy *et al*, 1987). Cotreatment with LPS at or near the time of administration of the toxicant generally results in a decreased toxic threshold; this may be due to injury from the inflammatory response such as reactive oxygen species and initiation of coagulation. This thought is based on the data indicating that inhibition of cyclooxygenase-2, Kupffer cells, neutrophils, and the coagulation cascade can attenuated LPS enhancement of xenobiotic hepatotoxicity (Ganey *et al*, 2001; Sneed *et al*, 1997; Barton *et al*, 2000). Therefore, whereas underlying inflammation induced by LPS exposure may enhance human health risk of xenobiotics, the timing of these two episodes is critical.

The potential for LPS to decrease the toxic threshold of xenobiotics found in the environment or work setting is a concern for human health. People are often exposed to small doses of LPS from many sources. Perhaps the most common source of LPS exposure is translocation through the gastrointestinal tract. This normally occurs in small quantities but is greatly increased during stresses such as abdominal surgery, drinking alcohol, and even running marathons

(Palmer *et al.* 1980; Deitch, 1994; Bode *et al*, 1987; Camus *et al*, 1997). Though large doses of LPS cause toxic injury, over the past several years small, nontoxic doses of LPS have been shown to potentiate the toxicity of many xenobiotics as described above. By seeking to understand the mechanism by which LPS potentiates the toxicity of xenobiotics, we may be able to predict which xenobiotic-induced toxicities LPS will enhance and perhaps inhibit the possible potentiation of drugs administered to patients susceptible to endotoxemia or underlying inflammation. To help elucidate this mechanism we chose to examine the mechanism of allyl alcohol-induced liver injury enhancement by LPS. Allyl alcohol is a well-studied model periportal hepatotoxicant with a great deal of literature describing the mechanism of its toxicity. A review of this literature is presented here.

1.3 Allyl Alcohol

Allyl alcohol (CH_2=CH-CH_2OH) is commonly used as an intermediate in the synthesis of many industrial compounds. It is also a constituent of some perfumes, food colorings, and pharmaceuticals. In addition, allyl alcohol can be formed from the hydrolysis of the allyl ester of weak acids (Kaye, 1973). As of 1977 there were no specific regulations for materials used in food flavorings for the United Kingdom. The Council of Europe classified allyl alcohol as "an artificial flavoring

substance that may be added temporarily to foodstuffs without hazard to public health", and it recommended it be further studied in acute and short-term experiments in animals of various species. It is a lachrymator causing eye, skin, and upper respiratory irritation. Humans can readily detect allyl alcohol by smell at 0.78 ppm, and it causes nose and eye irritation intolerably at 25 ppm. The symptoms usually go away after about 24-48 hours (Dunlap *et al*, 1958). "Typical signs of acute allyl alcohol toxicity in animals, regardless of the route of administration were pulmonary effusion, congestion of internal organs, marked hypotension, cloudy swelling of the kidney cells, and necrosis of the periportal areas of the liver" (Carpanini *et al*, 1978). The toxicity of allyl alcohol has been thoroughly studied to elucidate the mechanism.

To gain understanding of the mechanism for allyl alcohol toxicity an understanding of its metabolic pathways are necessary. The rate of metabolism for allyl alcohol was calculated to be about 23 mg/kg/h during constant *i.v.* infusion (Kodama *et al*, 1958). When rats were given an *i.p.* injection of ^{14}C-labeled allyl alcohol most of the label was found in the periportal region of the liver; however, when an inhibitor of alcohol dehydrogenase was added the amount of ^{14}C-labeled allyl alcohol in the liver was reduced by 80 percent. This large difference was not found in any other organs (Reid, 1972). This indicated that alcohol dehydrogenase is involved in the metabolism of allyl alcohol and causes

allyl alcohol to accumulate in the liver. This enzyme produces the major oxidative metabolite, acrolein, via the cofactor NAD+ (Patel *et al*, 1980, Serafini-Cessi, 1972). It is generally accepted that acrolein is the metabolite that is responsible for cellular damage. This was demonstrated by showing that inhibition of alcohol dehydrogenase activity blocks hepatic injury (Patel *et al*, 1980, Serafini-Cessi, 1972) and that addition of acrolein leads to cytotoxicity (Silva *et al*, 1989).

Acrolein is a very reactive aldehyde that is found in cigarette smoke and automobile exhaust and is a by-product of many industrial processes including burning oils. In fact, the word "acrolein" derives from the acrid smell of overheated frying oil (Rikans, 1987). It is also a metabolite of the common anticancer and immunosuppressant drug, cyclophosphamide. Acrolein has four main metabolic fates within the cell. First, about 15% of acrolein is converted to the non-reactive acrylic acid via the enzyme aldehyde dehydrogenase in the presence of NAD+ (Patel *et al*, 1980). Second, glutathione has a high affinity for binding the reactive acrolein molecules forming S-3-hydroxypropylglutathione, which is further broken down to 3-hydroxypropylmercapturic acid. Third, acrolein can be converted into an epoxide, glycidaldehyde, via liver microsomes in the presence of NADPH. Glycidaldehyde is then hydrolyzed to glyceraldehyde by epoxide hydrolase. Both glycidaldehyde and glycidol are substrates for glutathione-s-epoxide transferase (Patel

et al, 1980). Finally, acrolein will bind to protein sulfhydryl groups. These metabolic fates of acrolein act in concert to inhibit protein function through thiol binding, reduce cellular glutathione levels, and provoke the production of free radicals, which in culmination can lead to cellular injury.

Allyl alcohol is considered a classical, periportal hepatotoxicant. Necrotic doses of allyl alcohol cause a periportal-specific lesion which can then spread through the liver acinus toward the pericentral region as the dose of allyl alcohol is increased. The mechanism for the periportal specificity has yet to be determined. Originally it was thought that periportal hepatocytes had a higher alcohol dehydrogenase activity (Serafini-Cessi, 1972). This has since been shown to not be the case through several experiments. Penttila isolated pericentral and periportal hepatocytes and found not only that alcohol dehydrogenase activities were similar in these two populations, but also that periportal hepatocytes actually tended to be more resistant to allyl alcohol toxicity. This might have been due to their 26% higher GSH content after preincubation with L-methionine, because glutathione has been shown to be protective against allyl alcohol hepatotoxicity (Penttila, 1988). Liver sinusoids were also microdissected, and both alcohol dehydrogenase and aldehyde dehydrogenase activities were similar in periportal and pericentral regions (Sasse *et al*, 1991). In the isolated, perfused liver the

maximal increase in NADH fluorescence due to allyl alcohol metabolism was greater in the pericentral region (Belinsky *et al*, 1984). Cumulatively, these data clearly prove that allyl alcohol is not periportal-specific because of a higher alcohol dehydrogenase activity in this region.

The concentrations of allyl alcohol in the V. portae and V. cava were equal 30 minutes after dosing with allyl alcohol (Belinsky *et al*, 1984), which would suggest that both areas of the liver received the same amount of allyl alcohol. On repetition of this experiment, a different group of investigators found that the concentration of allyl alcohol at one minute after administration was 2800 μM in the V. portae and only 120 μM in the V. hepatica. By five minutes the V. portae concentration had decreased to 236 μM (Sasse *et al*, 1991). In addition, it was found that perfusing allyl alcohol in the retrograde direction caused pericentral-specific damage (Badr *et al*, 1986). From these experiments it is clear that there must be some concentration gradient within the liver causing the periportal hepatocytes to receive a larger dose of allyl alcohol.

Further experiments in the isolated, perfused liver demonstrated the importance of oxygen to allyl alcohol-induced hepatic injury. Because oxygen uptake was decreased only in the damaged region and the amount of injury was dependent on the oxygen content of the

perfused medium (Badr *et al*, 1986), it was suggested that oxygen concentration may play an important role in the zone-specificity of allyl alcohol. The concentration of oxygen did not affect the toxicity of allyl alcohol in isolated hepatocytes indicating that some other cell type is involved in the oxygen-dependence. The role of Kupffer cells, which are predominately found in the periportal region, in the oxygen-dependence of allyl alcohol injury was investigated by blocking Kupffer cell activity with gadolinium chloride ($GdCl_3$). Rats treated with $GdCl_3$ developed less hepatic injury from allyl alcohol suggesting that Kupffer cells may be involved. Interestingly, blocking Kupffer cell activity with $GdCl_3$ in the isolated, medium-perfused liver had no effect on the hepatotoxicity of allyl alcohol, suggesting that circulating blood elements may also contribute to the injury (Przybocki *et al*, 1992). This experiment was repeated by our laboratory and resulted in contradictory findings. Neither inhibition of Kupffer cells with $GdCl_3$ nor depletion of neutrophils with anti-neutrophil antibodies was protective against allyl alcohol hepatotoxicity (Ganey *et al*, 1995). Therefore, the importance of Kupffer cells in allyl alcohol hepatotoxicity may be dependent on other factors and is likely not the cause of periportal specificity.

It was hypothesized that glutathione concentrations could explain the periportal specificity of allyl alcohol hepatotoxicity. Glutathione was depleted throughout the liver within 20 minutes after allyl alcohol

injection, but liver injury was not markedly increased until 40 minutes and was observed only in the periportal region. Because glutathione levels were depleted in periportal and pericentral areas, glutathione depletion alone could not explain the specificity of allyl alcohol for the periportal region. Interestingly, after 60 minutes of allyl alcohol perfusion of isolated livers, the ATP/ADP ratio was only decreased in the periportal region, indicating the possibility that periportal cells had less energy to protect and repair themselves. However, it was argued that because decreasing the ATP/ADP ratio with potassium cyanide did not induce liver injury, the decrease in energy status did not explain the zone-specificity of allyl alcohol (Badr *et al*, 1986). Thus, the reason for the periportal localization remains unknown.

Equally mysterious is the actual cause of cell death from allyl alcohol toxicity. Some groups think that hepatocytes die from the direct effects of acrolein binding to and disrupting critical proteins. Others argue that acrolein indirectly causes toxicity by depleting glutathione stores, leaving the cell unprotected from oxidative injury. One of the cell's mechanisms for protection from protein thiol binding and oxidative injury is glutathione. Allyl alcohol caused a rapid decrease in reduced glutathione, without the formation of oxidized glutathione, and a decrease in protein thiols (Dogterom *et al*, 1988; Jaeschke *et al*, 1986; Silva *et al*, 1989; Comporti et al, 1991). Depletion of glutathione by 65%

with ethacrynic acid increased acrolein-induced hepatocyte toxicity (Silva *et al*, 1989). Hepatocytes incubated with acrolein and washed were protected from injury by the addition of dithiothreitol, glutathione, or N-acetylcysteine indicating that initial events in this cytotoxicity are reversible (Silva *et al*, 1989; Rikans *et al*, 1994).

The loss of protein thiol groups can lead to the inactivation of proteins necessary for maintaining cellular energy levels (i.e. ATP). This decrease in energy levels was thought to be a cause of cell death from allyl alcohol. In isolated, rat hepatocytes allyl alcohol (50 μM) caused a 15 percent decrease in ATP. Because a similar decrease in ATP concentration using sodium azide did not induce the same injury, it was concluded that alteration of energy homeostasis alone did not explain cell death from allyl alcohol (Rikans *et al*, 1995).

Another hypothesis is that lipid peroxidation is more important to cell death from allyl alcohol than protein loss. Vitamin E, an antioxidant, protected hepatocytes from lipid peroxidation and death from allyl alcohol (Dogterom *et al*, 1989, Comporti *et al*, 1991). The importance of lipid peroxidation was called into question with the observation that disulfiram, an inhibitor of aldehyde dehydrogenase, and its metabolite, diethyldithiocarbamate, protected from lipid peroxidation but not cell death in hepatocytes (Dogterom *et al*, 1988). This interpretation may have been flawed, however, because disulfiram alone increased cell

death. On the other hand, the prevention of lipid peroxidation with desferrioxamine, an iron chelator, was accompanied by decreased cell death and protein thiol loss of membranes with no decrease in thiol loss in the cytosol (Pompella *et al*, 1991; Dogterom *et al*, 1988; Jaeschke *et al*, 1987; Miccadei *et al*, 1988). This indicates the possibility that protein thiol loss in the membrane is in part from lipid-derived radicals.

Because of the protective effect of desferrioxamine, it was thought that the lipid peroxidation occurs through an iron-dependent mechanism. In support of this, ferrous sulfate increased allyl alcohol-induced damage. In addition, NADH and Fe3+ or NADH and ferritin induced lipid peroxidation in hepatocytes (Jaeschke *et al*, 1992). Furthermore, lipid peroxidation was observed only when glutathione stores were depleted by allyl alcohol to 15-30% of normal levels (Silva *et al*, 1989). This is less glutathione depletion than is thought necessary to allow endogenous lipid peroxidation to occur. There are other proposed sources of free radicals for the initiation of lipid peroxidation. Acrolein and its glutathione adduct, glutathionylpropionaldehyde, induce oxygen radical formation: the glutathionylpropionaldehyde metabolite of acrolein interacts with xanthine oxidase and aldehyde dehydrogenase to produce superoxide and hydroxyl radicals, and acrolein forms acroleinyl radical and superoxide (Adams *et al*, 1993).

The evidence presented suggests that allyl alcohol depletes glutathione stores which leaves the cell more susceptible to lipid peroxidation. Lipid peroxidation may be initiated through several sources including Kupffer cell- or neutrophil-derived reactive oxygen intermediates, released iron, xanthine oxidase, or aldehyde dehydrogenase activity. If these events that occur with larger, toxic doses of allyl alcohol also occur with threshold doses, then the reactive oxygen intermediates released from LPS-stimulated inflammatory cells may significantly increase injury.

1.4 The Potentiation of Allyl Alcohol-Induced Liver Injury

To understand better the mechanism by which LPS enhances the toxicity of many xenobiotics our lab has focused on the ability of LPS to potentiate the toxicity of allyl alcohol. Rats were used to produce a dose-response curve for LPS and allyl alcohol. Doses of each compound were found that did not cause toxicity alone. The rats were given a nonhepatotoxic dose of LPS two hours before a nonhepatotoxic dose of allyl alcohol. This combination resulted in a significant hepatotoxicity (Sneed *et al*, 1997). Furthermore, treatment of LPS-treated rats with gadolinium chloride abolished the LPS enhancement of allyl alcohol hepatotoxicity (Sneed *et al*, 1997). These studies demonstrate the potentiation of allyl alcohol-induced liver injury by LPS and the

importance of the activation state of the inflammatory system in this potentiation.

The LPS potentiation of allyl alcohol-induced liver injury was studied further to determine its mechanism. The conclusion that LPS potentiates the toxicity of allyl alcohol is based on histology. Injury from large doses of LPS is focused around the midzonal region, and the potentiated injury is focused around the periportal region similar to what occurs after a large dose of allyl alcohol (Sneed *et al*, 1997). For the potentiation, maximal liver injury occurs six hours after allyl alcohol administration as measured by the release of alanine aminotransferase (ALT), indicating that the injury is rapid in onset. This injury is dependent on the metabolism of allyl alcohol into the reactive metabolite acrolein through the enzyme alcohol dehydrogenase. An inhibitor of alcohol dehydrogenase, 4-methylpyrazole, blocked the potentiation of allyl alcohol hepatotoxicity by LPS (Sneed *et al*, 1997). In addition, LPS administration does not appear to affect the rate of allyl alcohol metabolism or the amount of glutathione found in the liver (Sneed et al, 1997). Thus, the basic mechanism of allyl alcohol toxicity appears to be unaltered by LPS, and LPS does not make the liver more susceptible to allyl alcohol by depleting protective glutathione.

The importance of other cell types was demonstrated through several experiments. In isolated hepatocytes, LPS did not shift the dose-

response curve for allyl alcohol, indicating that the potentiation observed *in vivo* could not be replicated with hepatocytes alone. TNF-α is involved in many mechanisms of injury; however, it is not thought to be involved in this potentiation due to the lack of protection from anti-TNF antibodies (Sneed *et al*, 2000). It remains possible that TNF-α acts in an autocrine fashion and that inhibition with anti-TNF-α antibodies could not scavenge the TNF-α before acting on a TNF-α receptor. However, when TNF was added to isolated hepatocyte cultures no alteration in allyl alcohol toxicity was observed, indicating that TNF is not involved in the potentiation through direct stimulation of hepatocytes (Sneed *et al*, 2000).

The ability of gadolinium chloride to attenuate the potentiation response demonstrates the importance of Kupffer cells to this injury (Sneed *et al*, 1997). Because the ability of gadolinium chloride to inhibit Kupffer cells specifically is the main evidence that indicates the importance of Kupffer cells in this model of potentiation, it is important that we have little question about the mechanism by which gadolinium chloride attenuates the potentiation of allyl alcohol toxicity by endotoxin. Gadolinium chloride is a rare earth metal that selectively inhibits Kupffer cell activity. The mechanism by which gadolinium chloride inhibits Kupffer cell activity is not completely understood. Gadolinium chloride is soluble in an acidic solution. When this acidic solution is injected into

the systemic circulation, the pH increases and the gadolinium chloride forms particulate clumps in the blood that are then phagocytosed by macrophages. Once inside the acidic lysosomal compartment the particulates may release free gadolinium into the macrophage that may then be toxic to the cell (reviewed in Adachi *et al*, 1994). The specificity of gadolinium chloride is likely due to the enormous phagocytic capacity of the Kupffer cell. Kupffer cells represent about 85 to 95 percent of the total intravascular phagocytic capacity (Ruttinger *et al*, 1996). Gadolinium chloride does have other effects including inhibition of stretch-activated ion channels and physiological responses of tissues to mechanical stimulation (Adding *et al*, 2001). However, in the liver the predominant effect of gadolinium chloride is to decrease phagocytic activity of Kupffer cells. Though gadolinium chloride treatment alone can cause a decrease in P450 activity and slight increases in serum markers of liver injury (ALT and AST), cytokine release (TNF-α and IL-6), bile flow, and the number of nonperfused sinusoids, there was no change in observable liver injury, mean arterial blood pressure, or amount of circulating endotoxin in gadolinium chloride treated animals (Ruttinger *et al*, 1996; Badger *et al*, 1997). At smaller doses, gadolinium chloride can cause Kupffer cell death and at larger doses Kupffer cells are absent from the liver, presumably because they have died and been washed out by circulating blood (Adachi *et al*, 1994). Gadolinium chloride

pretreatment of LPS-treated animals increased survival and decreased liver injury as well as cytokine release (Vollmar *et al*, 1996; Sarphie *et al*, 1996; Iimuro *et al*, 1994; Fujita *et al*, 1995).

Most investigators demonstrate inhibition of Kupffer cell activity by gadolinium chloride treatment by measuring either phagocytic capacity of the Kupffer cells or by counting the number of Kupffer cells in the liver. These are usually done in a separate experiment and do not demonstrate that Kupffer cells were inhibited during the experiment from which they drew conclusions. Furthermore, whereas they demonstrate the inhibition of Kupffer cells in a separate experiment they do not monitor many, if any, other effects that gadolinium chloride could have on the liver. Thus conclusions drawn from experiments using gadolinium chloride are based on the drug's main effect, inhibition of Kupffer cell activity. While there are other inhibitors of Kupffer cell activity, each has the same basic problems of secondary drug effects. To prove that Kupffer cells play a critical role would require further examination, such as *in vitro* experiments.

Experiments demonstrating the ability of gadolinium chloride to attenuate LPS enhancement of allyl alcohol toxicity did not demonstrate inhibition of Kupffer cells (Sneed *et al*, 1997). In these experiments the dose of gadolinium chloride used was able to inhibit the activity of Kupffer cells as reported by others (Adachi *et al*, 1994; Ganey *et al*,

1995; Brown *et al*, 1997). Furthermore, gadolinium chloride does not inhibit the activity of alcohol dehydrogenase (Przybocki *et al*, 1992), indicating that attenuation of LPS potentiation of allyl alcohol toxicity with gadolinium chloride is not through decreased metabolism of allyl alcohol into the reactive metabolite, acrolein. The inability of gadolinium chloride to inhibit the toxicity of allyl alcohol given alone indicates that gadolinium chloride does not have any effects on the toxicity of allyl alcohol. Thus, the inhibition of LPS-enhanced allyl alcohol hepatotoxicity by gadolinium chloride is likely due to its ability to inhibit Kupffer cell activity or some other LPS-mediated portion of the potentiation. To further examine the role of Kupffer cells in the potentiation of allyl alcohol toxicity by LPS, hepatocyte-Kupffer cell cocultures were used in experiments described below.

Neutrophils were also found to contribute to the potentiation of allyl alcohol toxicity by LPS. Depletion of neutrophils with an anti-neutrophil antibody afforded protection from liver injury (Sneed, 2000). The specificity of the anti-neutrophil antibody for depleting circulating neutrophils was determined by counting white blood cells. Whereas the number of neutrophils and monocytes was significantly decreased the number of lymphocytes was not significantly decreased. While the possibility remains that monocytes may play a critical role in the potentiation of allyl alcohol hepatotoxicity by LPS, injury develops very

rapidly in this model of toxicity, and neutrophils are known to elicit their actions before monocytic infiltration by several hours. Furthermore, neutrophils play a critical role in LPS-induced liver injury (Hewett *et al*, 1992). The possible contributions that neutrophils provide to the potentiation of allyl alcohol by LPS needs to be investigated.

The potentiation of allyl alcohol hepatotoxicity by endotoxin appears to involve a complicated mechanism that includes the activities of several cell types in the liver. The focus of this thesis is to elucidate further the mechanism by which endotoxin enhances the hepatotoxicity of allyl alcohol. This includes investigating the roles of Kupffer cells, neutrophils, and other factors involved in inflammation.

CHAPTER 2

Involvement of Kupffer Cells in the Potentiation of Allyl Alcohol Hepatotoxicity by LPS

2.1 Introduction

The mechanism underlying the enhancement of allyl alcohol-induced injury by LPS is unknown. Although LPS can alter the hepatic metabolism of many compounds through changes in cytochrome P450 levels (reviewed in Morgan, 2001), LPS did not affect the rate of allyl alcohol metabolism (Sneed RA *et al*, 1997). There is evidence to suggest that the LPS enhancement of allyl alcohol toxicity involves components of the inflammatory system. For example, hepatocytes (HCs) exposed to LPS were not more sensitive to allyl alcohol than naïve HCs, indicating that LPS does not increase toxicity by directly altering HCs (Sneed *et al*, 1997). In addition, inhibition of activity of Kupffer cells (KCs), the resident macrophage of the liver, significantly decreased the potentiation of allyl alcohol hepatotoxicity by LPS, indicating that KCs play a critical role in the potentiation (Sneed *et al*, 1997). Inhibition of Kupffer cells can attenuate the toxicity of many xenobiotics including: carbon tetrachloride (Pereira *et al*, 1997; Badger *et al*, 1996; Wueweera *et al*, 1996), vinylidene chloride (Wueweera *et al*, 1996), acetaminophen (Michael et al, 1999), D-galactosamine (Stachlewitz *et al*, 1999), 1,2-dichlorobenzene (Hoglen *et al*, 1998), LPS (Sarphie *et al*, 1996; Fujita *et al*, 1995; Iimuro *et al*, 1994), ethanol (Bautista *et al*, 1999; Adachi *et al*, 1994), and diethyldithiocarbamate (Ishiyama *et al*, 1995). Because

Kupffer cells are thought to play a critical role in the development of liver injury of many compounds we sought to understand better the involvement of Kupffer cells in the potentiation of allyl alcohol hepatotoxicity by LPS.

The ability of LPS to stimulate KCs to release many mediators of inflammation that can act directly or indirectly on HCs to alter their physiology led to the hypothesis that LPS-stimulated KCs sensitize HCs to allyl alcohol-induced toxicity through the release of inflammatory mediators. Results presented here to test this hypothesis demonstrated that the model is more complex than a simple interaction among KCs, LPS, and HCs.

2.2 Methods

2.2.1 Materials

Williams' Medium E, gentamicin, RPMI, and NCTC-109 medium were purchased from GibcoBRL, Rockville, MD. Fetal Calf Serum was purchased from Intergen, Purchase, NY. TNF-α ELISA kits were purchased from Biosource, Camarillo, CA. ProbeOn Plus microscope slides were purchased from Fisher Scientific, Pittsburgh, PA. All other materials were purchased from Sigma Chemical Co., St. Louis, MO.

2.2.2 Animals

Male, Sprague-Dawley rats (CD-Crl:CD-(SD)BR VAF/Plus; Charles River, Portage, MI) weighing 175-225 g were allowed food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water *ad libitum*. They were housed under conditions of controlled temperature, humidity and 12-hr light and dark cycle. All procedures on animals were carried out according to the humane guidelines of the AALAS and the University Laboratory Animal Research Unit at MSU.

2.2.3 Kupffer Cell Isolation

KCs were isolated using a method based on Knook, 1977. Rats were anesthetized with sodium pentobarbital (50 mg/kg). The portal vein was cannulated and perfused with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution to clear the blood from the liver. The liver was then perfused with 200 ml of collagenase type II (0.4 mg/ml) in Gey's balanced salt solution (GBS). Pronase (0.2% in GBS with 2 μg/ml DNAse) was then perfused in a recirculating manner for 10-15 minutes. The liver was removed and gently combed to loosen the cells. The cells were filtered through gauze and centrifuged (50 x g, 2 min) to remove HCs, and the supernatant was collected from centrifugation (50 X g, 2 min) of cells twice. The cells found in the supernatants were then pooled via centrifugation (600 x g, 5 min). These cells were resuspended in

GBS containing 2 μg/ml DNAse and loaded onto a centrifugal elutriator (Beckman J6-MI centrifuge with JE-6B elutriator rotor). GBS was pumped through the rotor at a rate of 12 ml/min for 250 ml. Flow rate was then increased to 24 ml/min for 150 ml to remove endothelial cells. Flow was increased again to 42 ml/min for 150 ml and collected. This fraction was spun in a centrifuge (600 x g, 5 min), and the pellet was resuspended in RPMI culture medium supplemented with 1% Medium NCTC-109 and 15% bovine calf serum. The KCs were then plated in 12-well Falcon Primaria culture plates at 1 X 10^6 cells/well (37°C, 7.5% CO_2, 92.5% air). KC purity was found to be greater than 90% via peroxidase staining and latex bead phagocytosis. KC responsiveness was assessed by measuring TNF-α levels in the medium 90 min after stimulation with LPS (10^5 EU/ml) using a rat TNF-α ELISA kit.

2.2.4 Hepatocyte Isolation

Hepatic parenchymal cells were isolated using methods established by Seglen, 1973. Briefly, rats were anesthetized with sodium pentobarbitol (50 mg/kg, i.p.). The liver portal vein was cannulated and perfused with Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution to remove red blood cells and then digested by perfusion with a solution containing 60 mg collagenase type II in 250 ml Williams' Medium-E for 30 min. The digested liver was gently combed, filtered

through sterile gauze, and centrifuged (50 X g, 2 min) to pellet the HCs. The cells were washed twice with Williams' Medium-E and resuspended in Williams' Medium-E containing 1% gentamycin and 10% fetal calf serum. Viability was assessed via trypan blue exclusion. If cells were greater than 80% viable, they were plated in 12-well Falcon Primaria culture plates at a density of 2.5 X 10^5 cells/well. The cells were then allowed to incubate (37°C, 7.5% CO_2, 92.5% air) for four hours before the cells were washed and used in an experiment.

2.2.5 *In Vitro* Coculture Experiments

Kupffer cells were isolated and plated as described above. After 20 hours of incubation medium was removed, and hepatocytes isolated as described above were plated with the Kupffer cells. After an additional four hours of incubation, cocultures were treated with LPS and allyl alcohol. Medium was collected 1.5 hours after addition of allyl alcohol, and cells were lysed with 1 ml of 1% Triton X. Cytotoxicity was assessed from release of alanine aminotransferase (ALT) into the medium. Activity of ALT was determined in cell-free supernatant fluids using Sigma Diagnostics Kit No. 52. Percent total ALT was calculated by dividing ALT activity in medium by the total ALT activity found in medium and cell lysates. Total ALT activity in the medium and cell lysates did not change with any culture treatment. Results were

analyzed using analysis of variance (ANOVA) blocked for each day of the experiment.

2.2.6 Slide Coculture Experiment

Hepatocytes and Kupffer cells were isolated as described above and plated on ProbeOn Plus microscope slides. At the time of the experiment two slides were clipped together face to face so that the cells were within the capillary space between the slides provided by the thick frosted corners of the ProbeOn Plus microscope slides. The slides were set vertically in a small trough with a section of filter paper set on top, which served to oxygenate and distribute the medium evenly over the length of the slide. Medium was then pumped in a circulating manner from the trough to the filter were it flowed between the slides and was collected in the trough. Cells were treated with LPS and allyl alcohol and assessed for injury as described for coculture experiments above.

2.2.7 Liver Homogenate Experiment

Hepatocytes were isolated and plated as described above. Non-parenchymal cells, consisting of the cells remaining in the supernatant after the liver homogenate was centrifuged (50 X g, 2 min), were plated in 12-well Falcon Primaria culture plates at 1 X 10^6 cells/well containing hepatocytes. After incubation the cells were treated with LPS and allyl

alcohol, and cytotoxicity was assessed from release of ALT as described above.

2.2.8 Isolation and Perfusion of Rat Livers

Rats were weighed, then anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the portal vein was exposed. Livers were perfused via portal vein cannulation with polyethylene tubing (PE 190, Clay Adams, Parsippany, NJ). The perfusion medium was Krebs-Henseleit bicarbonate buffer supplemented with 2% bovine serum albumin and saturated with 95% O_2 and 5% CO_2 gas. Flow was constant at rate of 0.14 ml/min/g body weight. The thoracic portion of the inferior vena cava was cannulated (PE 240 Clay Adams, Parsippany, NJ) for outflow. The liver was placed in a temperature-controlled cabinet maintained at 37°C as previously described (Moulin *et al*, 1996). The livers were allowed to stabilize for 10 minutes with single pass perfusion. A sample of the perfusion medium was then taken (time = 0), and the system was switched to recirculating perfusion with medium containing LPS (1.2 X 10^5 EU/kg body weight). Samples (500μL) of the perfusion medium were taken every 30 minutes for two hours. At two hours allyl alcohol (30, 25, or 20 mg/kg body weight) was added to the perfusion reservoir, and medium samples were taken every 15 minutes for another two hours. ALT activity in the samples was determined as described above.

Previous experience demonstrated that perfusion of naïve livers with this dose of LPS does not produce injury. Accordingly, livers for which ALT activity in the perfused medium at two hours was greater than 200 U/L were considered damaged during the isolation procedure and were removed from the experiment.

2.2.9 Statistical Analysis

Data are expressed as means ± S.E.M. For all results presented, N represents the number of individual experiments. Data with non-homogeneous variances were square root- or log-transformed prior to analysis. Data represented as percents were angular-transformed prior to analysis. Data were analyzed using repeated measures ANOVA provided in the statistics program NCSS 2000. Comparisons among groups were performed using Fisher's Least Squares Difference test. The criterion for statistical significance was $p \leq 0.05$.

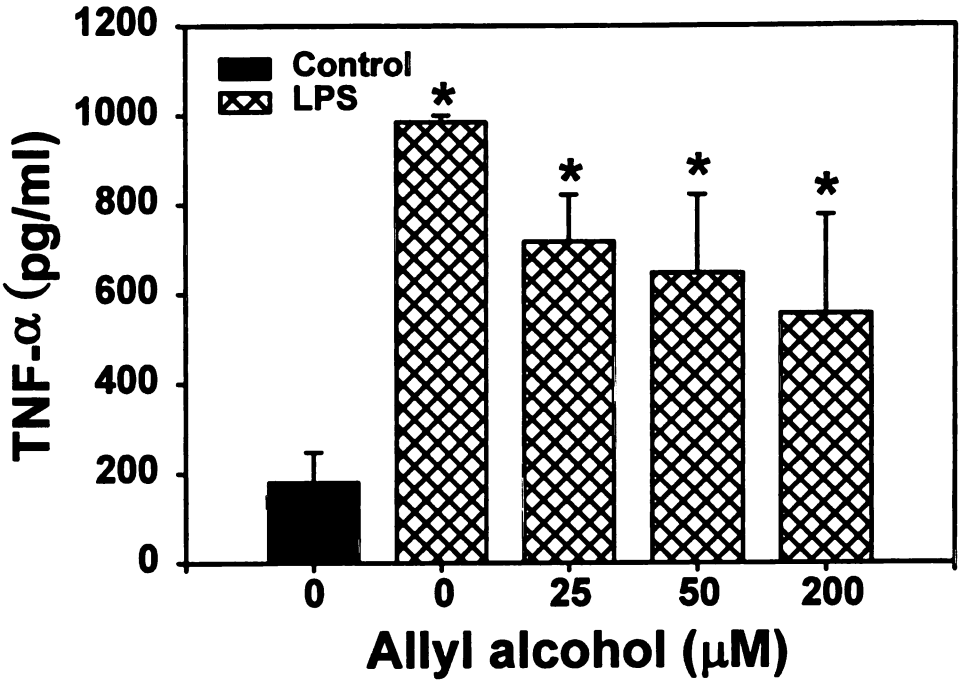
2.3 Results

KCs are critical to LPS enhancement of AA toxicity in vivo (Sneed *et al*, 1997), accordingly initial studies were designed to examine whether LPS-stimulated KCs or their mediators increased hepatocellular sensitivity to allyl alcohol. It has been reported that LPS stimulates the release of TNF-α from KCs (Lichtman *et al*, 1996). To ensure that isolated KCs to be used in these studies were functional and responsive to LPS, TNF-α concentration in medium of LPS-treated KCs was assessed. The concentration of TNF-α in the medium of untreated KCs was low (Figure 2.1). Treatment with LPS (10^5 EU/ml) increased TNF-α release significantly. The presence of allyl alcohol (25, 50, 200 μM) decreased the amount of LPS-stimulated TNF-α release in a concentration-dependent manner.

ALT release in KC/HC cocultures was lower than release in cultures of HCs alone by about 5 to 10% ALT release (data not shown). Exposure of cells to allyl alcohol induced a concentration-dependent increase in ALT release (Figure 2.2). Treatment of KC/HC cocultures with LPS ($1X10^5$ EU/ml) significantly decreased allyl alcohol induced ALT release compared to vehicle. A larger concentration of LPS ($4X10^5$ EU/ml), also did not induce a greater sensitivity to allyl alcohol toxicity *in vitro* (data not shown).

Figure 2.1. LPS stimulates release of TNF-α from KCs.

KCs were isolated via centrifugal elutriation and cultured for 24 hours before stimulation with LPS (10^5 EU/ml). TNF-α concentration was measured using an ELISA kit. Data are expressed as means ± S.E.M. N=3-7. *, significantly different from control.


1200
1000
800
600
400
200
0
TNF-α (pg/ml)
Control
LPS
*
*
*
*
0
0
25
50
200
Allyl alcohol (μM)

In vivo, the ratio of KCs to HCs is about 1:10 (Altin *et al*, 1988); however, toxic responses observed in KC/HC cocultures require a higher ratio of KCs to HCs (Lysz *et al*, 1990; Kausalya *et al*, 1993). Cocultures with a higher ratio of KCs:HCs produce less PGE_2 upon LPS stimulation than cocultures with lower ratios (Billiar *et al*, 1990). Because previous experiments have shown that prostaglandins can increase the toxicity of allyl alcohol in hepatocytes (Ganey *et al*, 2000), we did not want a decreased production of prostaglandins. To examine whether the lack of effect of LPS was related to the high ratio of KCs to HCs, cocultures were plated using different ratios. Allyl alcohol caused a concentration-dependent cytotoxicity, and LPS did not increase the sensitivity of HCs to allyl alcohol at any of the KC:HC ratios tested (Figure 2.3).

The lack of LPS effect on allyl alcohol hepatotoxicity could be dependent on reaching a threshold concentration of mediators or some labile mediator. To address this possibility a simulated sinusoid environment was used in a limited number of experiments. In this experiment hepatocytes and Kupffer cells were cocultured on glass slides placed a capillary distance apart. Although allyl alcohol caused a dose-dependent increase in ALT release, LPS treatment did not enhance the cytotoxicity of allyl alcohol (Figure 2.4).

Figure 2.2. LPS stimulation does not enhance allyl alcohol toxicity in cocultures of KCs and HCs.

Cocultures of KCs and HCs (4:1) were stimulated with LPS (10^5 EU/ml) and exposed to allyl alcohol at the concentrations indicated. ALT release was assessed 1.5 hours after allyl alcohol exposure as described in Methods. Data are expressed as means ± S.E.M. N=4. *, significantly different from control at the indicated concentration.

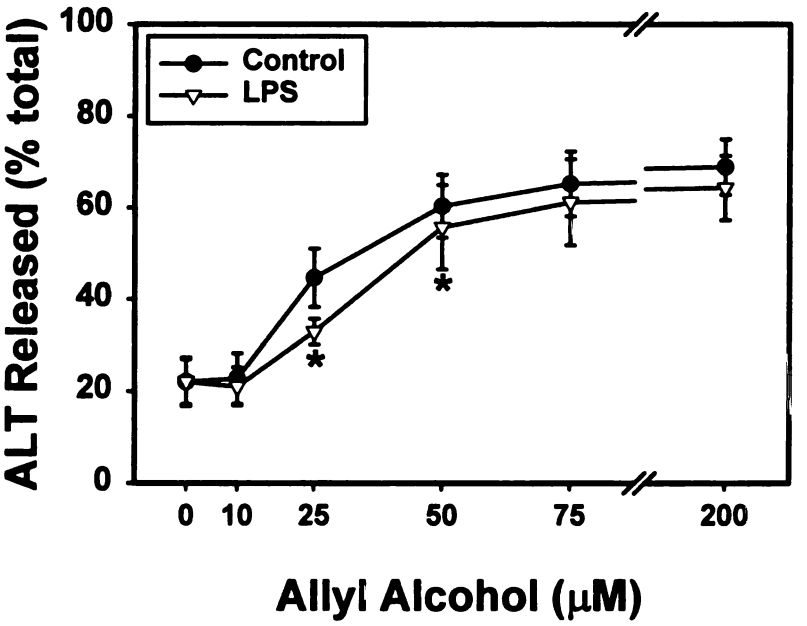

ALT Released (% total)
100
80
60
40
20
0
Control
LPS
*
*
0
10
25
50
75
200
Allyl Alcohol (μM)

Figure 2.3. Altering the ratio of KCs to HCs does not elicit an LPS-induced enhancement of allyl alcohol toxicity.

KCs were cocultured with HCs at a ratio of (A) 4 KCs to 1 HC (B) 1 KC to 1 HC or (C) 1 KC to 10 HCs and were exposed to vehicle (black bars) or LPS (10^5 EU/ml) (white bars) with increasing concentrations of allyl alcohol. ALT release was assessed 1.5 hours after allyl alcohol exposure as described in Methods. Data are expressed as means ± S.E.M. N=4. No significant differences between LPS-treated and control values were observed.

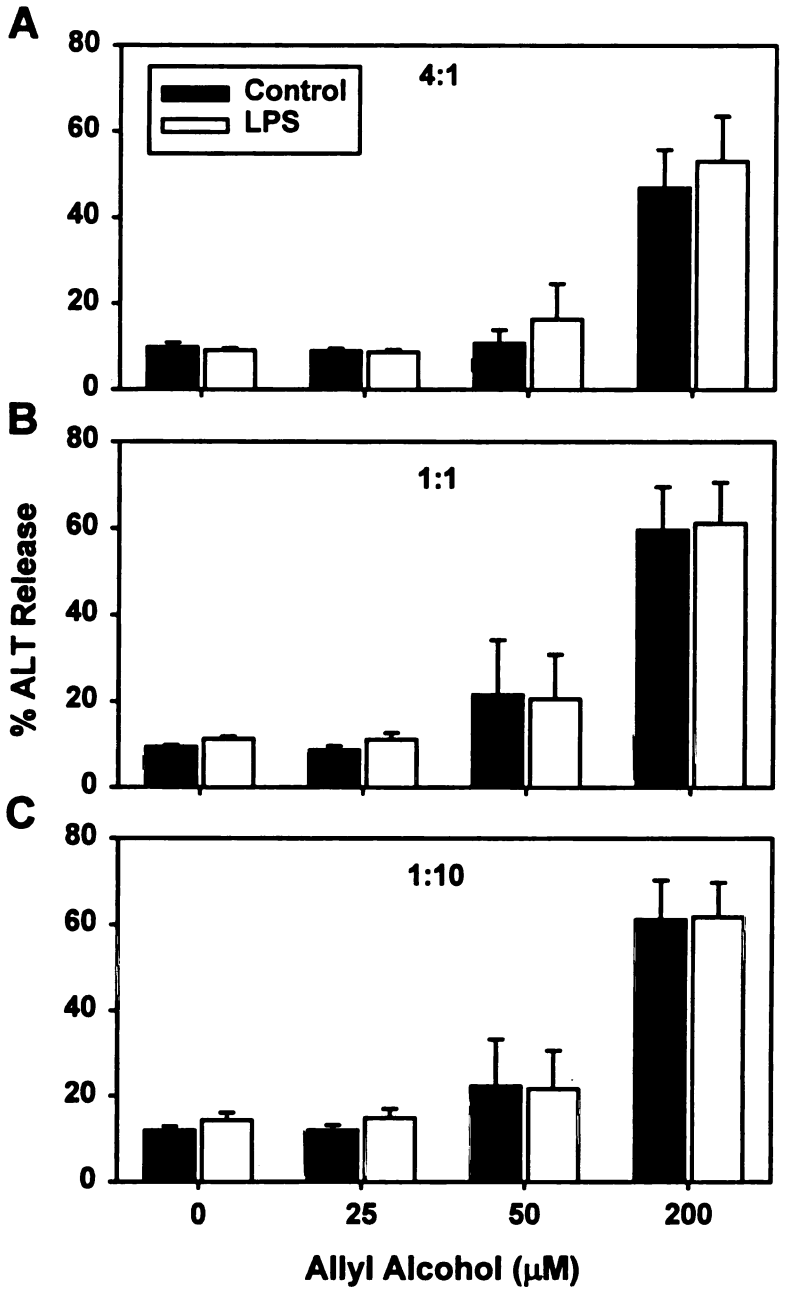
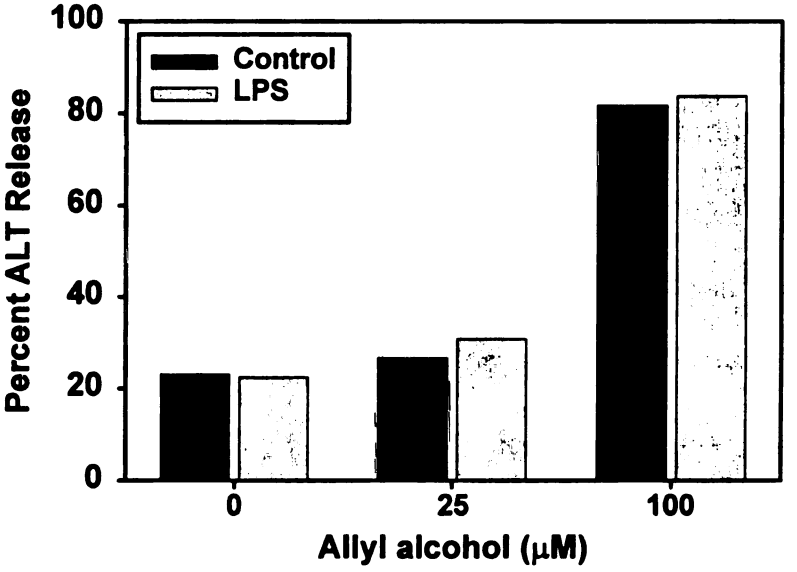

A
B
C
80
60
40
20
0
Control
LPS
4:1
1:1
1:10
% ALT Release
0
25
50
200
Allyl Alcohol (μM)

Figure 2.4. LPS-induced enhancement of allyl alcohol toxicity is not observed in simulated hepatic sinusoids.

KCs were cocultured with HCs on microscope slides placed a capillary distance apart with medium flowing between the slides in a circulating manner. Cells were exposed to vehicle (black bars) or LPS (10^5 EU/ml) (white bars) with increasing concentrations of allyl alcohol. ALT release was assessed 1.5 hours after allyl alcohol exposure as described in Methods. Data are expressed as means, N=2. No differences between LPS-treated and control values were observed.


100
80
60
40
20
0
Percent ALT Release
Control
LPS
0
25
100
Allyl alcohol (μM)

To determine whether the lack of LPS effect may be due to other cell types in the liver that are not found in the coculture, a liver nonparenchymal cell fraction was used in place of Kupffer cells. This system was also used to determine the effect of altering incubation times. To examine a time-course of cytotoxicity after exposure to allyl alcohol, ALT release was assessed 30, 60, and 90 minutes after addition of allyl alcohol. Allyl alcohol caused a dose dependent increase in hepatocellular injury. This injury was observed after 60 minutes of allyl alcohol incubation and increased slightly by 90 minutes. The response was unaffected by addition of LPS (Figure 2.5). In addition, the effect of the duration of exposure to LPS was examined by incubating homogenates with LPS for 0, 0.5, 2, or 8 hours. Though allyl alcohol caused a dose-dependent increase in hepatocellular injury, LPS did not cause a significant enhancement of allyl alcohol toxicity during any of the LPS-incubation times (Figure 2.6).

Though KCs were found to be a critical component in the potentiation of allyl alcohol toxicity by LPS *in vivo*, there may be other critical factors not present in the KC-HC coculture such as cell surface receptors that are cleaved during isolation or three-dimensional structural interactions. To assess these possibilities isolated, buffer-perfused livers from naïve rats were utilized. Allyl alcohol caused a time- and dose-dependent increase in ALT release in the perfused livers

Figure 2.5. Altering the time of incubation with allyl alcohol does not elicit an LPS-induced enhancement of allyl alcohol toxicity.

Liver homogenates were exposed to vehicle (gray bars) or LPS (10^5 EU/ml) (black bars) for 0 hours with increasing concentrations of allyl alcohol for 30, 60, or 90 minutes. ALT release was assessed at various times after allyl alcohol exposure as described in Methods. Data are expressed as means, N=2. No differences between LPS-treated and control values were observed.

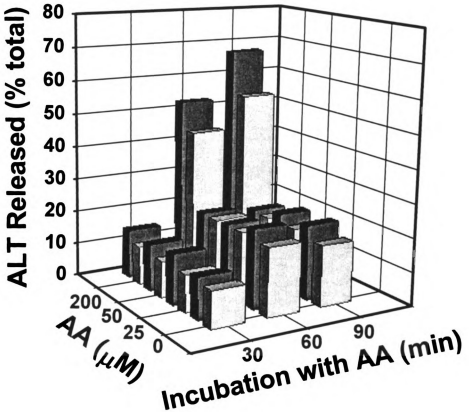
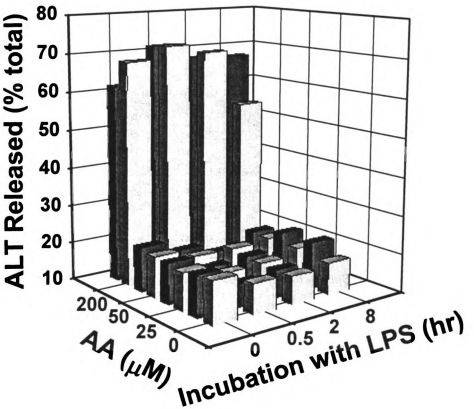

ALT Released (% total)
80
70
60
50
40
30
20
10
0
200
50
25
0
AA (μM)
30
60
90
Incubation with AA (min)

Figure 2.6. Altering the time of incubation with LPS does not elicit an LPS-induced enhancement of allyl alcohol toxicity.

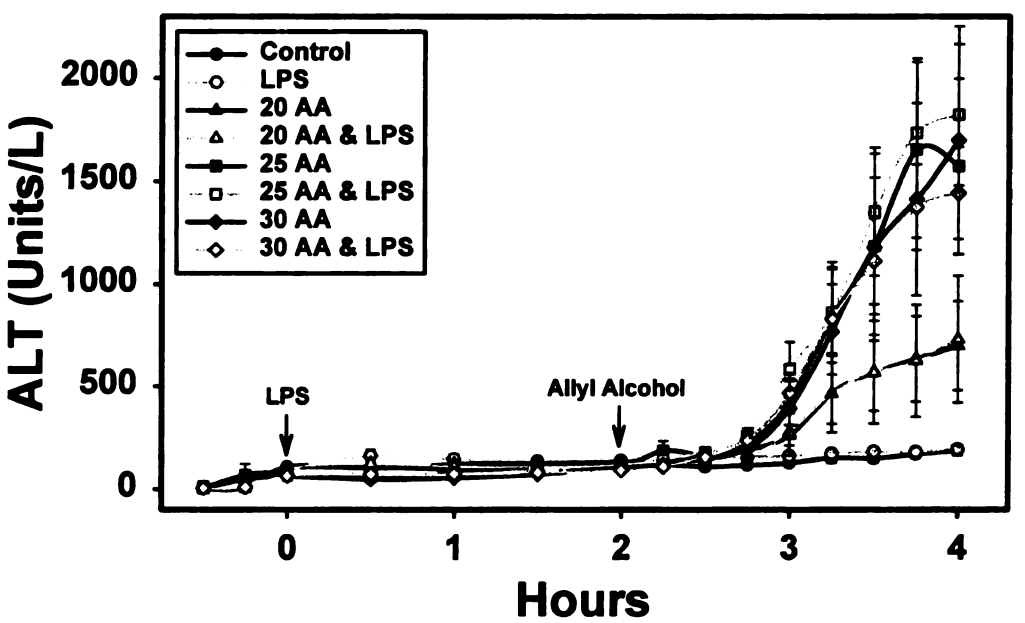
Liver homogenates were exposed to vehicle (gray bars) or LPS (10^5 EU/ml) (black bars) for various amounts of time before treatment with increasing concentrations of allyl alcohol. ALT release was assessed 1.5 hours after allyl alcohol exposure as described in Methods. Data are expressed as means, N=2. No differences between LPS-treated and control values were observed.


ALT Released (% total)
80
70
60
50
40
30
20
10
200
50
25
0
AA (μM)
0
0.5
2
8
Incubation with LPS (hr)

(Figure 2.7). LPS alone did not induce ALT release from isolated livers. LPS also did not significantly affect allyl alcohol-induced ALT release. These results suggest that an extra-hepatic factor is critical for the ability of LPS to increase allyl alcohol-induced liver injury.

Figure 2.7. Perfusion with LPS does not enhance allyl alcohol toxicity in the isolated liver.

Medium-perfused, rat livers were treated with LPS (1.2 X 10^5 EU/kg body weight) (gray lines) or vehicle (black lines) for two hours and subsequently exposed to allyl alcohol at the indicated doses (in mg/kg) for an additional two hours. ALT released into the medium was assessed over time as a marker of liver injury. Data are expressed as means ±SEM. N=6-14. No significant LPS effect was observed.


Control
LPS
20 AA
20 AA & LPS
25 AA
25 AA & LPS
30 AA
30 AA & LPS
ALT (Units/L)
2000
1500
1000
500
0
LPS
Allyl Alcohol
0
1
2
3
4
Hours

2.4 Discussion

Previous results indicate that KCs play a critical role in LPS enhancement of allyl alcohol-induced liver injury (Sneed *et al*, 1997). Studies presented here were designed to determine if KCs are sufficient for the LPS-induced enhancement of hepatocellular injury by allyl alcohol. Isolated KCs were activated by LPS as indicated by production of TNF upon exposure to LPS. However, under the conditions used for HC/KC coculture experiments, LPS-stimulated KCs did not increase the sensitivity of HCs to allyl alcohol.

Several alterations were made to the KC/HC cocultures in an attempt to reproduce the LPS-induced enhancement of allyl alcohol hepatotoxicity observed *in vivo*. For example, increasing the concentration of LPS did not increase KC/HC coculture sensitivity to allyl alcohol. In addition, the ratio of KCs to HCs was altered. The ratio of KCs to HCs can influence the ability of KCs to produce inflammatory mediators (Billiar *et al*, 1990), an effect that may be due to factors produced by HCs that control KC function. To account for this possibility we altered the KC/HC ratios and found that LPS did not affect the cytotoxicity of allyl alcohol to HCs at any of the KC/HC ratios examined.

The hypothesis that Kupffer cell mediators were not reaching a critical concentration for cellular activation, via either metabolism or diffusion in the culture plate medium, was tested using a simulated sinusoid. This system in effect increases cell number and decreases the diffusion space for metabolites. LPS did not enhance allyl alcohol cytotoxicity under these conditions. We simultaneously tested the hypotheses that other cells types in the liver may be involved, that LPS enhances the rate of allyl alcohol cytotoxicity, and that cells need to be exposed to LPS for some duration before addition of allyl alcohol using liver homogenates in place of isolated Kupffer cells. None of these alterations produced an LPS enhancement of allyl alcohol cytotoxicity. In summary, the lack *in vitro* of the LPS enhancement of allyl alcohol observed *in vivo* did not appear to be due to the concentration of LPS, the ratio of KCs to HCs, a decrease in mediator concentration, loss of other liver cell types, time of incubation with LPS, or time of incubation with allyl alcohol.

There are many other factors that may explain why KC/HC cocultures fail to mimic the LPS-induced enhancement of allyl alcohol toxicity found *in vivo*. An obvious possibility is that the coculture system lacks an element critical to the response. These factors may include the loss of Kupffer cell-derived reactive oxygen species. Kupffer cells are not stimulated to release reactive oxygen species upon LPS exposure *in vitro*,

although they are *in vivo*. This may be due to the loss of CD14 from Kupffer cells during isolation with collagenase-pronase perfusion (Ikejima *et al*, 1999). Additionally, the isolation process may remove critical receptors from the KCs or HCs, or factors released from the KCs could be highly labile and depend on the close association of KCs and HCs that is found *in vivo*. To account for these possibilities, the isolated, perfused liver was used as a model to reproduce the response observed *in vivo*. Exposure of livers to LPS did not enhance allyl alcohol-induced liver injury, suggesting that even in this system a critical factor was missing. This missing factor could be any of a number of factors including neutrophils, platelet activating factor, thrombin, and other components found in the circulatory system.

In summary, we found that LPS stimulation of Kupffer cells *in vitro* does not enhance the toxicity of allyl alcohol. This work demonstrates that although Kupffer cells play a critical role in the potentiation of allyl alcohol hepatotoxicity *in vivo*, they are not sufficient to enhance the sensitivity of hepatocytes to allyl alcohol. Other critical factors required for the potentiation of allyl alcohol toxicity by LPS may be present in the blood.

Chapter 3

Role of Reactive Oxygen Species in the Potentiation of Allyl Alcohol-Induced Liver Injury by LPS

3.1 Introduction

The sensitivity of rats to allyl alcohol-induced liver injury is dramatically increased by exposure to small doses of LPS (Sneed *et al*, 1997). This toxicity occurs rapidly and is maximal at 6 hours after allyl alcohol administration. Kupffer cells play a critical role in this potentiation, demonstrated by the protective effect of inhibiting Kupffer cell activity with gadolinium chloride (Sneed *et al*, 1997). However, the presence of Kupffer cells is not sufficient to cause LPS enhancement of allyl alcohol-induced liver injury as demonstrated by experiments in isolated perfused livers (Chapter 2).

An additional factor that may be required in this model of LPS-potentiated liver injury is the neutrophil (PMN). The toxicity caused by large doses of LPS is dependent on the presence of circulating neutrophils (Hewett *et al*, 1992; Sato *et al*, 1993) and smaller doses of LPS can cause neutrophil infiltration into the liver (Barton *et al*, 2000). Depletion of circulating neutrophils with anti-neutrophil antibodies protects against the potentiation of allyl alcohol-induced liver injury by LPS indicating that neutrophils are required for this model of potentiation (Sneed et al, 2000). In addition, LPS stimulates production of reactive oxygen species *in vivo* (Rahman *et al*, 1999; Robinson *et al*, 1997; Liu *et al*, 1995). Though, in addition to reactive oxygen species,

the development of hepatocellular injury may be induced by neutrophil derived proteases (Ganey *et al*, 1994), depletion of hepatocellular antioxidants may allow the development of injury from reactive oxygen species. Allyl alcohol is well known to cause reductive stress in hepatocytes through a rapid depletion of hepatocellular reduced glutathione stores without the formation of oxidized glutathione (Dogterom *et al*, 1988; Jaeschke *et al*, 1986; Silva *et al*, 1989; Comporti *et al*, 1991).

Since glutathione is used by cells to protect against oxidative injury, the ability of allyl alcohol to deplete hepatocellular glutathione stores may cause the hepatocytes to become susceptible to oxidative injury. The reactive oxygen species provided by neutrophils exposed to LPS may increase the toxicity of allyl alcohol. Accordingly, we tested the hypothesis that LPS-induced, neutrophil-derived reactive oxygen species increase the sensitivity of hepatocytes to the reductive stress of allyl alcohol.

3.2 Methods

3.2.1 Animals

Male, Sprague-Dawley rats [CD-Crl:CD-(SD)BR VAF/Plus]; Charles River, Portage, MI) weighing 200-300 g were used in all studies. The animals were allowed food (Rodent Chow, Teklad, Madison, WI) and

water *ad libitum*. They were maintained on a 12-hour light and dark cycle under conditions of controlled temperature and humidity.

3.2.2 Hepatocyte Isolation

Hepatic parenchymal cells were isolated using methods established by Seglen, 1973. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). The liver was perfused via the portal vein with Ca_2^+/Mg_2^+-free Hanks' balanced salt solution to remove red blood cells and then digested by perfusion with a collagenase type II (Sigma Chemical Co., St. Louis, MO) solution (240 μg/ml collagenase in Williams' Medium E) for 30 min. The digested liver was gently combed, filtered through sterile gauze, and centrifuged (50 X g, 2 min) to pellet the hepatocytes. The cells were washed twice with Williams' Medium E, resuspended in Williams' Medium E containing 1% gentamycin (GIBCO) and 10% fetal calf serum (Intergen, Purchase, NY). Viability was assessed via trypan blue exclusion. If cells were greater than 80% viable, they were plated in 12-well Falcon Primaria culture plates at a density of 2.5 X 10^5 cells/well. After a 4-hr incubation period (37°C, 7.5% CO_2, 92.5% air) the cells were washed and used in experiments.

3.2.3 Neutrophil Isolation

Glycogen-elicited neutrophils were isolated from the peritoneum of male, Sprague-Dawley (Charles River Laboratories, Portage, MI), retired breeder rats as described previously (Hewett et al. 1988). Briefly, rats were anesthetized with diethyl ether and given 1% glycogen (30 ml i.p.). After four hours, rats were anesthetized again, decapitated, and 0.01M PBS containing heparin (1 unit/ml) (30 ml i.p.) was injected into the peritoneum. The fluids of the peritoneal cavity were collected, filtered through gauze, and centrifuged at 500 X g for 7 min. Contaminating red blood cells were lysed with 15 ml of 0.15 M NH_4Cl for two minutes. The remaining neutrophils were washed one time with 0.1 M PBS and again with Williams' Medium E. The percentage of neutrophils and cell viability were routinely greater than 95%. Cells were diluted to 2.5 X 10^6 cells/ml for use in experiments.

3.2.4 Detection of GSH Concentration

The concentrations of glutathione (GSH) in cell preparations were determined using HPLC (Fariss and Reed, 1987; Jean *et al*, 1995). Cells were plated (5 X 10^6 cells/ml) in 6-well plates and treated as described below in the superoxide anion experiment. At the end of the experiment cells were lysed with 0.5 mL of 10% perchloric acid (v/v in milli-Q water) containing 1 mM bathophenanthroline-disulfonic acid and 0.5 mM

glutamyl glutamate (internal standard). The plates were scraped, and the contents of duplicate wells were combined. The lysates were centrifuged at 15,000 X g for 1 min, and 0.5 mL of the supernatant was collected. The lysate supernatants were derivatized by adding 50 μL of 100 mM iodoacetic acid containing 0.2 M m-cresol purple. This was then brought to pH 8-9 by the addition of 0.48 ml of KOH (2 M)-$KHCO_3$ (2.4 M) and allowed to incubate for 10 min. in the dark. Next 100 μL of 5% fluorodinitrobenzene (v/v in ethanol) was added and mixed with a vortex mixer. This was set in the dark in the refrigerator overnight.

The HPLC system consisted of two Waters HPLC 510 pumps, a Waters 717 WISP autosampler, a Waters 486 variable wavelength detector, and a 3-aminopropyl column (5μ Custom LC, Inc., Houston, TX). The Millenium 2010 Chromatography Manager software (Millipore Corp., Milford, MA) was used to control the HPLC and integrate peaks. Samples were diluted 50:50 with methanol, and 100 μL of sample was injected onto the column. Samples were eluted using a linear gradient of 80% methanol and 0.5 M sodium acetate in 64% methanol. The amount of GSH was calculated from the area under the UV absorbance curves.

3.2.5 Detection of Superoxide Anion

Superoxide anion generation by phorbol myristate acetate (PMA)-stimulated neutrophils was detected by the reduction of cytochrome c in the presence and absence of superoxide dismutase (SOD). Neutrophils in 96 well culture plates (5 X 10^5 cells/well in calcium- and magnesium-containing HBSS), containing cytochrome c, allyl alcohol, and ±SOD were stimulated with PMA (20 ng/ml). PMA is an effective stimulator of superoxide production in rat neutrophils. The absorbance of each well was measured at 550 nm every two minutes for up to 90 minutes. Superoxide anion production was calculated using an extinction coefficient of 18.5 $cm^{-1}mM^{-1}$ and the difference in absorbance of wells with and without SOD. Rate of superoxide anion production was estimated by change in superoxide anion production every two minutes. Area under the curve was calculated using the trapezoidal rule.

3.2.6 Effect of PMN-Derived Superoxide Anion on Hepatocellular Viability *In Vitro*

After 4 hours of incubation, hepatocyte cultures were washed with Williams' Medium E, and Williams' Medium E with or without freshly isolated neutrophils was added. The neutrophils were allowed to settle for 20 min, and the cultures were treated with allyl alcohol (0, 25, 50, 75, 100, 125, 150 and 200 μM). PMA (20 ng/ml) was added to the

cultures 20 minutes after the addition of allyl alcohol. Following 90 minutes of allyl alcohol treatment cell viability was assessed via ALT release from hepatocytes. Medium was collected, and cells were lysed with 1 ml of 1% Triton X100. Percent total ALT released was then calculated by dividing ALT activity released into medium by the total ALT activity found in medium and cell lysates. Results were analyzed using ANOVA blocked for each day of the experiment.

3.2.7 Effect of PMN-Derived Proteases on Hepatocellular Viability

Freshly isolated neutrophils in Williams' Medium E were exposed to cytochalasin B (10.4 nM), a primer for neutrophil degranulation, for 5 min before addition of formyl-methionyl-leucyl-phenylalanine (fMLP) (100 nM). The cell suspension was placed in a shaking water bath at 37°C for 30 min. The cells were then centrifuged at 500 X g for 10 min and the resulting conditioned medium was added to hepatocyte cultures that had been washed after 4 hours of incubation. Medium containing cytochalasin B and fMLP was used as a control. The cells were then treated with allyl alcohol (0, 25, 50, 75, 100, 125, 150 and 200 μM). Following 90 minutes of allyl alcohol treatment cell viability was assessed via alanine aminotransferase (ALT) release from hepatocytes as described above.

3.2.8 Treatment *In Vivo* with Antioxidants

Rats were given LPS (1.2 X 10^5 EU/kg body weight, i.v.) (*Escherichia coli*, serotype 0127:B8) or an equivalent volume of saline vehicle (Abbott Labs, Abbott Park, IL) one hour before treatment with polyethylene glycol-coupled superoxide dismutase (10,000 IU/kg i.v.), polyethylene glycol-coupled catalase (40,000 IU/kg i.v.) or an equivalent volume of saline. One hour later allyl alcohol (30 mg/kg ip) was administered. Six hours after treatment with allyl alcohol or saline control, rats were anesthetized with sodium pentobarbital (50 mg/kg ip), and blood was collected from the abdominal aorta into syringes containing 3.8% sodium citrate (final concentration 0.38%). The activity of ALT was determined in plasma. SOD activity was confirmed in plasma samples taken one hour after allyl alcohol administration by measuring superoxide production by neutrophils as described above in the presence of rat plasma diluted 1:3.

In a separate experiment rats were given apocynin (5 mg/kg, p.o.) or an equivalent volume of 5% ethanol vehicle 2 hours before treatment with of LPS (1.2 X 10^5 EU/kg body weight, i.v.) (*Escherichia coli*, serotype 0127:B8) or an equivalent volume of saline vehicle (Abbott Labs, Abbott Park, IL). Allyl alcohol (30 mg/kg i.p.) was administered two hours after LPS. Six hours after treatment with allyl alcohol or saline control, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and blood

was collected from the abdominal aorta into syringes containing 3.8% sodium citrate (final concentration 0.38%). Hepatotoxicity was determined via the activity of ALT in plasma.

3.2.9 Statistical Analysis

Data are expressed as means ± S.E.M. For all results presented, N represents the number of individual experiments. Data with non-homogeneous variances were square root- or log-transformed prior to analysis. Data were analyzed using repeated measures ANOVA provided in the statistics program NCSS 2000. Comparisons among groups were performed using Fisher's Least Squares Difference test. The criterion for statistical significance was $p \leq 0.05$.

3.3 Results

3.3.1 Effect of Superoxide on Allyl Alcohol-Treated Hepatocytes

To test the hypothesis that LPS-induced, neutrophil-derived reactive oxygen species increase the sensitivity of hepatocytes to the reductive stress of allyl alcohol leading to an enhanced hepatotoxicity, neutrophil-hepatocyte cocultures were used. Since the hypothesis was that glutathione depletion by allyl alcohol renders hepatocytes more sensitive to neutrophil-derived reactive oxygen species, it was important to stimulate neutrophils to produce superoxide anion at a time when glutathione was low. Accordingly, the time courses for glutathione depletion by allyl alcohol in hepatocytes and superoxide generation by PMA-stimulated neutrophils were determined. Allyl alcohol caused a concentration-dependent decrease in hepatocellular GSH concentrations within the first 5 minutes of exposure (Figure 3.1). GSH continued to decrease for 20 minutes and then began to increase slowly.

In freshly isolated neutrophils, PMA stimulated the production of superoxide anion, and the rate of production was maximal by 6 min (Figure 3.2). Based on these results, neutrophil-hepatocyte cocultures were treated with allyl alcohol 20 minutes before addition of PMA.

Figure 3.1. Glutathione depletion occurs within 20 minutes after allyl alcohol treatment of hepatocytes.

Isolated hepatocytes were treated with allyl alcohol at the indicated concentrations and assessed for glutathione content at the indicated times after allyl alcohol treatment. Percent initial glutathione concentration is based on the concentration of glutathione 5 minutes after treatment with 0 μM allyl alcohol. Data are represented as means. N=4.

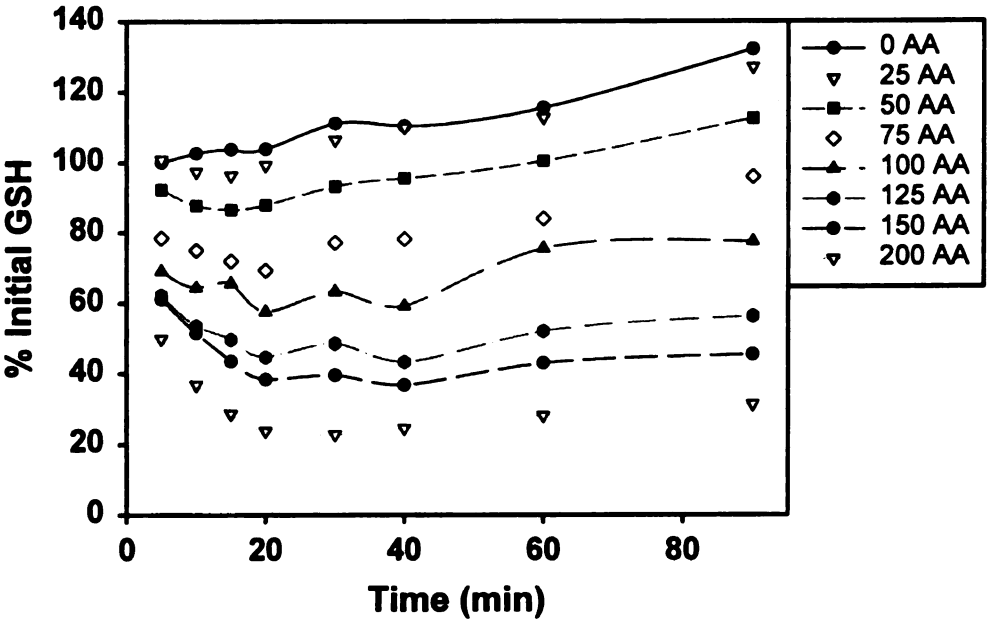
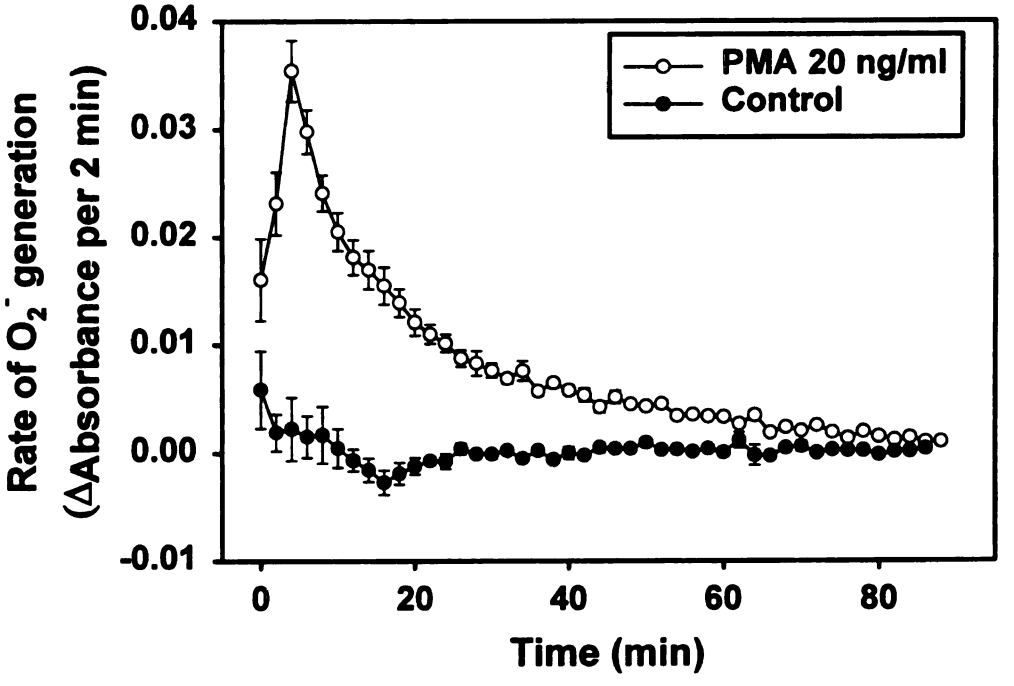

140
120
100
80
60
40
20
0
% Initial GSH
0
20
40
60
80
Time (min)
0 AA
25 AA
50 AA
75 AA
100 AA
125 AA
150 AA
200 AA

Figure 3.2. PMA stimulates maximal superoxide generation from neutrophils at 6 minutes.

Isolated neutrophils were plated in 96 well plates containing cytochrome c for determination of superoxide generation. Neutrophils were treated with either phorbol myristate acetate (PMA) at 20 ng/ml (open circle) or vehicle control (black circle). Data are represented as mean ± S.E.M. N=4.


0.04
0.03
0.02
0.01
0.00
-0.01
0
20
40
60
80
Time (min)
Rate of O_2^- generation
(ΔAbsorbance per 2 min)
PMA 20 ng/ml
Control

During each individual experiment, superoxide anion production from neutrophils was monitored in the presence of allyl alcohol. PMA caused a consistent production of superoxide anion from the isolated neutrophils. Allyl alcohol had a minimal effect on superoxide anion production by PMA-stimulated neutrophils (Figure 3.3). Only the highest concentration of allyl alcohol caused a small but statistically significant decrease in superoxide anion production.

The glutathione concentration of hepatocytes cultured alone or with PMNs was not altered by PMA (Figure 3.4). Allyl alcohol caused a concentration dependent decrease in cellular GSH. Neither the presence of PMNs nor PMA treatment had a significant effect on the concentration of GSH.

Allyl alcohol caused a concentration dependent increase in ALT release from both neutrophil-hepatocyte cocultures and hepatocyte cultures (Figure 3.5). PMA stimulation increased slightly but significantly the sensitivity of hepatocytes to allyl alcohol in the neutrophil cocultures but did not affect the hepatocytes cultured alone.

Figure 3.3. Superoxide generation by PMA-stimulated neutrophils is not attenuated by allyl alcohol.

Isolated neutrophils were plated in 96 well plates containing cytochrome c for determination of superoxide anion production. Total superoxide generation after 70 minutes of PMA (20 ng/ml) stimulation was calculated. Data are represented as mean ± S.E.M. N=4. *, significatly different from control (0 μM allyl alcohol).

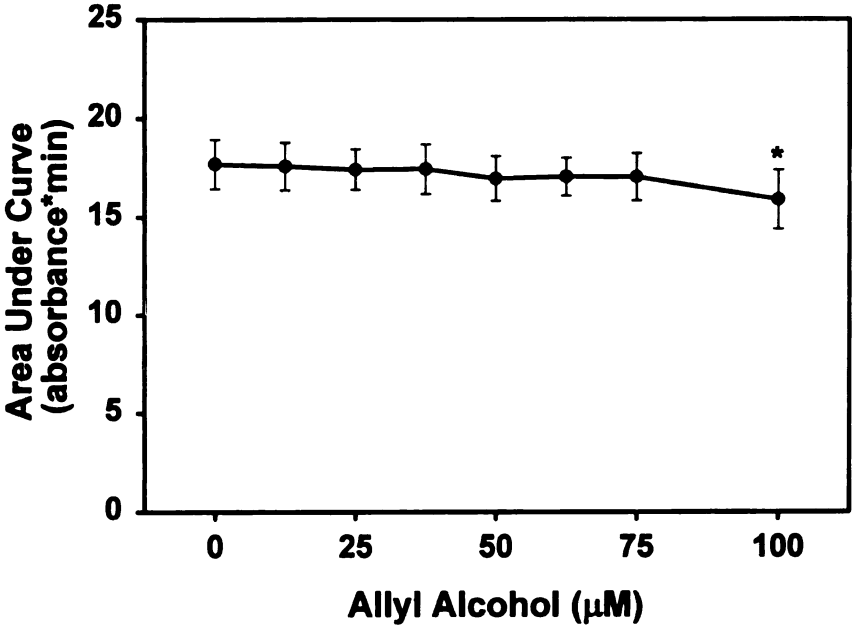

25
20
15
10
5
0
Area Under Curve
(absorbance*min)
*
0
25
50
75
100
Allyl Alcohol (μM)

Figure 3.4. Allyl alcohol-induced glutathione depletion is not enhanced by PMA-stimulated neutrophils.

Isolated hepatocytes were cocultured with or without isolated neutrophils (PMNs). The cells were treated with allyl alcohol at concentrations indicated and 20 minutes later treated with PMA (20 ng/ml) or vehicle control. After a 90 minute incubation with allyl alcohol, glutathione (GSH) concentration was determined. Data are represented as means, N = 3. No statistical differences among groups.

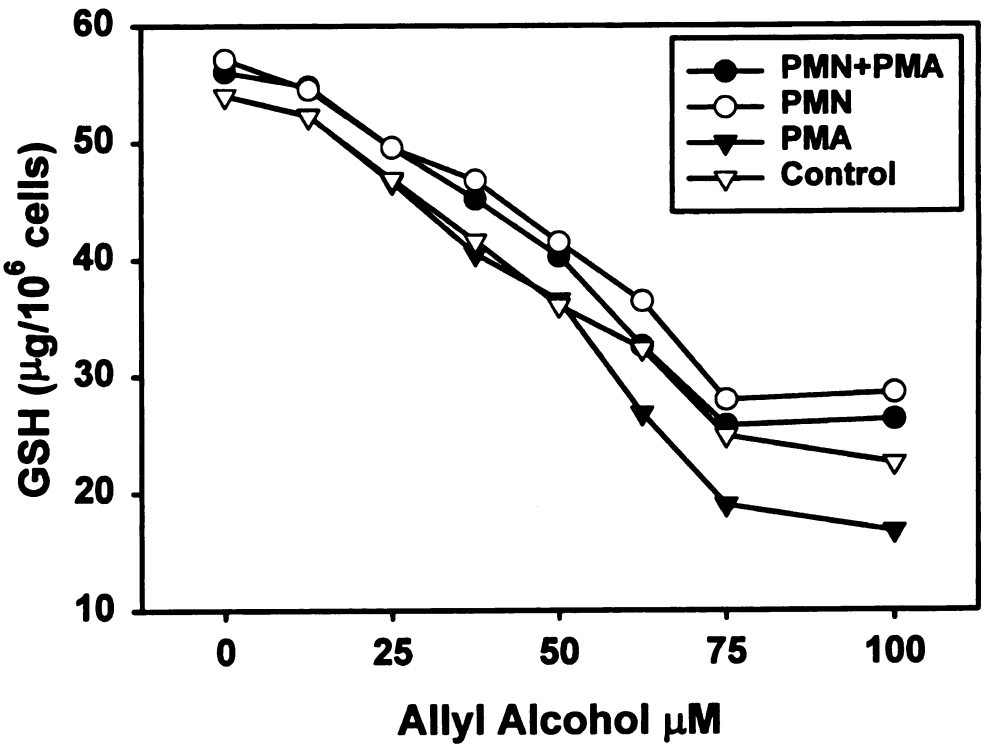
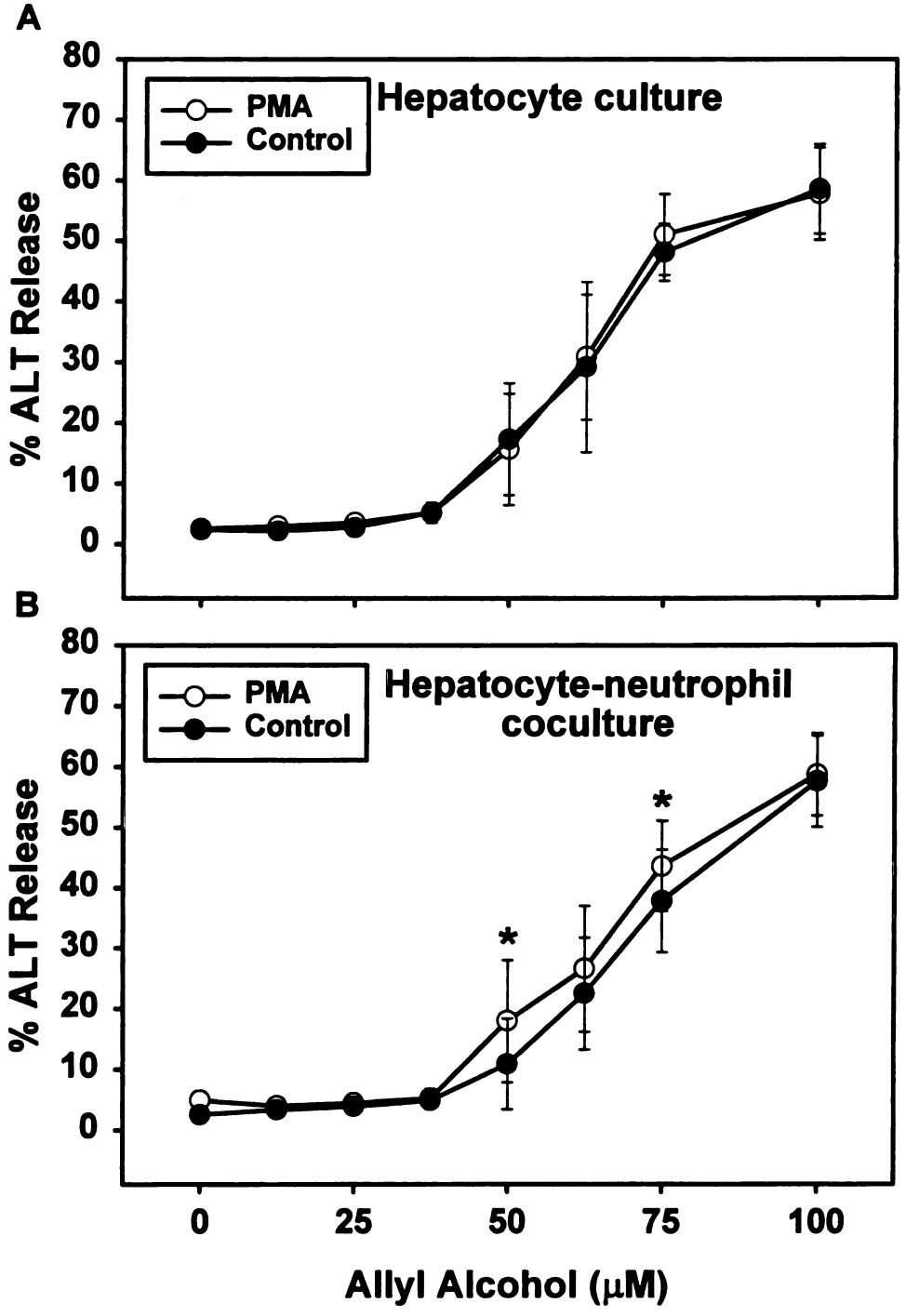

60
50
40
30
20
10
GSH (μg/10^6 cells)
0
25
50
75
100
Allyl Alcohol μM
PMN+PMA
PMN
PMA
Control

Figure 3.5. PMA-stimulated release of reactive oxygen species enhances allyl alcohol cytotoxicity in hepatocyte-neutrophil cocultures.

Isolated hepatocytes were cultured alone (A) or cocultured with neutrophils (B). Cells were treated allyl alcohol at the indicated concentrations and 20 minutes later with phorbol myristate acetate (PMA) (20 ng/ml) or vehicle. After 90 minutes of allyl alcohol incubation cell viability was assessed by measuring release of ALT activity. Data are represented as mean ± S.E.M. N=4. *, significantly different from control at same allyl alcohol concentration.


A
B
PMA
Control
Hepatocyte culture
Hepatocyte-neutrophil coculture
% ALT Release
0
10
20
30
40
50
60
70
80
*
*
0
25
50
75
100
Allyl Alcohol (μM)

3.3.2 Effect of Neutrophil-Derived Proteases on Allyl Alcohol-Treated Hepatocytes

Allyl alcohol caused a concentration dependent increase in ALT release from hepatocyte cultures (Figure 3.6). Conditioned medium from fMLP-stimulated neutrophils did not affect the amount of ALT released into the medium.

3.3.3 Effect of Antioxidants *In Vivo*

Animals treated with both LPS and allyl alcohol developed significant liver injury as assessed by plasma ALT activity. Pretreatment with superoxide dismutase and catalase did not offer any protection from the hepatotoxicity induced by LPS and allyl alcohol cotreatment (Figure 3.7). Plasma samples taken from rats one hour after treatment with allyl alcohol were analyzed for superoxide dismutase activity. Neutrophil-generated superoxide anion reduction of cytochrome C was decreased 60% by plasma from superoxide dismutase treated animals compared to vehicle treated animals (data not shown). Pretreatment with the NADPH oxidase inhibitor apocynin did not induce liver injury when administered alone (41 ± 7 Units/L plasma ALT activity) and did not afford protection from the cotreatment of LPS and allyl alcohol (Figure 3.8).

Figure 3.6. Conditioned medium does not enhance the cytotoxicity of allyl alcohol.

Isolated hepatocytes were cultured in naïve medium containing fMLP and cytochalasin B (black) or conditioned medium containing supernatant from cytochalasin B- and fMLP-treated neutrophils. Cells were treated with allyl alcohol at the indicated concentrations. After 90 minutes, cell viability was assessed by measuring ALT activity. Data are represented as mean ± S.E.M. N=4.

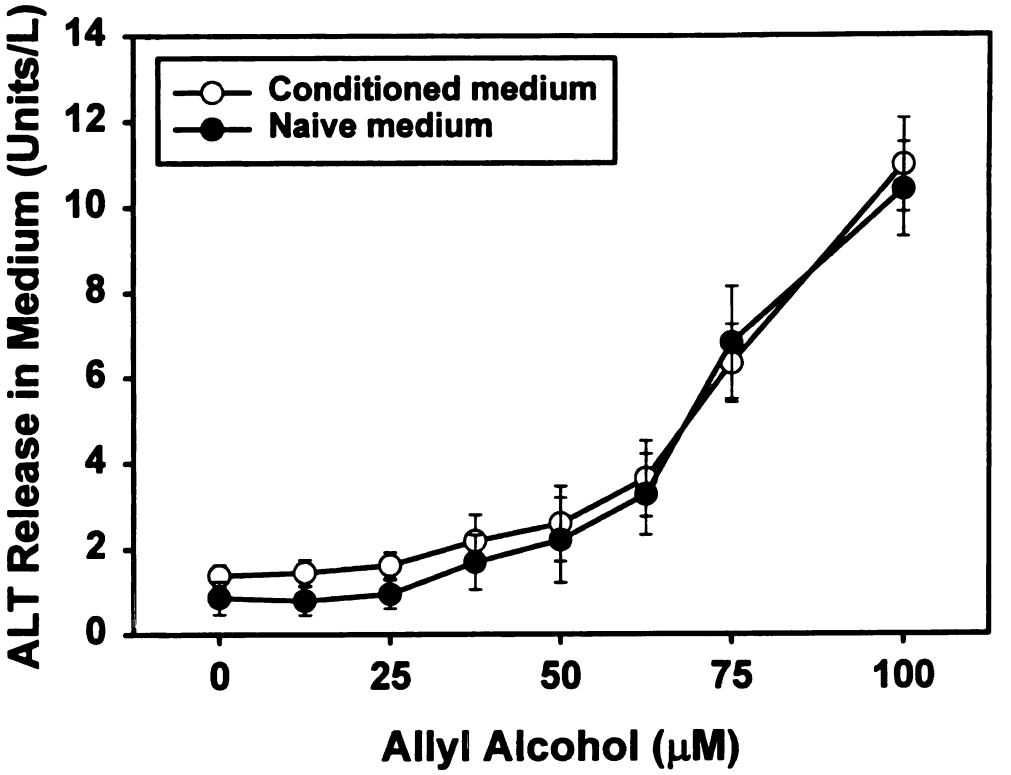

ALT Release in Medium (Units/L)
14
12
10
8
6
4
2
0
Conditioned medium
Naive medium
0
25
50
75
100
Allyl Alcohol (μM)

Figure 3.7. Superoxide dismutase and catalase do not attenuate LPS-enhanced allyl alcohol hepatotoxicity.

Rats were treated with LPS (1.2 X 10^5 EU/kg body weight, i.v.). One hour later rats were treated with polyethylene-coupled superoxide dismutase (10,000 units/kg, i.v.) and polyethylene-coupled catalase (40,000 units/kg, i.v.) (SOD/CAT) or vehicle. One hour later rats were treated with allyl alcohol (30 mg/kg, i.p.). After an additional six hours liver injury was assessed via plasma ALT activity. Data are represented as mean ± S.E.M. N = 4.

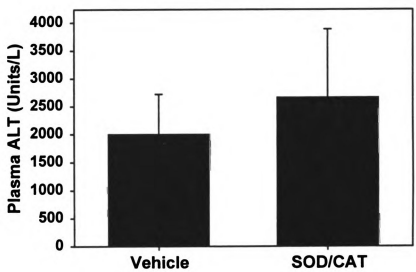
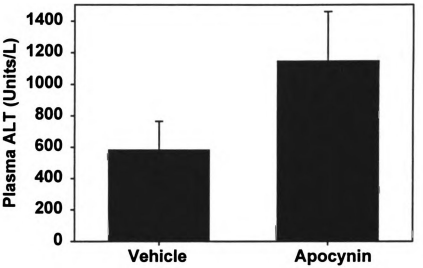

Plasma ALT (Units/L)
4000
3500
3000
2500
2000
1500
1000
500
0
Vehicle
SOD/CAT

Figure 3.8. Apocynin does not attenuate LPS-enhanced allyl alcohol hepatotoxicity.

Rats were given apocynin (5 mg/kg, p.o.) or an equivalent volume of 5% ethanol vehicle 2 hours before treatment with LPS (100 μg/kg, i.v.). Allyl alcohol (30 mg/kg i.p.) was administered two hours after LPS. Six hours after treatment with allyl alcohol liver injury was assessed via plasma ALT activity. Data are represented as mean ± S.E.M. N = 3.


Plasma ALT (Units/L)
1400
1200
1000
800
600
400
200
0
Vehicle
Apocynin

3.4 Discussion

Neutrophils are a critical component of liver injury in this potentiation model. Large numbers of neutrophils accumulate in the liver sinusoids. Removal of neutrophils with an anti-neutrophil antibody (Sneed, 2000) protects animals from LPS enhancement of allyl alcohol hepatotoxicity, confirming the important role neutrophils play in organ damage during LPS enhancement of allyl alcohol-induced injury.

It is widely held that neutrophils must become activated to contribute to tissue injury. One function of activated neutrophils is production of reactive oxygen species. Allyl alcohol decreases the cellular levels of GSH (Figure 3.1), which leaves the cells more susceptible to attack from reactive oxygen species. For example, depletion of hepatic GSH with maleic acid diethyl ester or phorone increases oxidative hepatic injury in rats (Carbonell *et al*, 2000; Moriya *et al*, 2000). Thus the hypothesis was posed that neutrophil-derived superoxide anion contributes to LPS enhancement of allyl alcohol toxicity.

To test this possibility *in vitro* experiments were performed with neutrophil-hepatocyte cocultures. PMA was used to stimulate the production of reactive oxygen species from PMNs at a time that corresponded with maximal GSH loss in allyl alcohol-treated

hepatocytes. A small but significant increase in ALT release was observed in cocultures of hepatocytes and PMA-stimulated neutrophils (Figure 3.5). The small magnitude of this increase in allyl alcohol toxicity raises questions about the pathophysiological relevance of this effect. The ability of PMA-stimulated neutrophils to enhance the cytotoxicity of allyl alcohol was greater on some days than others. This was not correlated with the amount of superoxide produced by PMNs or with the degree of glutathione depletion. The cytotoxicity-enhancing effect of PMA-stimulated neutrophils in cocultures might depend on other factors that were not monitored, such as vitamin E content, glutathione peroxidase and/or reductase activity, or release of proteases from PMA-stimulated neutrophils.

To examine the possibility that the increase in toxicity observed with PMA-stimulated neutrophils was related to preoteases released into the medium, experiments were performed with conditioned medium from fMLP-activated neutrophils. fMLP stimulates PMNs to degranulate and to produce reactive oxygen species. Because superoxide anion has a very short half-life and the PMNs are spun out of the conditioned medium, the conditioned medium was likely free of reactive oxygen species and contained degranulation products including proteases. The lack of effect of conditioned medium to increase the toxicity of allyl alcohol in this paper indicates that the observed enhancement of allyl

alcohol cytotoxicity by PMA-stimulated neutrophils may be attributed to superoxide anion production rather than PMA-stimulated protease release. Nonetheless, the inability of conditioned medium to enhance the cytotoxicity of allyl alcohol *in vitro* may not lead us to conclude a lack of importance for protease release *in vivo*. The lack of enhanced allyl alcohol cytotoxicity may be due to the short duration of the experiment. Our lab has previously found that the proteases cathepsin G and elastase are major contributors to hepatocellular injury from exposure *in vitro* to conditioned medium from fMLP-activated neutrophils; however this injury takes 16 hours to develop (Ganey *et al*, 1994; Ho *et al*, 1996). Thus, the role of neutrophil-derived proteases cannot be ruled out by experiments in this study.

Pretreatment of rats with two different antioxidants did not afford protection from the potentiation of allyl alcohol hepatotoxicity by LPS. The antioxidants superoxide dismutase and catalase act in concert to scavenge superoxide anion. Polyethylene glycol-coupled enzymes were used to increase the half-life of superoxide dismutase and catalase (Tamura *et al*, 1988). The lack of protection with this antioxidant treatment could be due to an insufficient concentration of antioxidants for effective oxidant scavenging. However, plasma from rats treated with polyethylene glycol-coupled superoxide dismutase and catalase demonstrated a significant superoxide anion scavenging activity.

Furthermore, the doses used in our experiments were similar to those used by other investigators for studies in which protective effects against oxidative injury without toxic effects of antioxidant treatment alone were observed (elSisi *et al*, 1993; Tamura *et al*, 1988; Thomson *et al*, 1990; Suzuki *et al*, 1992; Lehman *et al*, 1992; Liu *et al*, 1989). For example, vitamin A potentiation of carbon tetrachloride hepatotoxicity was attenuated by treating animals with the same dose of superoxide dismutase and catalase used in experiments presented here. The level of lipid peroxidation was also reduced as measured by ethane concentration in expired air (elSisi *et al*, 1993).

Lack of antioxidant-induced protection against LPS enhancement of allyl alcohol toxicity could be due to an insufficient concentration of antioxidants in the small space between the neutrophils and the hepatocytes to which they adhere. However, in a model of reperfusion injury in isolated rabbit hearts, injury was not as severe in hearts isolated from rabbits pretreated with polyethylene glycol-coupled superoxide dismutase. Because treatment with the antioxidant occurred *in vivo* and the perfusion medium did not contain the superoxide dismutase, the protective effect was most likely due to intramyocardial or membrane-bound superoxide dismutase. This indicates that polyethylene glycol linkage may facilitate intracellular access (Lehman *et al*, 1992). Thus, polyethylene glycol-coupled superoxide dismutase and

catalase may reside on or in the membranes within the space between the neutrophils and hepatocytes, and the inability of this antioxidant treatment to protect against the potentiation of allyl alcohol hepatotoxicity by LPS is not likely due to insufficient antioxidant capacity.

To further examine the involvement of neutrophil-derived reactive oxygen species in the potentiation of allyl alcohol hepatotoxicity by LPS another antioxidant was used. Apocynin is metabolically activated by reactive oxygen species and myeloperoxidase enzyme activity from activated phagocytes into a compound that potently inhibits NADPH oxidase assembly on the membrane (Stolk *et al*, 1994; Wang *et al*, 1994). Apocynin is thought to reduce the production of reactive oxygen species by activated neutrophils without affecting phagocytosis, exocytosis, or intracellular killing of bacteria (Wang *et al*, 1994). Though apocynin pretreatment did not attenuate the potentiation of allyl alcohol hepatotoxicity by LPS, a similar dose of apocynin was shown to attenuate oxidative injury in other models of tissue injury without causing toxicity on its own (Supinski *et al*, 1999; Salmon *et al*, 1998). For example, disruption of diaphragmatic function after endotoxin administration in rats is dependent on oxygen-derived free radicals. Administration of apocynin at doses similar to those used in our experiments was protective against endotoxin-induced decreases in force

production of diaphragm strips. Furthermore, this treatment regimen resulted in decreased formation of 4-hydroxynonenal (a marker of lipid peroxidation) and nitrotyrosine (a marker of free radical-mediated protein modification) (Supinski *et al*, 1999). Thus the inability of apocynin to attenuate the enhancement of allyl alcohol hepatotoxicity by LPS was not likely due to insufficient inhibition of NADPH oxidase. However, apocynin elicits other effects such as inhibition of thromboxane A2 release and the stimulation of release of prostaglandins E2 and F2 as well as increases in glutathione synthesis (Engels *et al*, 1992, Lapperre *et al*, 1999). The ability of apocynin to stimulate release of prostaglandins may have a toxic effect in this potentiation model, because a cyclooxygenase-2 inhibitor attenuates the potentiation of allyl alcohol hepatotoxicity by LPS (Ganey *et al*, 2001). This might explain the somewhat greater toxicity observed in allyl alcohol/LPS-cotreated rats given apocynin.

Although neutrophils are required for hepatotoxicity in this model, reactive oxygen species do not appear to play a critical role in LPS enhancement of allyl alcohol-induced hepatocellular injury. The small effect of reactive oxygen species observed *in vitro* may indicate that the presence of both reactive oxygen species and proteases is necessary to elicit the injury observed *in vivo*.

CHAPTER 4

The Role of Thrombin in the Potentiation of Allyl Alcohol-Induced Liver Injury by LPS

4.1 Introduction

The potentiation of allyl alcohol-induced liver injury by LPS is dependent on Kupffer cell activity. Inhibition of Kupffer cell activity with gadolinium chloride protects against the LPS enhancement of allyl alcohol toxicity. However, we have previously shown in Kupffer cell/hepatocyte cocultures and the isolated, perfused liver that LPS-stimulated Kupffer cells (KCs) do not sensitize hepatocytes (HCs) to injury from allyl alcohol (Chapter 2). These data indicate that KCs are not sufficient for LPS to enhance the toxicity of allyl alcohol and that other extrahepatic factors must play a critical role in the potentiation of allyl alcohol-induced liver injury by LPS.

Though liver injury from large doses of allyl alcohol alone is not dependent on the presence of extrahepatic factors, liver injury from large doses of LPS is critically dependent on the extrahepatic factor thrombin (Moulin *et al*, 1996). Thrombin induces liver injury in livers isolated from LPS pretreated rats. Thrombin elicits its damaging effects on liver through a thrombin receptor-mediated mechanism (Moulin, 1999). Studies presented here were designed to test whether thrombin plays a role in LPS enhancement of allyl alcohol injury that is similar to its role in liver damage from large doses of LPS. The hypothesis was that thrombin participates in the potentiation of allyl alcohol-induced liver

injury by LPS through a mechanism involving a thrombin receptor in the liver.

4.2 Methods

4.2.1 Materials

Primers for cytokine-induced neutrophil chemoattractant-1 (CINC-1) and cyclooxygenase-2 (COX-2) were purchased from Biosource International, Camarillo, CA and Ambion, Austin, TX respectively. Tri Reagent was from Molecular Research Center, Cincinnati, OH. RETROscript protocol and Quantum RNA kit were from Ambion, Austin, TX. All other materials were purchased from Sigma Chemical Co., St. Louis, MO.

4.2.2 Animals

Male, Sprague-Dawley rats (CD-Crl:CD-(SD)BR VAF/Plus; Charles River, Portage, MI) weighing 175-225 g were allowed food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and water *ad libitum*. They were housed under conditions of controlled temperature, humidity and 12-hr light and dark cycle.

4.2.3 *In Vivo* Experiments with Heparin

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or an equivalent volume of saline as control one hour before administration of LPS. Rats were treated with LPS ($1.2X10^5$ EU/kg body weight, i.v.), and two hours later they were treated with allyl alcohol (30 mg/kg body weight, i.p.). At 2, 3, and 8 hours after LPS administration rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for removal of blood (in 0.38% sodium citrate) and liver samples. Plasma samples were analyzed for ALT, aspartate aminotransferase (AST) and alkaline phosphatase activities using Sigma Diagnostic Kits No. 52, 51, and 245 respectively, and for fibrinogen concentration using a BBL fibrometer (Becton, Dickinson and Company, Hunt Valley, MD). Livers were weighed, and a portion of the liver was frozen immediately in liquid nitrogen and stored at –80°C for preparation of RNA (see below). The remaining liver sample was fixed in formalin and processed for histological evaluation. Liver sections were stained for PMNs as described previously (Pearson *et al*, 1995) using an anti-PMN antibody prepared as described previously (Hewett *et al*, 1992). Stained cells were counted under light microscopy in 20 random fields at 400X magnification.

4.2.4 Determination of CINC-1 and COX-2 mRNA Expression Levels

Total RNA was isolated from frozen liver tissue using Tri Reagent (Molecular Research Center, Cincinnati, OH) as described by Chomczynski (Chomczynski, 1993; Chomczynski and Mackey, 1995). The concentration and purity of RNA was determined from absorbance at 260 and 280 nm. RNA was adjusted to 50μg/ml with RNAse-free water. First strand cDNA was synthesized using the RETROscript protocol (Ambion, Austin, TX). Semiquantitative polymerase chain reaction (PCR) was performed using the Quantum RNA kit (Ambion). 18S rRNA was used as the internal control. Primers for CINC-1 and COX-2 were obtained from Biosource International and Ambion respectively. Samples were denatured for 90 seconds at 94°C, followed by 35 cycles of 30 seconds at 94°C, 45 seconds at 60°C, and 45 seconds at 72°C with a final 7 minute extension step at 72°C. PCR products were separated via electrophoresis on a 1.5 % agarose gel containing ethidium bromide. Band intensity was quantified using Quantity One quantitation software (version 4, BIO-RAD). Band intensities for COX-2 and CINC-1 were normalized to the band intensity of the 18S rRNA internal standard.

4.2.5 *In Vivo* Experiments with a Hepatotoxic Dose of Allyl Alcohol

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or an equivalent volume of saline as control. Three hours after heparin, rats were treated with allyl alcohol (50 mg/kg body weight, i.p.). At 9 hours after heparin administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for removal of blood (in 0.38% sodium citrate). Blood plasma samples were analyzed for ALT and fibrinogen as described above.

4.2.6 *In Vivo* Experiments with Warfarin

Rats were treated with warfarin (20 mg/kg body weight, p.o.) or vehicle twice, 24 hours apart. 18 hours after the last treatment, rats were given LPS ($1.2X10^5$ EU/kg body weight, i.v.) or saline vehicle, and two hours after LPS they were treated with allyl alcohol (30 mg/kg body weight, i.p.) or vehicle. 8 hours after LPS administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for removal of blood (in 0.38% sodium citrate) and liver samples. Plasma samples were analyzed for ALT and fibrinogen concentration as described above.

4.2.7 Isolation and Perfusion of Rat Livers

Rats were weighed and then treated with LPS ($1.2X10^5$ EU/kg body weight, i.v.) or an equivalent volume of saline as control. After two hours, the rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the portal vein was exposed. Livers were perfused via portal vein cannulation with polyethylene tubing (PE 190, Clay Adams, Parsippany, NJ). The perfusion medium was Krebs-Henseleit bicarbonate buffer supplemented with 2% bovine serum albumin and saturated with 95% O_2 and 5% CO_2 gas. Flow was constant at a rate of 0.14 ml/min/g body weight. The thoracic portion of the inferior vena cava was cannulated (PE 240 Clay Adams, Parsippany, NJ) for outflow. The liver was placed in a temperature-controlled cabinet maintained at 37°C as previously described (Moulin *et al*, 1996). The livers were allowed to stabilize for 10 minutes with single pass perfusion. A sample of the perfusion medium was then taken (time = SPP), and the system was switched to recirculating perfusion. Thrombin (70 units/rat) or vehicle control was added to the perfusion medium before the addition of allyl alcohol (32.5 mg/kg body weight) or vehicle control. Medium samples (350μL) were taken every 15 minutes for two hours. ALT activity in the samples was determined as described above.

4.2.8 Statistical Analysis

Data are expressed as means ± S.E.M. For all results presented, N represents the number of individual experiments. Data with non-homogeneous variances were square root- or log-transformed prior to analysis. Data were analyzed using repeated measures ANOVA provided in the statistics program NCSS 2000. Comparisons among groups were performed using Fisher's Least Squares Difference test. The criterion for statistical significance was $p \leq 0.05$.

4.3 Results

One extrahepatic factor that is critical for liver injury induced by large doses of LPS alone is thrombin (Hewett *et al*, 1995; Moulin *et al*, 1996). To begin to assess the importance of thrombin in the LPS enhancement of allyl alcohol hepatotoxicity, rats were treated with heparin (2000 Units/kg) one hour prior to LPS. Heparin is an anticoagulant that enhances the binding of antithrombin III to thrombin, inhibiting the formation of active thrombin. Plasma fibrinogen concentration was monitored as a marker of thrombin activity and drug effectiveness. Thrombin proteolytically cleaves fibrinogen to fibrin; thus activation of thrombin is associated with a decrease in circulating fibrinogen.

The plasma fibrinogen concentration of animals treated only with LPS did not decrease, whereas that of animals treated with allyl alcohol alone decreased a small but insignificant amount. Plasma fibrinogen concentrations in animals co-treated with LPS and allyl alcohol were significantly decreased 3 hours after LPS administration (Table 4.1). Pretreatment with heparin significantly blocked the decreases in plasma fibrinogen concentration. Plasma ALT activity was not elevated above those of animals receiving saline alone in any of the treatment groups indicating that significant liver injury had not developed by 3 hours after LPS administration. Plasma AST activity was slightly but significantly

Table 4.1. Plasma ALT, AST, and fibrinogen values in rats 3 hours after LPS administration.

Rats were treated with heparin (Hep; 2000 Units/kg body weight, i.v.) or saline one hour before LPS (1.2×10^5 EU/kg body weight, i.v.) or saline. Two hours after LPS, rats received allyl alcohol (AA; 30 mg/kg body weight, i.p.) or saline. Plasma ALT and AST activities, and fibrinogen concentration were measured 3 hours after LPS administration. Data are expressed as means ± S.E.M. N = 3-5. *, significantly different from saline only.

Hep	LPS	AA	Fibrinogen (mg/dL)	ALT (Units/L)	AST (Units/L)
-	-	-	200 ± 18	120 ± 25	76 ± 23
+	-	-	225 ± 21	121 ± 15	103 ± 12
-	+	-	233 ± 26	138 ± 29	107 ± 25
+	+	-	203 ± 27	144 ± 31	85 ± 18
-	-	+	152 ± 41	107 ± 27	142 ± 36
+	-	+	226 ± 8	128 ± 27	87 ± 15
-	+	+	* 39 ± 14	139 ± 24	* 226 ± 66
+	+	+	214 ± 35	173 ± 34	149 ± 41

elevated in animals receiving LPS and allyl alcohol co-treatment compared to animals receiving saline only.

The effectiveness of heparin at inhibiting thrombin was also demonstrated by measuring plasma fibrinogen concentrations at 8 hours post LPS administration (Figure 4.1). Fibrinogen was depleted only in the LPS-allyl alcohol cotreatment group. Heparin pretreatment significantly attenuated this depletion of fibrinogen, indicating that an effective dose of heparin was used. Liver injury was assessed via the release of AST and ALT into the plasma 8 hours after LPS administration. Neither LPS nor allyl alcohol alone induced significant ALT release; however, LPS-allyl alcohol cotreatment induced significant ALT release at 8 hours. Similar results were observed with AST activity. There were no significant changes in alkaline phosphatase activity or liver weight/body weight index at any time after treatment (data not shown). Treatment with heparin significantly attenuated the LPS-enhanced toxicity of allyl alcohol as assessed by plasma ALT and AST activities at 8 hours post LPS administration (Figure 4.2).

Heparin has been shown to inhibit various processes of inflammation (Downing *et al*, 1998; Darien *et al*, 1998; Shin *et al*, 1997). To assess its effect on inflammation in our study, the number of neutrophils in the liver, expression of the chemokine, CINC-1 and

Figure 4.1. Heparin pretreatment attenuates fibrinogen depletion in rats cotreated with LPS and allyl alcohol.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle one hour before LPS (1.2×10^5 EU/kg body weight, i.v.) or vehicle (saline). Two hours after LPS, rats received allyl alcohol (30 mg/kg body weight, i.p.) or vehicle. Plasma fibrinogen was measured 8 hours after LPS administration. Data are expressed as means ± S.E.M. N = 3-5. *, significantly different from saline only; †, significantly different from LPS/AA in the absence of heparin.

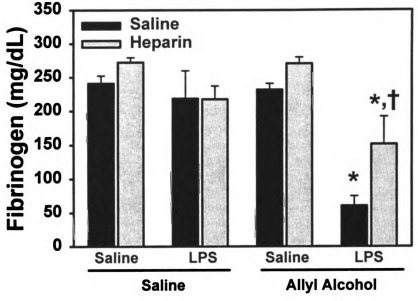
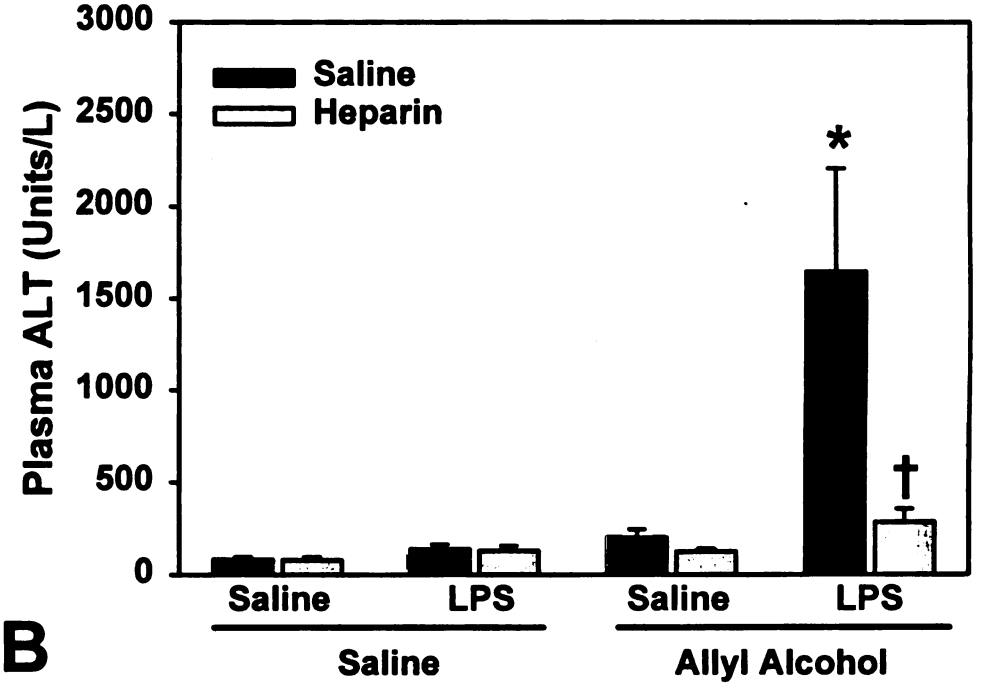

Fibrinogen (mg/dL)
350
300
250
200
150
100
50
0
Saline
Heparin
*,†
*
Saline
LPS
Saline
LPS
Saline
Allyl Alcohol

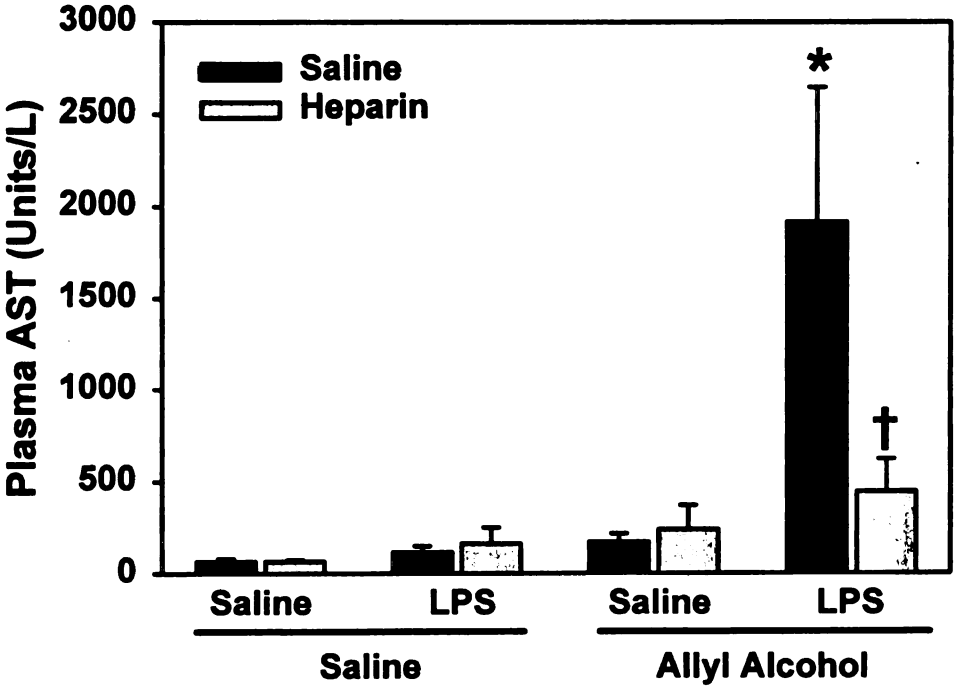
Figure 4.2. Heparin pretreatment attenuates LPS-enhanced allyl alcohol hepatotoxicity *in vivo*.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle (control) one hour before LPS (1.2 X 10^5 EU/kg body weight, i.v.) or vehicle (saline). Two hours after LPS, rats received allyl alcohol (30 mg/kg body weight, i.p.) or vehicle (saline). (A) ALT and (B) AST activities were measured in plasma samples as markers of liver toxicity 8 hours after LPS administration. Data are expressed as means ± S.E.M. N = 6. *, significantly different from saline only (control). †, Significantly different from respective value in the absence of heparin.

A


3000
2500
2000
1500
1000
500
0
Plasma ALT (Units/L)
Saline
Heparin
*
†
Saline
LPS
Saline
LPS
Saline
Allyl Alcohol

B


3000
2500
2000
1500
1000
500
0
Plasma AST (Units/L)
Saline
Heparin
*
†
Saline
LPS
Saline
LPS
Saline
Allyl Alcohol

expression of COX-2 were measured in LPS-treated groups. In livers from control rats, few neutrophils (PMNs) were present (Figure 4.3). Treatment with LPS in the absence or presence of allyl alcohol resulted in accumulation of PMNs in liver tissue (Figure 4.3). Heparin pretreatment did not attenuate the LPS-dependent increase in the number of PMNs in the liver. CINC-1 mRNA expression was increased by treatment with LPS in the absence or presence of allyl alcohol, and heparin pretreatment significantly increased its expression level (Figure 4.4). Allyl alcohol treatment alone did not enhance the expression of CINC-1 mRNA. COX-2 mRNA expression was increased by treatment with LPS in the absence or presence of allyl alcohol, and heparin pretreatment did not significantly alter its expression level (Figure 4.5). Allyl alcohol treatment alone did not significantly enhance the expression of COX-2 mRNA.

To determine whether heparin would affect the toxicity of allyl alcohol in the absence of LPS, a large dose of allyl alcohol (50 mg/kg) was administered to rats that had been treated with heparin or vehicle 3 hours earlier. Whereas allyl alcohol caused significant liver injury, pretreatment with heparin did not significantly attenuate the injury (ALT activity in plasma was 631 ± 189 and 393 ± 102, respectively) (Figure 4.6). In addition, the concentration of plasma fibrinogen from rats treated with a large dose of allyl alcohol did not decrease from normal

Figure 4.3. Neutrophil infiltration is not attenuated by heparin.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle one hour before LPS (1.2×10^5 EU/kg body weight, i.v.) or vehicle. Two hours after LPS, rats received allyl alcohol (30 mg/kg body weight, i.p.) or vehicle. Liver sections were taken 3 and 8 hours after LPS administration and stained for neutrophils (PMNs). The number of stained cells were counted under light microscopy in 20 fields at 400X magnification. Data are expressed as means ± S.E.M. N = 3-6. *, significantly different from saline only (control).

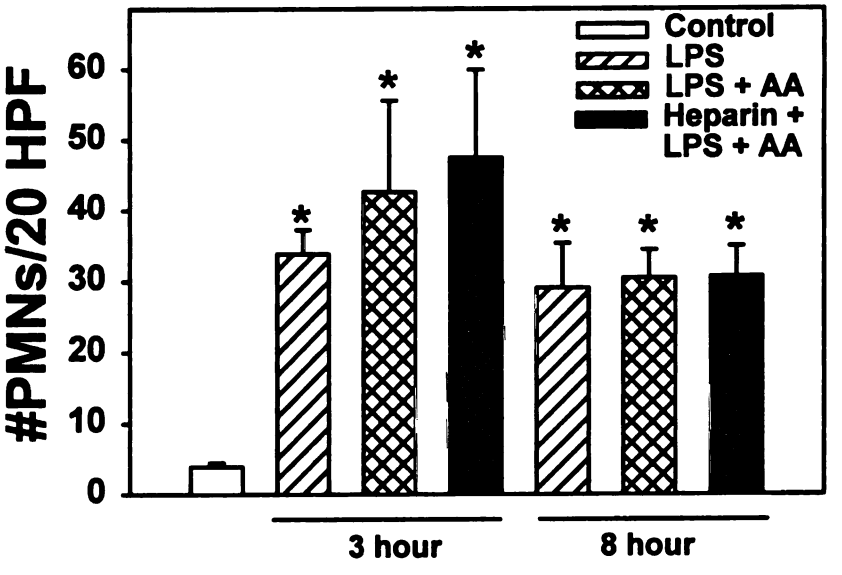

#PMNs/20 HPF
60
50
40
30
20
10
0
Control
LPS
LPS + AA
Heparin +
LPS + AA
*
*
*
*
*
*
3 hour
8 hour

Figure 4.4. CINC-1 mRNA expression is not attenuated by heparin.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle one hour before LPS (1.2 X 10^5 EU/kg body weight, i.v.) or vehicle. Two hours after LPS, rats received allyl alcohol (30 mg/kg body weight, i.p.) or vehicle. Liver sections were taken 3 hours after LPS administration and assessed for CINC-1 mRNA by RT-PCR. CINC-1 band intensity was normalized to 18S rRNA band intensity. Data are expressed as means ± S.E.M. N = 4. *, significantly different from saline only (control); †, significantly different from LPS/AA in the absence of heparin.

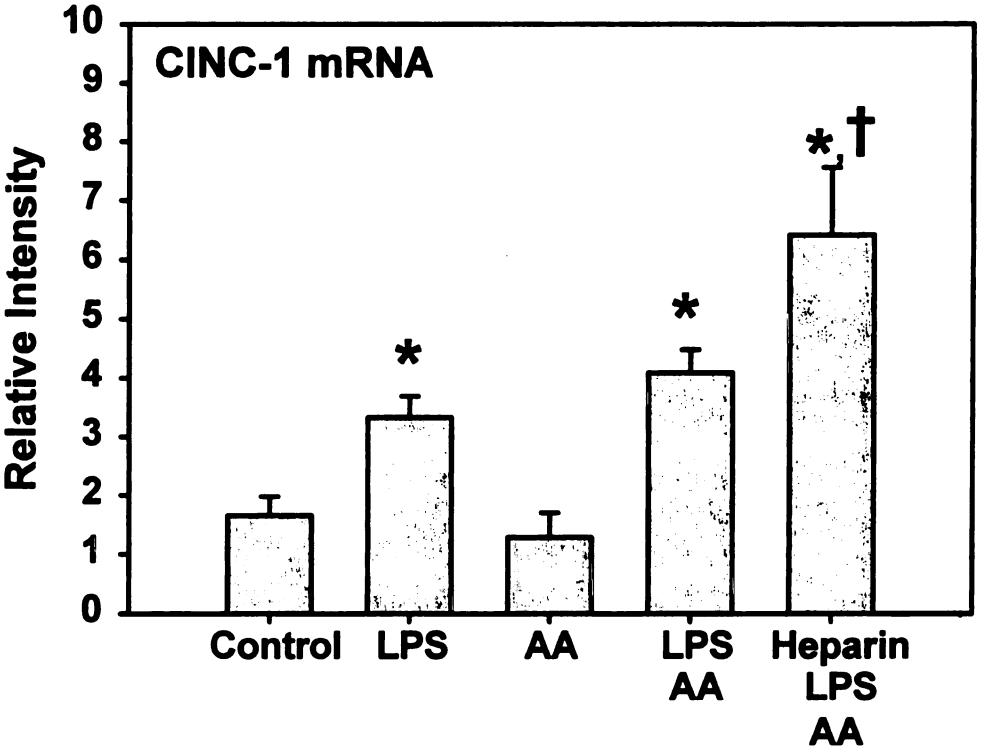

CINC-1 mRNA
Relative Intensity
10
9
8
7
6
5
4
3
2
1
0
*
*
*†
Control
LPS
AA
LPS AA
Heparin LPS AA

Figure 4.5. COX-2 mRNA expression is not attenuated by heparin.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle one hour before LPS (1.2×10^5 EU/kg body weight, i.v.) or vehicle. Two hours after LPS, rats received allyl alcohol (30 mg/kg body weight, i.p.) or vehicle. Liver sections were taken 3 hours after LPS administration and assessed for COX-2 mRNA by RT-PCR. COX-2 band intensity was normalized to 18S rRNA band intensity. Data are expressed as means ± S.E.M. N = 4. *, significantly different from saline only (control).

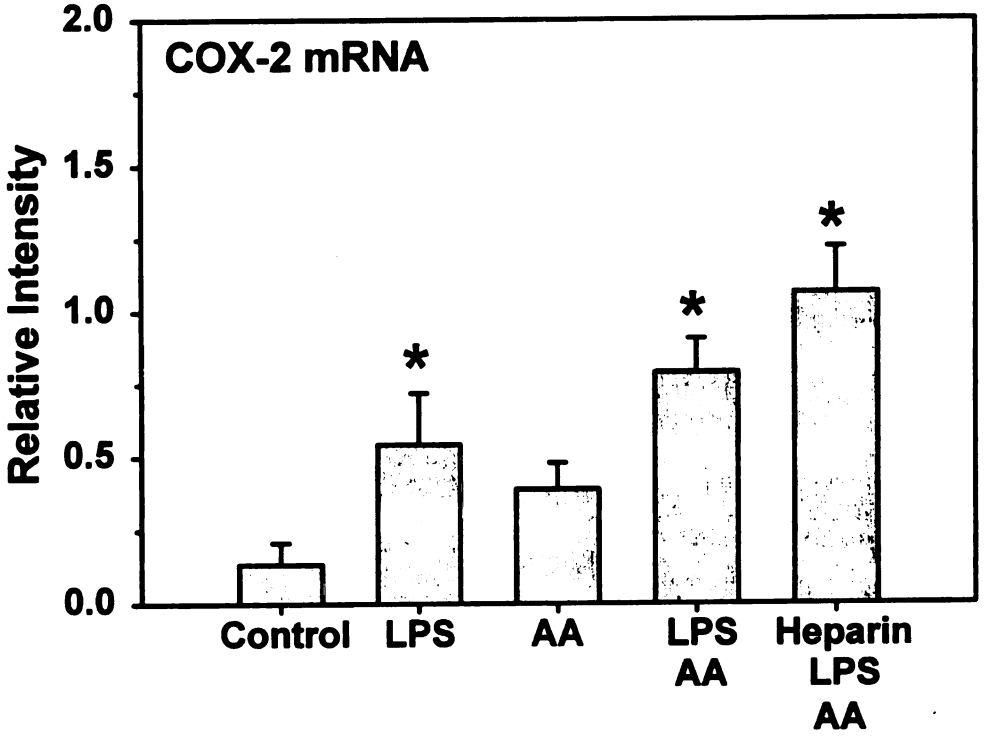

COX-2 mRNA
Relative Intensity
2.0
1.5
1.0
0.5
0.0
*
*
*
Control
LPS
AA
LPS
AA
Heparin
LPS
AA

Figure 4.6. Heparin does not inhibit the toxicity of allyl alcohol alone.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle three hours before allyl alcohol (50 mg/kg body weight, i.p.). Plasma samples were taken 6 hours after allyl alcohol administration for determination of ALT activity. Data are represented as mean ± S.E.M. N = 14-15.

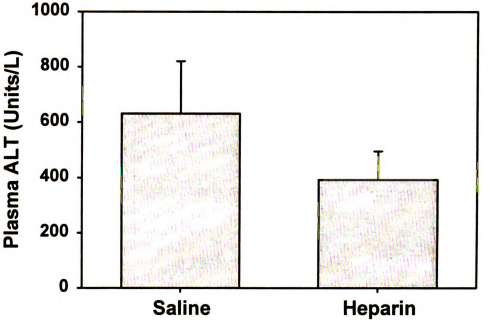
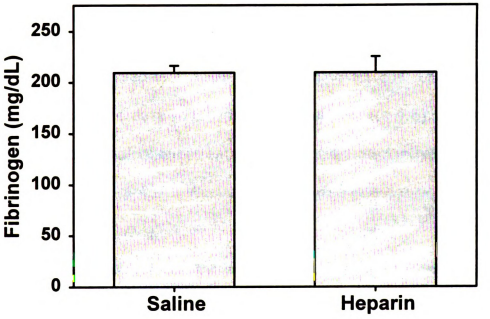

Plasma ALT (Units/L)
1000
800
600
400
200
0
Saline
Heparin

Figure 4.7. Allyl alcohol does not decrease circulating fibrinogen concentration.

Rats were treated with heparin (2000 Units/kg body weight, i.v.) or vehicle three hours before allyl alcohol (50 mg/kg body weight, i.p.). Plasma samples were taken 6 hours after allyl alcohol administration for determination of fibrinogen concentration. Data are represented as mean ± S.E.M. N = 7-8.


Fibrinogen (mg/dL)
250
200
150
100
50
0
Saline
Heparin

levels and was not altered by pretreatment with heparin (plasma fibrinogen concentration was 209 ± 7 and 210 ± 15, respectively) (Figure 4.7).

To confirm that the attenuation of LPS-enhanced allyl alcohol hepatotoxicity was due to inhibition of coagulation by heparin another anticoagulant was used, Warfarin. Warfarin inhibits the coagulation cascade through inhibition of the synthesis of functional coagulation factors VII, IX, X, and II (prothrombin). Plasma fibrinogen concentration was significantly decreased in rats treated with both LPS and allyl alcohol, and pretreatment with warfarin blocked the LPS and allyl alcohol-induced decrease in plasma fibrinogen (Figure 4.8). Liver injury from LPS and allyl alcohol cotreatment was attenuated in rats pretreated with warfarin (Figure 4.9). Warfarin treatment alone did not cause liver injury.

To determine whether thrombin plays a role in the potentiation of allyl alcohol toxicity by LPS through a mechanism mediated by a thrombin-receptor in the liver, as it does in LPS-induced liver injury, the isolated, perfused liver model was used. Rats were treated *in vivo* with LPS, to allow neutrophil accumulation in the liver, and two hours later their livers were isolated and perfused with buffer in a recirculating manner. Two hours corresponds to the time that rats would receive allyl alcohol during *in vivo* experiments. Medium-perfused livers were treated

Figure 4.8. LPS-enhanced, allyl alcohol-induced decrease in plasma fibrinogen is blocked by warfarin pretreatment.

Rats were treated with warfarin (20 mg/kg body weight, p.o.) or saline vehicle twice 24 hours apart. Then 18 hours after the last warfarin treatment rats were treated with LPS ($1.2X10^5$ EU/kg body weight, i.v.), and two hours after LPS they were treated with allyl alcohol (30 mg/kg body weight, i.p.). Eight hours after LPS administration plasma samples were analyzed for fibrinogen concentration. Data are represented as mean ± S.E.M. N = 4-6. *, significantly different from saline/saline control.

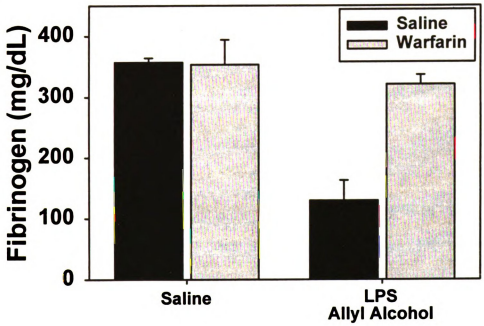

Saline
Warfarin
Fibrinogen (mg/dL)
400
300
200
100
0
Saline
LPS
Allyl Alcohol

Figure 4.9. Warfarin attenuates LPS-enhanced allyl alcohol hepatotoxicity.

Rats were treated with warfarin (20 mg/kg body weight, p.o.) or saline vehicle twice 24 hours apart. Then 18 hours after the last warfarin treatment rats were treated with LPS ($1.2X10^5$ EU/kg body weight, i.v.) and two hours later with allyl alcohol (30 mg/kg body weight, i.p.). Eight hours after LPS administration plasma samples were analyzed for ALT activity. Data are represented as mean ± S.E.M. N = 4-15. *, significantly different from control at $p<0.05$.

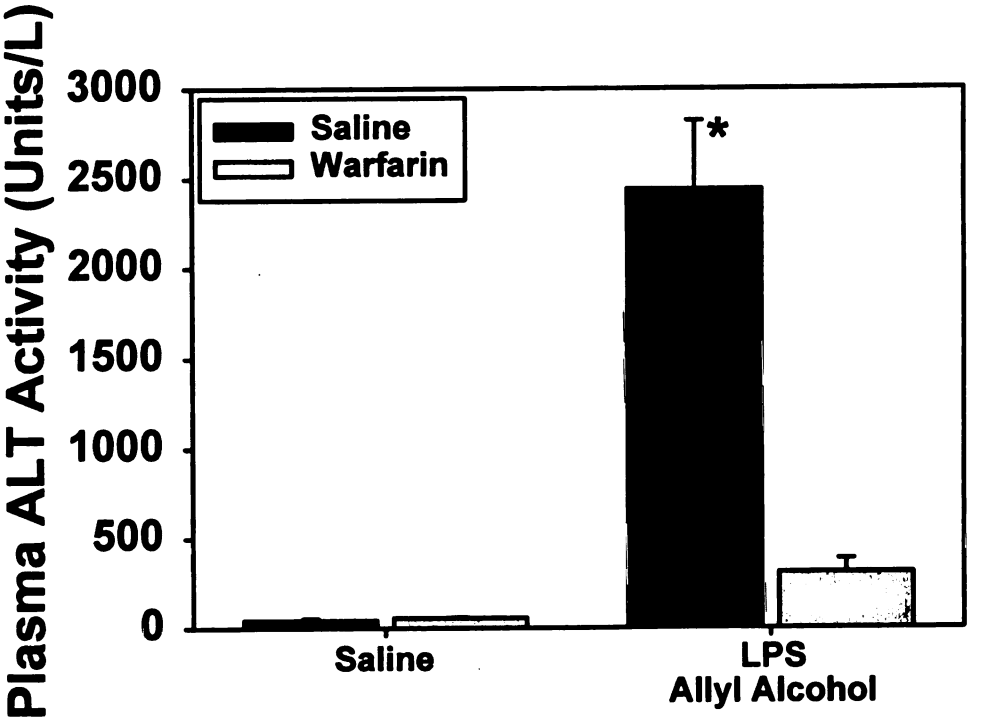
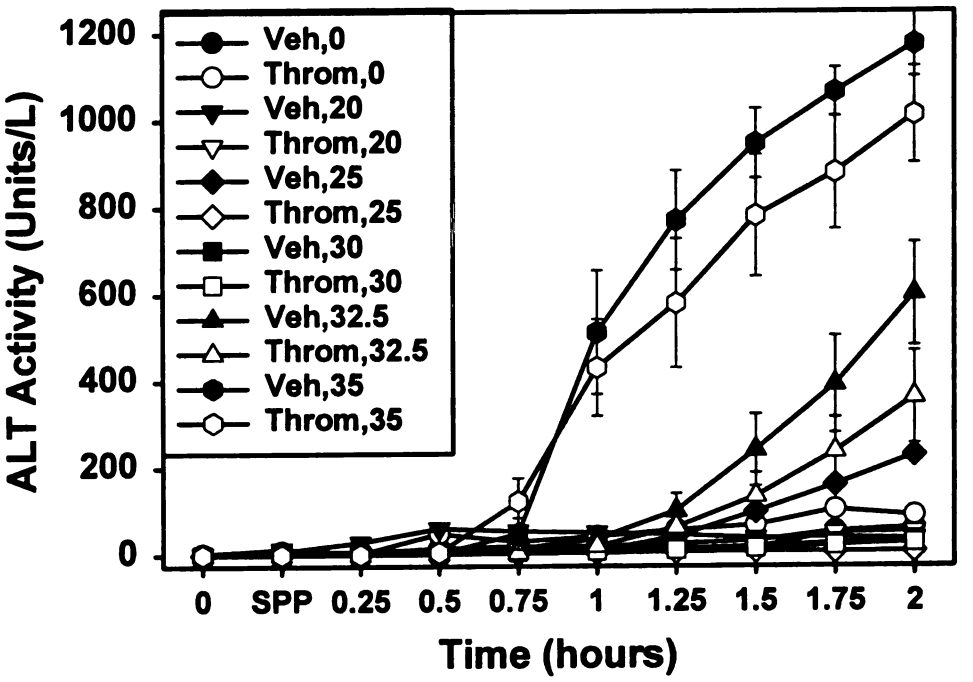

Plasma ALT Activity (Units/L)
3000
2500
2000
1500
1000
500
0
Saline
Warfarin
*
Saline
LPS
Allyl Alcohol

Figure 4.10. Thrombin does not enhance the hepatotoxicity of allyl alcohol in isolated, buffer-perfused livers from LPS-treated rats.

Livers were isolated from rats 2 hours after treatment with LPS (1.2 X 10^5 EU/kg body weight) and subsequently perfused with allyl alcohol at the indicated doses (numbers in legend are mg/kg) and with either 70 units/liver thrombin (open symbols) or vehicle (filled symbols) for an additional two hours. ALT released into the medium was assessed over time as a marker of liver injury. Data are expressed as means ±S.E.M. N=2-8. No significant thrombin effect was observed.


ALT Activity (Units/L)
1200
1000
800
600
400
200
0
Veh,0
Throm,0
Veh,20
Throm,20
Veh,25
Throm,25
Veh,30
Throm,30
Veh,32.5
Throm,32.5
Veh,35
Throm,35
0
SPP
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Time (hours)

with thrombin and the indicated concentrations of allyl alcohol (Figure 4.10). Liver injury was assessed from the ALT activity of the perfusion medium. Allyl alcohol-induced liver injury was found to be both time- and concentration-related. However, addition of thrombin did not enhance the hepatotoxicity of allyl alcohol at any concentration of allyl alcohol used.

In a separate isolated, perfused liver experiment the effect of LPS on allyl alcohol-induced hepatotoxicity was determined. Rats were treated *in vivo* with LPS or saline, and two hours later their livers were isolated and perfused in a recirculating manner as described above. Allyl alcohol (32.5 mg/kg body weight) was then added to the perfusion medium, and samples were taken for an additional two hours. Liver injury as assessed by ALT activity increased with time during perfusion with allyl alcohol. Livers from rats pretreated with LPS developed significantly greater injury than livers from rats pretreated with saline (Figure 4.11).

Thrombin did not increase injury in livers isolated from LPS-treated rats. It is possible that activation of thrombin *in vivo* prior to isolation of the livers could have caused thrombin receptors to become refractory to further thrombin stimulus. To determine whether this was a possibility, plasma was collected from rats two hours after administration of LPS or saline (i.e., at the time livers were isolated for

Figure 4.11. Livers isolated from LPS-treated rats are more sensitive to allyl alcohol-induced injury than control livers.

Livers were isolated from rats 2 hours after treatment with LPS (1.2 X 10^5 EU/kg body weight) (closed squares) or saline (open circles). Subsequently, livers were perfused with medium containing allyl alcohol (32.5 mg/kg) for a period of two hours. ALT released into the medium was assessed over time as a marker of liver injury. Data are expressed as means ± S.E.M. N = 5-6. *, significantly different from saline at corresponding time.

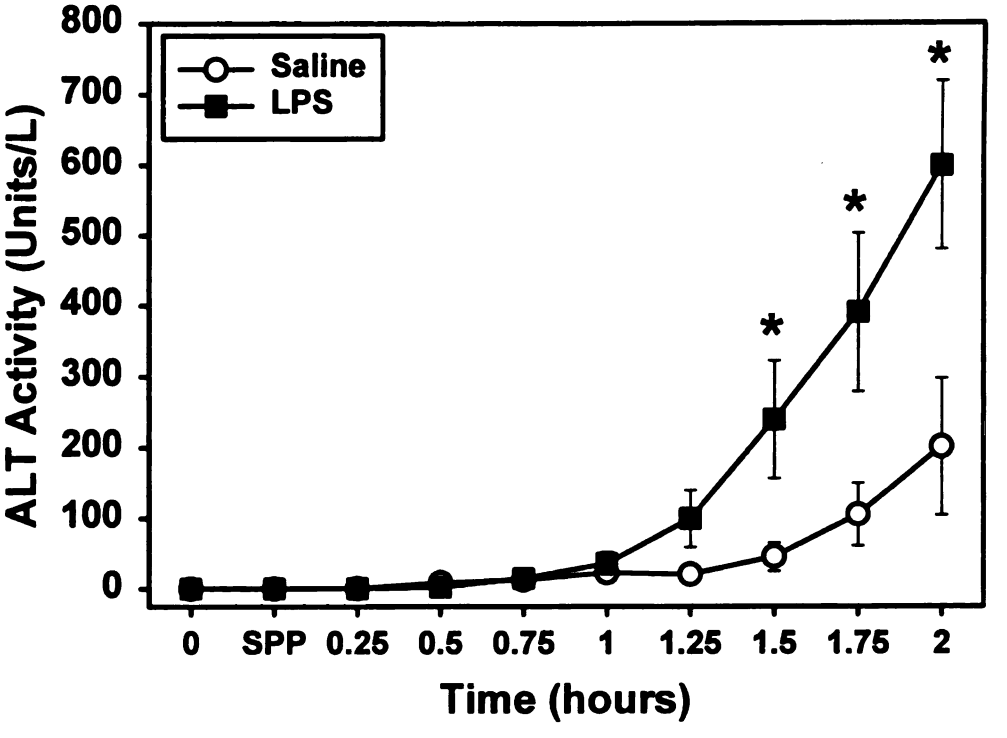
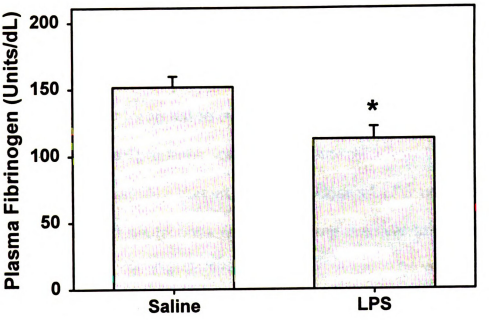

800
700
600
500
400
300
200
100
0
ALT Activity (Units/L)
Saline
LPS
*
*
*
0
SPP
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Time (hours)

Figure 4.12. Thrombin is activated by 2 hours in LPS-treated rats.

Blood plasma was isolated from rats 2 hours after treatment with LPS (1.2 X 10^5 EU/kg body weight) or saline control. Plasma fibrinogen concentration was determined as described in Methods. Data are expressed as means ± S.E.M. N = 15. *, significantly different from saline control.

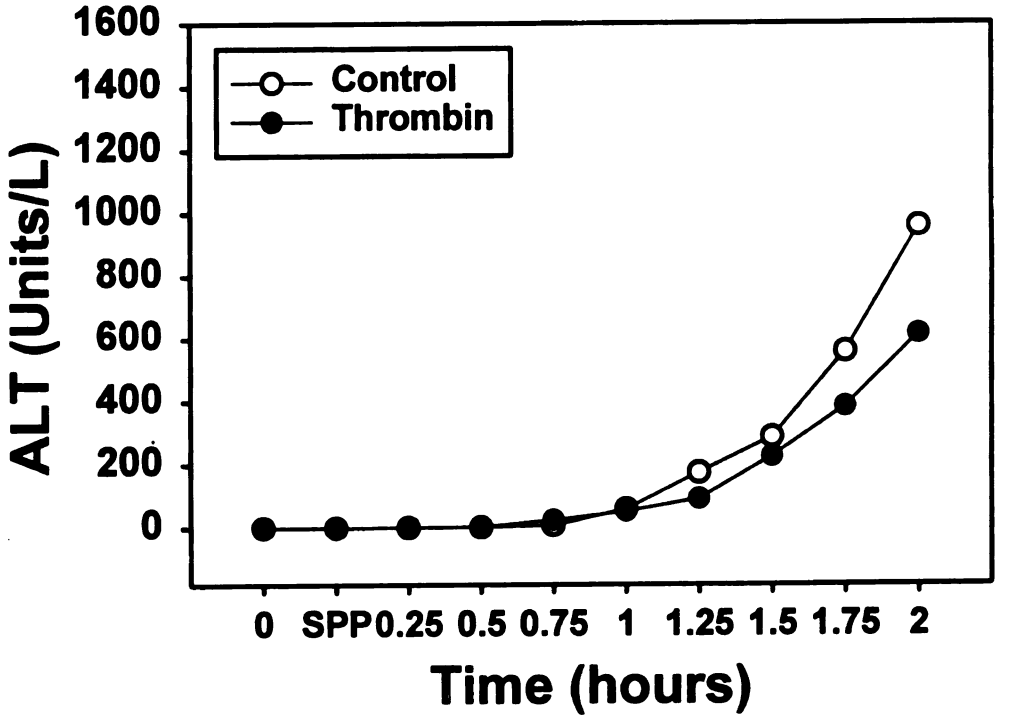

Plasma Fibrinogen (Units/dL)
200
150
100
50
0
*
Saline
LPS

perfusion), and plasma fibrinogen concentration was determined. A significant decrease in plasma fibrinogen concentration was observed (saline, 152 ± 8 mg/dL; LPS, 113 ± 9 mg/dL) (Figure 4.12). To test whether this activation *in vivo* prevented the effect of thrombin in isolated livers, rats were pretreated with heparin (2000 Units/kg body weight) one hour before LPS treatment *in vivo*. Two hours later the livers were isolated and perfused with medium containing allyl alcohol in the absence or presence of thrombin. Though allyl alcohol caused an increase in ALT release over time, addition of thrombin did not enhance the hepatotoxicity of allyl alcohol (Figure 4.13).

The ability of thrombin to induce liver injury in the isolated liver is dependent on the presence of neutrophils (Moulin, 1999). We considered the possibility that at the small dose of LPS used in these studies, not enough neutrophils were infiltrating the liver to support this effect of thrombin in the isolated, perfused liver. To examine this possibility the number of neutrophils was counted in liver sections from animals two hour after treatment with LPS. Hepatic neutrophil numbers were also determined just after single-pass perfusion of isolated livers from LPS-treated rats and at the end of isolated, perfused liver experiments. In livers from LPS-treated rats there were about 33 neutrophils per 20X field at two hours (i.e. the time livers are isolated). After isolation of the livers and single-pass perfusion for 15 minutes the

Figure 4.13. Thrombin does not enhance the hepatotoxicity of allyl alcohol in isolated, perfused livers from rats treated with heparin and LPS.

Livers were isolated from rats 2 and 3 hours after treatment with LPS (1.2 X 10^5 EU/kg body weight) and heparin (2000 Units/kg body weight), respectively. Subsequently, livers were perfused with allyl alcohol (32.5 mg/kg) and either thrombin (70 Units/rat) (closed circle) or vehicle control (open circle) for a period of two hours. ALT released into the medium was assessed over time as a marker of liver injury. Data are expressed as means. N = 2.


1600
1400
1200
1000
800
600
400
200
0
ALT (Units/L)
Control
Thrombin
0
SPP
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Time (hours)

number of neutrophils decreased to 13 neutrophils per 20X field. By the end of the two-hour recirculating perfusion experiment about 7 neutrophils per 20X field remained in the liver. This is much greater than the 0.2 neutrophils per 20X field found in livers from naïve rats.

4.4 Discussion

In previous studies isolated, perfused livers from naïve rats were perfused with buffer containing LPS or saline for two hours and allyl alcohol for an additional two hours (Chapter 2). The inability of LPS to enhance the hepatotoxicity of allyl alcohol as observed *in vivo* led to the possibility that extrahepatic factors play a critical role in the potentiation of allyl alcohol-induced liver injury by LPS. Injury from larger doses of allyl alcohol alone is not dependent on extrahepatic factors as evidenced by the lack of protection offered by heparin or neutrophil depletion (Ganey *et al*, 1995) and the ability of allyl alcohol to injure isolated hepatocytes (Sneed *et al*, 1997). However, LPS-induced liver injury from large doses of LPS is critically dependent on extrahepatic factors (Moulin *et al*, 1996; Hewett *et al*, 1992). One extrahepatic factor that plays a critical role in LPS-induced liver injury is thrombin. In a rat model of liver injury induced by large doses of LPS, inhibitors of the coagulation cascade such as heparin, warfarin, or a specific inhibitor of thrombin, hirudin, each attenuated LPS-induced liver injury (Hewett *et al*, 1995;

Pearson *et al*, 1996). Thus it was of interest to examine a possible role for thrombin in the LPS enhancement of allyl alcohol hepatotoxicity.

To begin to assess whether thrombin or the coagulation cascade plays a role in LPS-enhanced allyl alcohol hepatotoxicity, heparin was used to inhibit thrombin activity. Heparin enhances the ability of anti-thrombin III to bind to thrombin, thereby inhibiting the activity of thrombin. Pretreatment with heparin reduced liver injury from the cotreatment of allyl alcohol and LPS *in vivo*. These data suggest that thrombin participates in the response. However, although heparin inhibits thrombin it also has other actions. Heparin has been shown to inhibit the infiltration of PMNs into tissues and the production of superoxide anion and nitric oxide from stimulated neutrophils (Downing *et al*, 1998; Darien *et al*, 1998; Shin *et al*, 1997; Riesenberg *et al*, 1995; Beltran *et al*, 1999). To determine if parts of the inflammatory response were decreased in our experiment several aspects of inflammation were assessed. CINC-1 is a chemokine that is released during inflammation and helps to bring neutrophils into tissues (Zhang *et al*, 1995). Neither the expression of CINC-1 mRNA nor LPS-induced neutrophil accumulation in liver was attenuated by pretreatment with heparin. In addition, LPS and other inflammatory stimuli upregulate the inducible cyclooxygenase enzyme, COX-2 (Ruetten *et al*, 1997). We know from studies in our laboratory that expression of COX-2 plays a critical role in

the potentiation of allyl alcohol hepatotoxicity by LPS (Ganey *et al*, 2001). Expression of COX-2 mRNA was increased in livers from rats cotreated with LPS and allyl alcohol, but this increase was unaffected by pretreatment with heparin. These findings demonstrate that heparin pretreatment did not have an inhibitory effect on several aspects of the inflammatory response in our experiment. Therefore the protective effect of heparin against liver injury was likely not due to an inhibition of the inflammatory response but rather to its ability to block the coagulation cascade.

Though the results of heparin pretreatment suggest that the mechanism of protection was through its inhibition of coagulation, we confirmed this by using another anticoagulant that works through a completely different mechanism to inhibit the coagulation cascade. Warfarin is an anticoagulant that inhibits the regeneration of reduced vitamin K. Reduced vitamin K is critical for the carboxylation of glutamate to γ-carboxyglutamate residues on precursor protein factors VII, IX, X, and II (prothrombin). Thus warfarin inhibits the coagulation cascade by inhibiting the formation of functional coagulation factors. Using this alternate method of inhibiting the coagulation cascade, the potentiation of allyl alcohol toxicity by LPS also was attenuated. Collectively these data suggest that the coagulation cascade plays a critical role in the potentiation of allyl alcohol hepatotoxicity by LPS.

To confirm that attenuation of LPS-enhanced allyl alcohol-induced liver injury was specific to the potentiation and not simply attenuation of the toxicity of allyl alcohol, heparin pretreated rats were treated with a large dose of allyl alcohol. Not only did heparin fail to decrease allyl alcohol-induced liver injury at this large dose, but this dose of allyl alcohol did not activate the coagulation cascade as assessed by plasma fibrinogen concentration. This indicates that the observed protective effect of heparin against LPS enhancement of allyl alcohol-induced liver injury is unique to the potentiation model and provides evidence that the mechanism of injury in the potentiation model differs from allyl alcohol-induced liver injury. Further, it provides evidence that heparin did not decrease the bioactivation of allyl alcohol.

As mentioned above thrombin plays a critical role in liver injury from large doses of LPS. The mechanism by which thrombin contributes to LPS-induced injury involves a specific receptor for thrombin, protease-activated receptor-1 (PAR-1) found in the liver (Moulin *et al*, 1996; Moulin, 1999). To examine the possibility of thrombin acting through a receptor-mediated mechanism in the LPS-induced potentiation of allyl alcohol, isolated livers from LPS-treated rats were perfused with allyl alcohol and either thrombin or vehicle. Addition of thrombin to the medium did not enhance the hepatotoxicity of allyl alcohol. However, livers from LPS-treated rats were more sensitive to allyl alcohol-induced

injury than livers from saline-treated rats. Analysis of plasma fibrinogen concentration from rats two hours after saline or LPS injection revealed that LPS caused a slight but significant activation of the coagulation cascade in this time frame. This indicates that thrombin had been activated *in vivo* by LPS before isolation of the livers. Thus, one explanation for the lack of effect of thrombin in the isolated, perfused liver is that prior thrombin activation caused a downregulation or internalization of thrombin receptors in the liver preventing their subsequent activation in the isolated, perfused liver. To test this hypothesis, a limited number of rats was pretreated with heparin one hour before LPS, and livers were isolated and perfused as in the previous experiments. Thrombin did not enhance allyl alcohol-induced injury in these livers. This suggests that prior activation of thrombin and possible internalization of thrombin receptors does not explain the inability of thrombin to enhance the hepatotoxicity of allyl alcohol. Taken together the data suggest that enhancement of allyl alcohol-induced liver injury is dependent on thrombin activation of blood components or some other components of the coagulation cascade. One potential component is the neutrophil.

Though there were a greater number of neutrophils in livers from LPS treated rats than in livers from naïve rats, it is possible that there were not enough neutrophils present to induce injury upon thrombin

stimulus. In experiments demonstrating the ability of thrombin to induce injury in livers from LPS-treated rats, maximal injury occurred in the presence of neutrophils (Moulin, 1999). At the end of a two hour recirculating experiment, these livers were found to contain about 41 neutrophils per 20X field (Moulin and Copple, unpublished), which is four times as many neutrophils in the liver at the end of the experiment than there were at the beginning of experiments presented here. Though the larger number of neutrophils may be due to a three-fold larger dose of LPS than was used in the results presented here, it raises the possibility that a minimum number of neutrophils was needed to observe a thrombin-mediated injury and that this minimum number of neutrophils was not present in the livers for studies presented here. Furthermore, the potentiation of allyl alcohol hepatotoxicity by LPS *in vivo* is dependent on the presence of neutrophils (Sneed, 2000). Thus, it is possible that the lack of thrombin effect was due to a decrease in the number of neutrophils. The number of liver-infiltrating neutrophils found *in vivo* may be further increased after the administration of allyl alcohol through added stress and increased chemokine release. Therefore, it may be that thrombin-induced liver injury requires a critical number of infiltrating neutrophils.

Livers from LPS-treated rats were more sensitive to allyl alcohol hepatotoxicity than livers from saline-treated rats. If not enough

neutrophils were present in the livers, then by what mechanism did LPS enhance the hepatotoxicity of allyl alcohol? Kupffer cells play a critical role in the potentiation of allyl alcohol by LPS (Sneed *et al*, 1997). Therefore it is possible that LPS-stimulated Kupffer cells released mediators into the liver that altered the hepatocytes to become more sensitive to allyl alcohol hepatotoxicity. However, enhanced allyl alcohol hepatotoxicity was not observed during perfusion of isolated livers from naïve rats with LPS. Accordingly, the enhancement of allyl alcohol hepatotoxicity in livers from LPS-treated rats is not likely due solely to the release of Kupffer cell-derived mediators into the perfusion medium. Therefore, it is possible that there are enough neutrophils present in the isolated livers from LPS treated rats to develop injury and that LPS treatment *in vivo* activates components of the coagulation cascade which alter the liver to make it more susceptible to allyl alcohol. In this scenario pretreatment with heparin would inhibit the activation of coagulation. Therefore, the inability of thrombin to enhance the toxicity of allyl alcohol is likely due to the absence of other critical factors of the coagulation cascade or other thrombin-activated factors present in the blood.

Results presented in this paper indicate that thrombin does not appear to play a role in the potentiation of allyl alcohol-induced liver injury by LPS through a liver receptor-mediated mechanism. However,

the ability of anticoagulants to attenuate the potentiation indicate that some component of the coagulation cascade does play a critical role in the ability of LPS to enhance allyl alcohol-induced liver injury. The role of thrombin in the potentiation of allyl alcohol hepatotoxicity by LPS may be dependent on other coagulation factors or components of the blood.

CHAPTER 5

Summary and Conclusions

5.1 Summary

Humans are exposed to many different drugs and chemicals from day to day. Investigators have shown that endotoxin exposure can enhance the toxicity of various xenobiotics. Because endotoxin is well known to be a potent stimulator of the inflammatory system, the ability of endotoxin to enhance the toxicity of xenobiotics may be due to the stimulated inflammatory system. Humans exposed to a xenobiotic during a simultaneous inflammatory bout may be at greater risk for toxicity.

To elucidate the mechanism by which inflammation can enhance the toxicity of xenobiotics we chose allyl alcohol as a model xenobiotic and endotoxin to stimulate an inflammatory state. The ability of LPS to enhance the hepatotoxicity of allyl alcohol has been investigated previously. Early findings demonstrated that endotoxin acts to enhance the sensitivity of the liver to the toxicity of allyl alcohol based on the observation that the lesion remains similar to that observed with a large dose of allyl alcohol. Furthermore, exposure of isolated hepatocytes to LPS did not alter the hepatotoxicity of allyl alcohol. This indicated that some other cell type or cellular mediator is required for LPS to enhance the toxicity of allyl alcohol. Inhibition of Kupffer cell activity attenuated LPS enhancement of the toxicity of allyl alcohol. This implicated a critical role for Kupffer cells in LPS-enhanced allyl alcohol hepatotoxicity.

The purpose of the present body of work was to investigate further the mechanism by which underlying inflammation induced by a small dose of LPS enhances the hepatotoxicity of allyl alcohol.

In Chapter 2 the critical role of Kupffer cells in the potentiation of allyl alcohol hepatotoxicity by LPS was investigated. Because the role of Kupffer cells would most easily be studied *in vitro*, a Kupffer cell-hepatocyte coculture system was utilized. However, many alterations to the coculture system failed to replicate the potentiation observed *in vivo*. We also discovered that LPS could not potentiate the hepatotoxicity of allyl alcohol in isolated, buffer-perfused livers from naïve rats. This observation indicated that a Kupffer cell-hepatocyte coculture system would not be sufficient to study the role of Kupffer cells. Furthermore, the critical role of Kupffer cells in the potentiation was dependent on other extrahepatic factors. Thus, work was conducted to determine which extrahepatic factors play a critical role in the potentiation.

Because allyl alcohol-induced hepatotoxicity is not known to be dependent on extrahepatic factors, the mechanism of LPS-induced hepatotoxicity was reviewed. LPS-induced hepatotoxicity is critically dependent on neutrophils and thrombin, both of which are extrahepatic factors (Hewett *et al*, 1992; Moulin *et al*, 1996). The work presented in Chapters 3 and 4 investigated the roles of neutrophils and thrombin, respectively, in the potentiation of allyl alcohol hepatotoxicity by LPS.

The critical role of neutrophils in the potentiation of allyl alcohol hepatotoxicity by LPS was discovered by Sneed (Sneed, 2000). This led to the hypothesis that allyl alcohol-induced depletion of glutathione renders hepatocytes more susceptible to LPS-induced, neutrophil-derived reactive oxygen species. Experiments presented in Chapter 3 tested this hypothesis. Neutrophil-hepatocyte cocultures were used to test *in vitro* whether neutrophil-derived reactive oxygen species could enhance the hepatotoxicity of allyl alcohol. The results from this set of experiments demonstrated that neutrophil-derived reactive oxygen species were capable of enhancing the toxicity of allyl alcohol; however, the magnitude of effect was very small and cast doubt on the role of reactive oxygen in this response in the whole animal.

The role of reactive oxygen species in the potentiation of allyl alcohol-induced liver injury by LPS was assessed *in vivo* using two different inhibitors of reactive oxygen species. In one experiment rats were pretreated with polyethylene-coupled superoxide dismutase and catalase. This inhibitor would neutralize the reactive oxygen species formed by activated neutrophils. Though the dose used in the experiments presented in Chapter 3 caused a 66 percent decrease in superoxide detection and was similar to the dose used in a previous study demonstrating protection from vitamin A potentiation of carbon tetrachloride hepatotoxicity (elSisis *et al*, 1993), we did not observe any

protection from injury in the LPS/allyl alcohol model. These data suggested that reactive oxygen species do not play a critical role in the potentiation of allyl alcohol toxicity by LPS. It could be argued, however, that the dose of polyethylene-coupled superoxide dismutase and catalase was not large enough to neutralize the generated superoxide sufficiently. In particular, the small space between the sequestered neutrophil and the hepatocyte could contain a very high concentration of superoxide in comparison to the concentration of superoxide dismutase. Therefore another inhibitor of reactive oxygen species was used.

Apocynin is a drug that inhibits the assembly of the NADPH oxidase enzyme complex on the surface of cells. It is this enzyme that is responsible for the production of superoxide anion by activated cells. Thus, by using apocynin we could prevent reactive oxygen species from being formed. Using a dose that was reported to protect against neutrophil-induced respiratory muscle dysfunction during sepsis (Supinski *et al*, 1999), we did not find a significant attenuation of liver injury. These data further demonstrated that superoxide does not play a critical role in the potentiation of allyl alcohol-induced liver injury by LPS.

This result has been found in other models of liver injury that involve neutrophils. It was surprising that allyl alcohol, being an agent that causes reductive stress in cells, did not become very toxic in the

presence neutrophil-derived superoxide anion. However, our laboratory has found that hepatocellular injury is also dependent on neutrophil-derived proteases, specifically cathepsin G and elastase, via their ability to induce hepatocellular injury from activated neutrophils (Ho *et al*, 1996). This may also be the mechanism by which neutrophils play a critical role in the potentiation of allyl alcohol hepatotoxicity by LPS.

In Chapter 4 we investigated the potential role of thrombin in the potentiation of allyl alcohol toxicity by LPS. Thrombin acts through a receptor-mediated mechanism in a model of large-dose, LPS-induced liver injury (Moulin, 1999). This led to the hypothesis that thrombin plays a critical role in the potentiation of allyl alcohol hepatotoxicity by LPS through a receptor-mediated mechanism. The ability of heparin and warfarin to inhibit the potentiation of allyl alcohol damage by LPS indicated that thrombin was playing a role in the potentiation of allyl alcohol. However, neither heparin nor warfarin is a specific inhibitor of thrombin. These drugs also inhibit other factors within the coagulation cascade. To demonstrate the specific effect of thrombin on the liver the isolated, perfused liver model was used. Addition of thrombin to isolated, perfused livers from LPS-treated rats did not enhance the hepatotoxicity of allyl alcohol, indicating that thrombin was not acting through a liver receptor-mediated mechanism. Thus the role of the coagulation cascade in the potentiation of allyl alcohol heptotoxicity by

LPS could be dependent on other factors of the coagulation cascade or other factors within the blood that thrombin can activate. Alternatively, it is possible that too many neutrophils were flushed out of the livers from LPS-treated rats and that the role of thrombin is dependent on some critical number of neutrophils in the liver.

The mechanism by which underlying inflammation induced by low doses of LPS enhances the hepatotoxicity of allyl alcohol remains unknown. It is clear from this body of work that this is a complex mechanism which is critically dependent on several different aspects of inflammation including Kupffer cells, neutrophils, and the coagulation cascade. It is interesting that while none of these factors is critical for the hepatotoxicity of allyl alcohol alone, all of these factors play a critical role in the hepatotoxicity of large doses of LPS. From this point of view it would appear that allyl alcohol enhances the hepatotoxicity of LPS. The liver lesion from the potentiation of allyl alcohol and LPS is located in an area representative of allyl alcohol hepatotoxicity. This could be due to the toxicity of LPS that is being enhanced in the region of allyl alcohol-induced stress. In the simplest terms the mechanism of the potentiation with LPS and allyl alcohol may be due to the combination of a primed inflammatory system with a hepatocellular stress. The stress of allyl alcohol toxicity, though not enough to induce cell death, may induce release of cellular mediators that can trigger the already primed

immune system to become active whereas under normal circumstances the immune system would not be activated.

5.2 Revised Proposed Mechanism

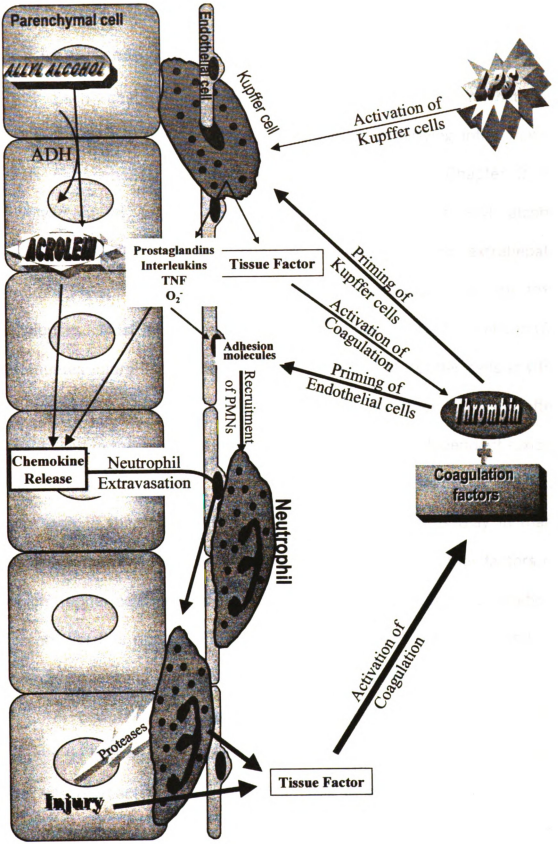
Using information from this thesis and the literature a simplified mechanism by which LPS enhances the toxicity of allyl alcohol is proposed. Upon LPS exposure, Kupffer cells are activated to release many different mediators including prostaglandins, interleukins, TNF, and reactive oxygen species. The effects of activated Kupffer cells in the liver include decreased protein synthesis, synthesis of acute phase proteins which include chemokines, recruitment of neutrophils, and activation of coagulation through tissue factor expression. The initiation of coagulation acts as a proinflammatory stimulus that can further activate and prime Kupffer cells, endothelial cells, and neutrophils. The proinflammatory actions of coagulation may be due to thrombin. Thrombin activates endothelial cells to release platelet activating factor, adhesion molecules, and reactive oxygen species. Thrombin also enhances the release of mediators from both Kupffer cells and neutrophils. Though inhibition of thrombin activity does not block neutrophil infiltration, it does protect against neutrophil-dependent liver injury (Pearson *et al*, 1996). Thus, thrombin may play a major role in the propagation of liver injury.

Endothelial cell activation by either LPS, Kupffer cell-derived mediators, or thrombin can led to expression of adhesion molecules such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin. Neutrophils also begin to express adhesion molecules and infiltrate into the liver. The liver is now primed to protect itself from invading bacteria. However, as reviewed by Jaeschke (Jaeschke, 1997) the presence of neutrophils in the liver does not mean that they are contributing to the injury. There are many examples in which neutrophils must accumulate in the liver for several hours before injury results and still others in which liver injury does not occur despite a large number of neutrophils in the liver. Only neutrophils that adhere to hepatocytes appear to be involved in injury. For neutrophils to extravasate there must be some signal from the hepatocytes to call them in such as CINC-1. This may be the point at which allyl alcohol toxicity may act as the trigger for neutrophil damage. The slight injury or insult induced by small doses of allyl alcohol may cause the hepatocyte to release chemokines such as CINC-1 which would call neutrophils into the extravascular space, allowing direct contact between the hepatocyte and the neutrophil. This contact with a partially damaged cell may trigger the neutrophil to release its antimicrobial milieu resulting in damage to the hepatocyte. In addition the activated neutrophil is capable of releasing tissue factor both from itself and from the injured hepatocyte.

The increased release of tissue factor would serve to activate further the coagulation cascade. In addition, activation of the coagulation cascade would prime further the inflammatory response leading to propagation of the injury (Figure 5.1).

Figure 5.1 Proposed mechanism for LPS-enhancement of allyl alcohol hepatotoxicity.

Cartoon representation of a liver sinusoid exposed to LPS and allyl alcohol. Allyl alcohol acts directly on hepatocytes and causes stress that may enhance the production of chemokines. LPS stimulates Kupffer cells which then release mediators such as prostaglandins, cytokines, reactive oxygen species, and tissue factor that stimulate neutrophil infiltration and release of chemokines from hepatocytes. Chemokine release from hepatocytes induces neutrophils to extravasate and subsequently injure the hepatocytes. Release of tissue factor from stimulated cells activates the coagulation cascade which then exacerbates the inflammatory response and consequent injury. Arrow thickness represents an approximate order of events with thin arrows representing early events and thick arrows representing later events.


Parenchymal cell
ALLYL ALCOHOL
ADH
ACROLEIN
Endothelial cell
Kupffer cell
Activation of Kupffer cells
LPS
Prostaglandins
Interleukins
TNF
O_2^-
Tissue Factor
Priming of Kupffer cells
Activation of Coagulation
Priming of Endothelial cells
Thrombin
Coagulation factors
Adhesion molecules
Recruitment of PMNs
Chemokine Release
Neutrophil Extravasation
Neutrophil
Activation of Coagulation
Proteases
Injury
Tissue Factor

5.3 Importance of Work

Each chapter of this thesis has helped to further our understanding of the mechanism by which an underlying inflammatory state can enhance the toxicity of a xenobiotic. In Chapter 2 the involvement of Kupffer cells in the potentiation of allyl alcohol hepatotoxicity by LPS was found to be dependent on extrahepatic factors. Kupffer cells play a critical role in many types of toxic responses. Most investigators find that inhibition of Kupffer cells *in vivo* attenuates injury and do not investigate the role of Kupffer cells *in vitro*. Thus, the critical dependence of Kupffer cells on other factors is often overlooked in the literature. Prevention of Kupffer cell-dependent toxicity via inhibition of Kupffer cells may not be beneficial or a practical approach to therapy due to the critical role these cells play in many other normal physiological functions. Inhibition of the other factors on which Kupffer cells depend, such as thrombin, may be a more beneficial target. For this reason, understanding the nature of the factors and the interdependence of Kupffer cells with them is important.

In Chapter 3 we found that the enhancement of allyl alcohol toxicity by LPS was not due to reactive oxygen species. For many years the role of reactive oxygen intermediates in the development of allyl alcohol-induced liver injury has been debated in the literature. While

some have shown that protein adduct formation and consequent loss of protein function is the cause of allyl alcohol-induced liver injury, others have presented evidence that cell damage is dependent on oxidative injury. If allyl alcohol-induced cell damage were dependent on oxidative injury then the addition of reactive oxygen species would be expected to increase cell damage. Our findings indicating that enhancement of allyl alcohol hepatotoxicity by LPS is not dependent on the release of reactive oxygen species from activated inflammatory cells suggest that the toxicity of allyl alcohol alone is also not dependent on oxidative injury. Therefore, the data presented here lend support toward the mechanism of allyl alcohol-induced liver injury being dependent on protein adduct formation and loss of protein function.

In Chapter 4 we found that the coagulation cascade plays a critical role in the potentiation of allyl alcohol-induced liver injury by LPS while it does not play a role in the toxicity of allyl alcohol alone. The ability of anticoagulants to afford protection could potentially be used to identify idiosyncratic drug interactions that can be explained by an underlying inflammatory state. The literature could be searched to compare the number of idiosyncratic drug interactions that occur with anticoagulants compared to the number of idiosyncratic drug interactions that occur with several other drug types. A smaller number of idiosyncratic

interactions with anticoagulants may indicate that anticoagulants protect against underlying inflammation as a cause for adverse interactions.

Work presented here has advanced our current knowledge of the mechanism by which underlying inflammation can enhance the toxicity of a xenobiotic agent. Each of the mechanisms found to play a role in enhanced toxicity has increased our current understanding of liver toxicology. Although studies in which inhibition of Kupffer cells *in vivo* alters a response can implicate these cells in a biological effect, they do not reveal the complexity of the contribution of these cells. Their complexity is only partially uncovered when attempting to recreate the response in an *in vitro* system. In this model of toxicity Kupffer cells appear to play a critical role in the development of injury through their ability to induce neutrophil accumulation and activate the coagulation cascade. Though Kupffer cells may be involved in the potentiation of allyl alcohol hepatotoxicity by endotoxin through other mechanisms, this body of work demonstrates the complex nature of toxic responses. Furthermore, results presented here have laid a foundation upon which further investigations can be based to further elucidate the mechanism by which LPS exposure can enhance the hepatotoxicity of allyl alcohol. By understanding factors involved in the enhancement of xenobiotic toxicity we may be better prepared to predict conditions that could cause possible enhancement of xenobiotic toxicity and take measures to

protect against such enhancements through inhibition of critical steps in the mechanism.

BIBLIOGRAPHY

BIBLIOGRAPHY

Adachi, Y., Bradford, B.U., Gao, W., Bojes, H.K., & Thurman, R.G. (1994). Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology*, **20**, 453-460.

Adams, J.D.J. & Klaidman, L.K. (1993). Acrolein-induced oxygen radical formation. *Free Radic.Biol Med*, **15**, 187-193.

Adding, L.C., Bannenberg, G.L., & Gustafsson, L.E. (2001). Basic experimental studies and clinical aspects of gadolinium salts and chelates. *Cardiovasc Drug Rev*, **19**, 41-56.

Akashi, S., Ogata, H., Kirikae, F., Kirikae, T., Kawasaki, K., Nishijima, M., Shimazu, R., Nagai, Y., Fukudome, K., Kimoto, M., & Miyake, K. (2000). Regulatory roles for CD14 and phosphatidylinositol in the signaling via toll-like receptor 4-MD-2. *Biochem Biophys Res Commun*, **268**, 172-177.

Altin, J.G. & Bygrave, F.L. (1988). Prostaglandin F_{2a} and the thromboxane A_2 analogue ONO-11113 stimulate Ca^{2+} fluxes and other physiological responses in rat liver. *Biochem.J.*, **249**, 677-685.

Antal-Szalmas, P. (2000). Evaluation of CD14 in host defence. *Eur J Clin Invest*, **30**, 167-179.

Antal-Szalmas, P., Strijp, J.A., Weersink, A.J., Verhoef, J., & Van Kessel, K.P. (1997). Quantitation of surface CD14 on human monocytes and neutrophils. *J Leukoc Biol*, **61**, 721-728.

Arditi, M., Zhou, J., Dorio, R., Rong, G.W., Goyert, S.M., & Kim, K.S. (1993). Endotoxin-mediated endothelial cell injury and activation: role of soluble CD14. *Infect Immun*, **61**, 3149-3156.

Arthur, M.J., Bentley, I.S., Tanner, A.R., Saunders, P.K., Millward-Sadler, G.H., & Wright, R. (1985). Oxygen-derived free radicals promote hepatic injury in the rat. *Gastroenterology*, **89**, 1114-1122.

Badger, D.A., Kuester, R.K., Sauer, J.M., & Sipes, I.G. (1997). Gadolinium chloride reduces cytochrome P450: relevance to chemical-induced hepatotoxicity. *Toxicology*, **121**, 143-153.

Badger, D.A., Sauer, J.M., Hoglen, N.C., Jolley, C.S., & Sipes, I.G. (1996). The role of inflammatory cells and cytochrome P450 in the potentiation of CCl4-induced liver injury by a single dose of retinol. *Toxicol Appl Pharmacol*, **141**, 507-519.

Badr, M.Z., Belinsky, S.A., Kauffman, F.C., & Thurman, R.G. (1986). Mechanism of hepatotoxicity to periportal regions of the liver lobule due to allyl alcohol: Role of oxygen and lipid peroxidation. *Journal of Pharmacology and Experimental Therapeutics*, **238**, 1138-1142.

Balsa, D., Merlos, M., Giral, M., Ferrando, R., Garcia-Rafanell, J., & Forn, J. (1997). Effect of endotoxin and platelet-activating factor on rat vascular permeability: role of vasoactive mediators. *J Lipid Mediat Cell Signal*, **17**, 31-45.

Bar-Shavit, R., Benezra, M., Sabbah, V., DeJana, E., Vlodavsky, I., & Wilner, G.D. (1992). functional domains in thrombin outside the catalytic site. In L.J. Berliner (Ed.), *thrombin: structure and function* (pp. 315New York: Plenum Press.

Barton, C.C., Barton, E.X., Ganey, P.E., Kunkel, S.L., & Roth, R.A. (2000). Bacterial lipopolysacchardie enhances aflatoxin B1 hepatotoxicity in rats by a mechanism that depends on tumor necrosis factor-alpha. *Hepatology*,

Barton, C.C., Ganey, P.E., & Roth, R.A. (2000). Lipopolysaccharide Augments Aflatoxin B(1)-Induced Liver Injury through Neutrophil-Dependent and -Independent Mechanisms. *Toxicol Sci*, **58**, 208-215.

Barton, C.C., Hill, D.A., Yee, S.B., Barton, E.X., Ganey, P.E., & Roth, R.A. (2000). Bacterial lipopolysaccharide exposure augments aflatoxin B(1)-induced liver injury. *Toxicol Sci*, **55**, 444-452.

Bautista, A.P., Meszaros, K., Bojta, J., & Spitzer, J.J. (1990). Superoxide anion generation in the liver during the early stage of endotoxemia in rats. *J Leukoc Biol*, **48**, 123-128.

Bautista, A.P. & Spitzer, J.J. (1999). Role of Kupffer cells in the ethanol-induced oxidative stress in the liver. *Front Biosci*, **4**, D589-D595

Bazil, V., Baudys, M., Hilgert, I., Stefanova, I., Low, M.G., Zbrozek, J., & Horejsi, V. (1989). Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. *Mol Immunol*, **26**, 657-662.

Beamer, L.J., Carroll, S.F., & Eisenberg, D. (1997). Crystal structure of human BPI and two bound phospholipids at 2.4 angstrom resolution. *Science*, **276**, 1861-1864.

Belinsky, S.A., Matsumura, T., Kauffman, F.C., & Thurman, R.G. (1983). Rates of allyl alcohol metabolism in periportal and pericentral regions of the liver lobule. *Molecular Pharmacology*, **25**, 158-164.

Beltran, A.E., Concepcion, F., Manzanares, D., Garrido, G., Glaria, L.A., & Rojas, A. (1999). Heparin and low molecular weight heparin decrease nitric oxide production by human polymorphonuclear cells. *Arch Med Res*, **30**, 116-119.

Beutler, B. (2000). Endotoxin, toll-like receptor 4, and the afferent limb of innate immunity. *Curr Opin Microbiol*, **3**, 23-28.

Billiar, T.R., Lysz, T.W., Curran, R.D., Bentz, B.G., Machiedo, G.W., & Simmons, R.L. (1990). Hepatocyte modulation of Kupffer cell prostaglandin E_2 production in vitro. *Journal of Leukocyte Biology*, **47**, 304-311.

Bizios, R., Lai, L., Fenton, J.W., II, & Malik, A.B. (1986). Thrombin-induced chemotaxis and aggregation of neutrophils. *Journal of Cellular Physiology.*, **128**, 485-490.

Bode, C., Kugler, V., & Bode, J.C. (1987). Endotoxemia in patients with alcoholic and non-alcoholic cirrhosis and in subjects with no evidence of chronic liver disease following acute alcohol excess. *J Hepatol*, **4**, 8-14.

Brightbill, H.D., Libraty, D.H., Krutzik, S.R., Yang, R.B., Belisle, J.T., Bleharski, J.R., Maitland, M., Norgard, M.V., Plevy, S.E., Smale, S.T., Brennan, P.J., Bloom, B.R., Godowski, P.J., & Modlin, R.L. (1999). Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science*, **285**, 732-736.

Brown, A.P., Harkema, J.R., Schultze, A.E., Roth, R.A., & Ganey, P.E. (1997). Gadolinium chloride pretreatment protects against hepatic injury but predisposes the lungs to alveolitis after lipopolysaccharide administration. *Shock*, **7**, 186-192.

Calvano, S.E., Thompson, W.A., Marra, M.N., Coyle, S.M., de Riesthal, H.F., Trousdale, R.K., Barie, P.S., Scott, R.W., Moldawer, L.L., & Lowry, S.F. (1994). Changes in polymorphonuclear leukocyte surface and plasma bactericidal/permeability-increasing protein and plasma lipopolysaccharide binding protein during endotoxemia or sepsis. *Arch Surg*, **129**, 220-226.

Camara, D.S., Caruana, J.A.J., Schwartz, K.A., Montes, M., & Nolan, J.P. (1983). D-Galactosamine liver injury: absorption of endotoxin and protective effect of small bowel and colon resection in rabbits. *Proc Soc Exp Biol Med*, **172**, 255-259.

Camara, D.S., Caruana, J.A., Jr., Schwartz, K.A., Montes, M., & Nolan, J.P. (1983). D-Galactosamine liver injury: absorption of endotoxin and protective effect of small bowel and colon resection in rabbits. *Proceedings of the Society for Experimental Biology and Medicine*, **172**, 255-259.

Camus, P., Joshi, U.M., Lockard, V.G., Petrini, M.F., Lentz, D.L., & Mehendale, H.M. (1989). Effects of drug-induced pulmonary phospholipidosis on lung mechanics in rats. *J Appl Physiol*, **66**, 2437-2445.

Canty, T.G.J., Boyle, E.M.J., Farr, A., Morgan, E.N., Verrier, E.D., & Pohlman, T.H. (1999). Oxidative stress induces NF-kappaB nuclear translocation without degradation of IkappaBalpha. *Circulation*, **100**, II361-II364

Cao, Z., Xiong, J., Takeuchi, M., Kurama, T., & Goeddel, D.V. (1996). TRAF6 is a signal transducer for interleukin-1. *Nature*, **383**, 443-446.

Carbonell, L.F., Nadal, J.A., Llanos, M.C., Hernandez, I., Nava, E., & Diaz, J. (2000). Depletion of liver glutathione potentiates the oxidative stress and decreases nitric oxide synthesis in a rat endotoxin shock model. *Crit Care Med*, **28**, 2002-2006.

Carney, D.H. (1992). postclotting cellular effects of thrombin mediated by interaction with high-affinity thrombin receptors. In L.J. Berliner (Ed.), *Thrombin: structure and function* (pp. 351New York: plenum Press.

Carpanini, F.M.B., Gaunt, I.F., Hardy, J., Gangolli, S.D., Butterworth, K.R., & Lloyd, A.G. (1978). Short-term toxicity of allyl alcohol in rats. *Toxicology*, **9**, 29-45.

Cebula, T.A., El-Hage, A.N., & Ferrans, V.J. (1984). Toxic interactions of benzyl alcohol with bacterial endotoxin. *Interaction and Immunity*, **44(1)**, 91-96.

Chan, K.L., Bizios, R., & Malik, A.B. (1988). Thrombin enhances opsonized zymosan-induced chemiluminescence of neutrophils. *Tissue Cell*, **20**, 13-17.

Chaudhary, P.M., Ferguson, C., Nguyen, V., Nguyen, O., Massa, H.F., Eby, M., Jasmin, A., Trask, B.J., Hood, L., & Nelson, P.S. (1998). Cloning and characterization of two Toll/Interleukin-1 receptor-like genes TIL3 and TIL4: evidence for a multi-gene receptor family in humans. *Blood*, **91**, 4020-4027.

Chi, L., Li, Y., Stehno-Bittel, L., Gao, J., Morrison, D.C., Stechschulte, D.J., & Dileepan, K.N. (2001). Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplified by

endotoxin and tumor necrosis factor-alpha. *J Interferon Cytokine Res*, **21**, 231-240.

Chow, J.C., Young, D.W., Golenbock, D.T., Christ, W.J., & Gusovsky, F. (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J Biol Chem*, **274**, 10689-10692.

Comporti, M., Maellaro, E., Del Bello, B., & Casini, A.F. (1991). Glutathione depletion: its effects on other antioxidant systems and hepatocellular damage. *Xenobiotica*, **21**, 1067-1076.

Coughlan, A.F., Hau, H., Dunlop, L.C., Berndt, M.C., & Hancock, W.W. (1994). P-selectin and platelet-activating factor mediate initial endotoxin-induced neutropenia. *J.Exp.Med.*, **179**, 329-334.

Couturier, C., Haeffner-Cavaillon, N., Caroff, M., & Kazatchkine, M.D. (1991). Binding sites for endotoxins (lipopolysaccharides) on human monocytes. *J Immunol*, **147**, 1899-1904.

Cunningham, M.D., Seachord, C., Ratcliffe, K., Bainbridge, B., Aruffo, A., & Darveau, R.P. (1996). Helicobacter pylori and Porphyromonas gingivalis lipopolysaccharides are poorly transferred to recombinant soluble CD14. *Infect Immun*, **64**, 3601-3608.

Cunningham, M.D., Shapiro, R.A., Seachord, C., Ratcliffe, K., Cassiano, L., & Darveau, R.P. (2000). CD14 employs hydrophilic regions to "capture" lipopolysaccharides. *J Immunol*, **164**, 3255-3263.

Czaja, M.J., Xu, J., Ju, Y., Alt, E., & Schmiedeberg, P. (1994). Lipopolysaccharide-neutralizing antibody reduces hepatocyte injury from acute hepatotoxin administration. *Hepatology*, **19**, 1282-1289.

Dahm, L.J., Schultze, A.E., & Roth, R.A. (1991). Activated neutrophils injure the isolated, perfused rat liver by an oxygen radical-dependent mechanism. *American Journal of Pathology*, **139(5)**, 1009-1020.

Darien, B.J., Fareed, J., Centgraf, K.S., Hart, A.P., MacWilliams, P.S., Clayton, M.K., Wolf, H., & Kruse-Elliott, K.T. (1998). Low molecular weight heparin prevents the pulmonary hemodynamic and pathomorphologic effects of endotoxin in a porcine acute lung injury model. *Shock*, **9**, 274-281.

Darveau, R.P. (1998). Lipid A diversity and the innate host response to bacterial infection. *Curr Opin Microbiol*, **1**, 36-42.

Daun, J.M. & Fenton, M.J. (2000). Interleukin-1/Toll receptor family members: receptor structure and signal transduction pathways. *J Interferon Cytokine Res*, **20**, 843-855.

Day, J.R., Albers, J.J., Lofton-Day, C.E., Gilbert, T.L., Ching, A.F., Grant, F.J., O'Hara, P.J., Marcovina, S.M., & Adolphson, J.L. (1994). Complete cDNA encoding human phospholipid transfer protein from human endothelial cells. *J Biol Chem*, **269**, 9388-9391.

de Jonge, E., Dekkers, P.E., Creasey, A.A., Hack, C.E., Paulson, S.K., Karim, A., Kesecioglu, J., Levi, M., van Deventer, S.J., & van Der, P. (2000). Tissue factor pathway inhibitor dose-dependently inhibits coagulation activation without influencing the fibrinolytic and cytokine response during human endotoxemia. *Blood*, **95**, 1124-1129.

Decker, K. (1997). Bacterial Endotoxin. In R.S. McCuskey & D.L. Earnest (Eds.), *Hepatic and Gastrointestinal Toxicology* (pp. 433New York: Elsevier Science Inc.

Deitch, E.A. (1994). Bacterial translocation: the influence of dietary variables. *Gut*, **35**, S23-S27

Detmers, P.A., Zhou, D., Powell, D., Lichenstein, H., Kelley, M., & Pironkova, R. (1995). Endotoxin receptors (CD14) are found with CD16 (Fc gamma RIII) in an intracellular compartment of neutrophils that contains alkaline phosphatase. *J Immunol*, **155**, 2085-2095.

Dogterom, P., Mulder, G.J., & Nagelkerke, J.F. (1989). Lipid peroxidation-dependent and -independent protein thiol modifications in

isolated rat hepatocytes: differential effects of vitamin E and disulfiram. *Chemico-Biological Interactions*, **71**, 291-306.

Dogterom, P., Nagelkerke, J.F., Van Steveninck, J., & Mulder, G.J. (1988). Inhibition of lipid peroxidation by disulfiram and diethyldithiocarbamate does not pervent hepatotoxin-induced cell death in isolated rat hepatocytes. a study with allyl alcohol, *tert*-butyl hydroperoxide, diethyl maleate, bromoisovalerylurea and carbon tetrachloride. *Chemico-Biological Interactions*, **66**, 251-265.

Downing, L.J., Strieter, R.M., Kadell, A.M., Wilke, C.A., Greenfield, L.J., & Wakefield, T.W. (1998). Low-dose low-molecular-weight heparin is anti-inflammatory during venous thrombosis. *J Vasc Surg*, **28**, 848-854.

ElSisi, A.E.D., Earnest, D.L., & Sipes, I.G. (1993a). Vitamin A potentiation of carbon tetrachloride hepatotoxicity: enhanced lipid peroxidation without enhanced biotransformation. *Toxicology and Applied Pharmacology*, **119**, 289-294.

ElSisi, A.E.D., Earnest, D.L., & Sipes, I.G. (1993b). Vitamin A potentiation of carbon tetrachloride hepatotoxicity: role of liver macrophages and active oxygen species. *Toxicology and Applied Pharmacology*, **119**, 295-301.

Engels, F., Renirie, B.F., Hart, B.A., Labadie, R.P., & Nijkamp, F.P. (1992). Effects of apocynin, a drug isolated from the roots of Picrorhiza kurroa, on arachidonic acid metabolism. *FEBS Lett*, **305**, 254-256.

Fariss, M.W. & Reed, D.J. (1987). High-performance liquid chromatography of thiols and disulfides: dinitrophenol derivatives. *Methods Enzymol.*, **143:101-9**, 101-109.

Feng, L., Xia, Y., Garcia, G.E., Hwang, D., & Wilson, C.B. (1995). Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-alpha, and lipopolysaccharide. *J Clin Invest*, **95**, 1669-1675.

Fenton, M.J. & Golenbock, D.T. (1998). LPS-binding proteins and receptors. *J Leukoc Biol*, **64**, 25-32.

Ferrero, E. & Goyert, S.M. (1988). Nucleotide sequence of the gene encoding the monocyte differentiation antigen, CD14. *Nucleic Acids Res*, **16**, 4173

Flo, T.H., Halaas, O., Lien, E., Ryan, L., Teti, G., Golenbock, D.T., Sundan, A., & Espevik, T. (2000). Human toll-like receptor 2 mediates monocyte activation by Listeria monocytogenes, but not by group B streptococci or lipopolysaccharide. *J Immunol*, **164**, 2064-2069.

Formal, S.B., Noyes, H.E., & Schneider, H. (1960). Experimental Shigella infections. III. Sensitivity of normal, starved and carbon tetrachloride treated guinea pigs to endotoxin. *Proceedings of the Society for Experimental Biology and Medicine*, **103**, 415-418.

Fox, E.S., Broitman, S.A., & Thomas, P. (1990). Bacterial endotoxins and the liver. *Lab.Invest.*, **63(6)** , 733-739.

Franco, R.F., de Jonge, E., Dekkers, P.E., Timmerman, J.J., Spek, C.A., van Deventer, S.J., van Deursen, P., van Kerkhoff, L., van Gemen, B., ten Cate, H., van der Poll, T., & Reitsma, P.H. (2000). The in vivo kinetics of tissue factor messenger RNA expression during human endotoxemia: relationship with activation of coagulation. *Blood*, **96**, 554-559.

Frey, E.A., Miller, D.S., Jahr, T.G., Sundan, A., Bazil, V., Espevik, T., Finlay, B.B., & Wright, S.D. (1992). Soluble CD14 participates in the response of cells to lipopolysaccharide. *J Exp Med*, **176**, 1665-1671.

Fujita, S., Aril, S., Monden, K., Adachi, Y., Funaki, N., Higashitsuji, H., Furutani, M., Mise, M., Ishiguro, S., & Kitao, T. (1995). Participation of hepatic macrophages and plasma factors in endotoxin-induced liver injury. *J.Surg.Res.*, **59**, 263-270.

Galanos, C., Freudenberg, M.A., & Reutter, W. (1979). Galactosamine-induced sensitization to the lethal effects of endotoxin. *Proc Natl Acad Sci U S A*, **76**, 5939-5943.

Gallay, P., Heumann, D., Le Roy, D., Barras, C., & Glauser, M.P. (1993). Lipopolysaccharide-binding protein as a major plasma protein responsible for endotoxemic shock. *Proc Natl Acad Sci U S A*, **90**, 9935-9938.

Gallay, P., Heumann, D., Le Roy, D., Barras, C., & Glauser, M.P. (1994). Mode of action of anti-lipopolysaccharide-binding protein antibodies for prevention of endotoxemic shock in mice. *Proc Natl Acad Sci U S A*, **91**, 7922-7926.

Ganey, P.E., Bailie, M.B., & Roth, R.A. (1993). Modulation of Kupffer cell function with gadolinium chloride does not alter methylene dianiline or allyl alcohol hepatotoxicity. *The Toxicologist*, **13**, 429(Abstract)

Ganey, P.E., Bailie, M.B., VanCise, S., Colligan, M.E., Madhukar, B.V., Robinson, J.P., & Roth, R.A. (1994). Activated neutrophils from rat injured isolated hepatocytes. *Lab.Invest.*, **70(1)**, 53-60.

Ganey, P.E., Barton, Y.W., Kinser, S., Sneed, R.A., Barton, C.C., & Roth, R.A. (2001). Involvement of cyclooxygenase-2 in the potentiation of allyl alcohol-induced liver injury by bacterial lipopolysaccharide. *Toxicol Appl Pharmacol*, **174**, 113-121.

Ganey, P.E. & Schultze, A.E. (1995). Depletion of neutrophils and modulation of Kupffer cell function in allyl alcohol-induced hepatotoxicity. *Toxicology*, **99**, 99-106.

Gay, N.J. & Keith, F.J. (1991). Drosophila Toll and IL-1 receptor. *Nature*, **351**, 355-356.

Golenbock, D.T., Liu, Y., Millham, F.H., Freeman, M.W., & Zoeller, R.A. (1993). Surface expression of human CD14 in Chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. *J Biol Chem*, **268**, 22055-22059.

Goyert, S.M., Ferrero, E.M., Seremetis, S.V., Winchester, R.J., Silver, J., & Mattison, A.C. (1986). Biochemistry and expression of myelomonocytic antigens. *J Immunol*, **137**, 3909-3914.

Gray, P.W., Flaggs, G., Leong, S.R., Gumina, R.J., Weiss, J., Ooi, C.E., & Elsbach, P. (1989). Cloning of the cDNA of a human neutrophil bactericidal protein. Structural and functional correlations. *J Biol Chem*, **264**, 9505-9509.

Grossman, N. & Leive, L. (1984). Complement activation via the alternative pathway by purified Salmonella lipopolysaccharide is affected by its structure but not its O-antigen length. *J Immunol*, **132**, 376-385.

Guigui, B., Rosenbaum, J., Preaux, A.-M., Martin, N., Zafrani, E.S., Dhumeaux, D., & Mavier, P. (1988). Toxicity of phorbol myristate acetate-stimulated polymorphonuclear neutrophils against rat hepatocytes. Demonstration and mechanism. *Lab.Invest.*, **59(6)**, 831-837.

Gumerlock, P.H., Edwards, B.F., Deitch, A.D., & Meyers, F.J. (1988). Human renal clear cell carcinoma: establishment and characterization of a new cell line (G-2101). *In Vitro Cellular and Developmental Biology*, **24(5)**, 429-434.

Haeffner-Cavaillon, N., Caroff, M., & Cavaillon, J.M. (1989). Interleukin-1 induction by lipopolysaccharides: structural requirements of the 3-deoxy-D-manno-2-octulosonic acid (KDO). *Mol Immunol*, **26**, 485-494.

Haeffner-Cavaillon, N., Carreno, M.P., Aussel, L., & Caroff, M. (1999). Molecular aspects of endotoxins relevant to their biological functions. *Nephrol Dial Transplant*, **14**, 853-860.

Hailman, E., Lichenstein, H.S., Wurfel, M.M., Miller, D.S., Johnson, D.A., Kelley, M., Busse, L.A., Zukowski, M.M., & Wright, S.D. (1994a). Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med*, **179**, 269-277.

Hailman, E., Lichenstein, H.S., Wurfel, M.M., Miller, D.S., Johnson, D.A., Kelley, M., Busse, L.A., Zukowski, M.M., & Wright, S.D. (1994b). Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med*, **179**, 269-277.

Harbrecht, B.G., Billiar, T.R., Curran, R.D., Stadler, J., & Simmons, R.L. (1993). Hepatocyte injury by activated neutrophils *in vitro* is mediated by proteases. *Annals of Surgery*, **218(2)**, 120-128.

Haziot, A., Chen, S., Ferrero, E., Low, M.G., Silber, R., & Goyert, S.M. (1988). The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J Immunol*, **141**, 547-552.

Haziot, A., Ferrero, E., Kontgen, F., Hijiya, N., Yamamoto, S., Silver, J., Stewart, C.L., & Goyert, S.M. (1996). Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity*, **4**, 407-414.

Haziot, A., Ferrero, E., Lin, X.Y., Stewart, C.L., & Goyert, S.M. (1995). CD14-deficient mice are exquisitely insensitive to the effects of LPS. *Prog Clin Biol Res*, **392**, 349-351.

Haziot, A., Rong, G.W., Silver, J., & Goyert, S.M. (1993). Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. *J Immunol*, **151**, 1500-1507.

Heemann, U., Szabo, A., Hamar, P., Muller, V., Witzke, O., Lutz, J., & Philipp, T. (2000). Lipopolysaccharide pretreatment protects from renal ischemia/reperfusion injury : possible connection to an interleukin-6-dependent pathway. *Am J Pathol*, **156**, 287-293.

Heine, H., Kirschning, C.J., Lien, E., Monks, B.G., Rothe, M., & Golenbock, D.T. (1999). Cutting edge: cells that carry A null allele for toll-like receptor 2 are capable of responding to endotoxin. *J Immunol*, **162**, 6971-6975.

Hewett, J.A. & Roth, R.A. (1988). Dieldrin activates rat neutrophils *in vitro*. *Toxicology and Applied Pharmacology*, **96**, 269-278.

Hewett, J.A. & Roth, R.A. (1993). Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol Rev*, **45**, 382-411.

Hewett, J.A. & Roth, R.A. (1995). The coagulation system, but not circulating fibrinogen, contributes to liver injury in rats exposed to lipopolysaccharide from gram-negative bacteria. *J Pharmacol Exp Ther*, **272**, 53-62.

Hewett, J.A., Schultze, A.E., VanCise, S., & Roth, R.A. (1992). Neutrophil depletion protects against liver injury from bacterial endotoxin. *Lab Invest*, **66**, 347-361.

Hisama, N., Yamaguchi, Y., Ishiko, T., Miyanari, N., Ichiguchi, O., Goto, M., Mori, K., Watanabe, K., Kawamura, K., Tsurufuji, S., & Ogawa, M. (1996). Kupffer cell production of cytokine-induced neutrophil chemoattractant following ischemia/reperfusion injury in rats. *Hepatology*, **24**, 1193-1198.

Hitchcock, P.J., Leive, L., Makela, P.H., Rietschel, E.T., Strittmatter, W., & Morrison, D.C. (1986). Lipopolysaccharide nomenclature--past, present, and future. *J Bacteriol*, **166**, 699-705.

Ho, J.S., Buchweitz, J.P., Roth, R.A., & Ganey, P.E. (1996). Identification of factors from rat neutrophils responsible for cytotoxicity to isolated hepatocytes. *J Leukoc Biol*, **59**, 716-724.

Hoffman, M. & Cooper, S.T. (1995). Thrombin enhances monocyte secretion of tumor necrosis factor and interleukin-1 beta by two distinct mechanisms. *Blood Cells Mol Dis*, **21**, 156-167.

Hoffmann, J.N., Vollmar, B., Inthorn, D., Schildberg, F.W., & Menger, M.D. (2000). The thrombin antagonist hirudin fails to inhibit endotoxin-induced leukocyte/endothelial cell interaction and microvascular perfusion failure. *Shock*, **14**, 528-534.

Hoglen, N.C., Younis, H.S., Hartley, D.P., Gunawardhana, L., Lantz, R.C., & Sipes, I.G. (1998). 1,2-Dichlorobenzene-induced lipid peroxidation in male Fischer 344 rats is Kupffer cell dependent. *Toxicol Sci* , **46**, 376-385.

Holland, J.A., Meyer, J.W., Chang, M.M., O'Donnell, R.W., Johnson, D.K., & Ziegler, L.M. (1998). Thrombin stimulated reactive oxygen species production in cultured human endothelial cells. *Endothelium*, **6**, 113-121.

Ihara, I., Harada, Y., Ihara, S., & Kawakami, M. (1982). A new complement-dependent bactericidal factor found in nonimmune mouse sera: specific binding to polysaccharide of Ra chemotype Salmonella. *J Immunol*, **128**, 1256-1260.

Iimuro, Y., Yamamoto, M., Kohno, H., Itakura, J., Fujii, H., & Matsumoto, Y. (1994). Blockade of liver macrophages by gadolinium chloride reduces lethality in endotoxemic rats-analysis of mechanisms of lethality in endotoxemia. *Journal of Leukocyte Biology*, **55**, 723-728.

Ikejima, K., Enomoto, N., Seabra, V., Ikejima, A., Brenner, D.A., & Thurman, R.G. (1999). Pronase destroys the lipopolysaccharide receptor CD14 on Kupffer cells. *Am J Physiol*, **276**, G591-G598

Ishiyama, H., Ogino, K., & Hobara, T. (1995). Role of Kupffer cells in rat liver injury induced by diethyldithiocarbamate. *Eur.J Pharmacol*, **292**, 135-141.

Jaeschke, H. (1997). Cellular adhesion molecules: regulation and functional significance in the pathogenesis of liver diseases. *Am J Physiol*, **273**, G602-G611

Jaeschke, H., Bautista, A.P., Spolarics, Z., & Spitzer, J.J. (1992). Superoxide generation by neutrophils and Kupffer cells during in vivo reperfusion after hepatic ischemia in rats. *Journal of Leukocyte Biology*, **52**, 377-382.

Jaeschke, H., Kleinwaechter, C., & Wendel, A. (1987). The role of acrolein in allyl alcohol-induced lipid peroxidation and liver cell damage in mice. *Biochem.Pharmacol.*, **36(1)**, 51-57.

Jann, K. & Jann, B. (1984). Structure and biosynthesis of O-antigens. *Chemistry of Endotoxin*, **1**, 138.

Jean, P.A., Bailie, M.B., & Roth, R.A. (1995). 1-Naphthylisothiocyanate-induced elevation of biliary glutathione. *Biochem.Pharmacol.*, **49**, 197-202.

Jirillo, E., De Simone, C., Covelli, V., Kiyono, H., McGhee, J.R., & Antonaci, S. (1990). LPS-mediated triggering of T lymphocytes in immune response against gram-negative bacteria. *Adv Exp Med Biol*, **256**, 417-425.

Juan, T.S., Kelley, M.J., Johnson, D.A., Busse, L.A., Hailman, E., Wright, S.D., & Lichenstein, H.S. (1995). Soluble CD14 truncated at amino acid 152 binds lipopolysaccharide (LPS) and enables cellular response to LPS. *J Biol Chem*, **270**, 1382-1387.

Kausalya, S., Malla, N., Ganguly, N.K., & Mahajan, R.C. (1993). Leishmania donovani: in vitro evidence of hepatocyte damage by Kupffer cells and immigrant macrophages in a murine model. *Exp Parasitol*, **77**, 326-333.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., & Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. *Immunity*, **11**, 115-122.

Kaye, C.M. (1973). Biosynthesis of mercapturic acids from allyl alcohol, allyl esters and acrolein. *Biochem.J.*, **134**, 1093-1101.

Kirn, A., Gut, J.P., & Gendrault, J.L. (1982). Interaction of viruses with sinusoidal cells. *Prog Liver Dis*, **7**, 377-392.

Kirschning, C.J., Wesche, H., Merrill, A.T., & Rothe, M. (1998). Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J Exp Med*, **188**, 2091-2097.

Kitchens, R.L. & Munford, R.S. (1995). Enzymatically deacylated lipopolysaccharide (LPS) can antagonize LPS at multiple sites in the LPS recognition pathway. *J Biol Chem*, **270**, 9904-9910.

Kitchens, R.L., Ulevitch, R.J., & Munford, R.S. (1992). Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J Exp Med*, **176**, 485-494.

Knook, D.L. & Sleyster, E.C. (1977). Preparation and characterization of kupffer cells from rat and mouse liver. pp. 273-88. *In: Wisse.E., Knook.DL., ed.Kupffer.cells and other liver sinusoidal.cells.Amsterdam., Elsevier./North-Holland.Biomedical.,* **:**

Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C.A., & Ghosh, S. (1999). ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. *Genes Dev*, **13**, 2059-2071.

Kruger, C., Schutt, C., Obertacke, U., Joka, T., Muller, F.E., Knoller, J., Koller, M., Konig, W., & Schonfeld, W. (1991). Serum CD14 levels in polytraumatized and severely burned patients. *Clin Exp Immunol* , **85**, 297-301.

Kulikov, V.I. & Muzya, G.I. (1998). The bioregulatory role of platelet-activating factor in intracellular processes and cell-cell interactions. *Biochemistry (Mosc)*, **63**, 47-54.

Lapperre, T.S., Jimenez, L.A., Antonicelli, F., Drost, E.M., Hiemstra, P.S., Stolk, J., MacNee, W., & Rahman, I. (1999). Apocynin increases glutathione synthesis and activates AP-1 in alveolar epithelial cells. *FEBS Lett*, **443**, 235-239.

Lee, J.D., Kato, K., Tobias, P.S., Kirkland, T.N., & Ulevitch, R.J. (1992). Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. *J Exp Med*, **175**, 1697-1705.

Lehman, J.D., Dyke, C., Abd-Elfattah, A., Yeh, T.J., Ding, M., Ezrin, A., & Wechsler, A.S. (1992). Polyethylene glycol-conjugated superoxide dismutase attenuates reperfusion injury when administered twenty-four hours before ischemia. *J Thorac Cardiovasc Surg*, **104**, 1597-1601.

Lehmann, V., Streck, H., Minner, I., Krammer, P.H., & Ruschmann, E. (1980). Selection of bacterial mutants from Salmonella specifically recognizing determinants on the cell surface of activated T lymphocytes. A novel system to define cell surface structures. *Eur J Immunol*, **10**, 685-693.

Lichtman, S.N., Wang, J., Zhang, C., & Lemasters, J.J. (1996). Endocytosis and Ca2+ are required for endotoxin-stimulated TNF-alpha release by rat Kupffer cells. *Am J Physiol*, **271**, G920-G928

Lind, R.C., Gandolfi, A.J., Sipes, I.G., & Brown, B.R. (1984). The involvement of endotoxin in halothane-associated liver injury. *Anesthesiology*, **61**, 544-550.

Lindberg, A. A., Wollin, R., Bruse, G., Ekwall, E., Svenson, S.B. (1983). Immunology and immunochemistry of synthetic and semisynthetic Salmonella O-antigen specific glycoconjugates, *ACS Symp. Ser.*, **231**, 83.

Liu, J., Sendelbach, L.E., Parkinson, A., & Klaassen, C.D. (2000). Endotoxin pretreatment protects against the hepatotoxicity of acetaminophen and carbon tetrachloride: role of cytochrome P450 suppression. *Toxicology*, **147**, 167-176.

Liu, P., McGuire, G.M., Fisher, M.A., Farhood, A., Smith, C.W., & Jaeschke, H. (1995). Activation of Kupffer cells and neutrophils for reactive oxygen formation is responsible for endotoxin-enhanced liver injury after hepatic ischemia. *Shock*, **3**, 56-62.

Liu, T.H., Beckman, J.S., Freeman, B.A., Hogan, E.L., & Hsu, C.Y. (1989). Polyethylene glycol-conjugated superoxide dismutase and catalase reduce ischemic brain injury. *Am J Physiol*, **256**, H589-H593

Lo, C.J., Cryer, H.G., Fu, M., & Kim, B. (1997). Endotoxin-induced macrophage gene expression depends on platelet-activating factor. *Arch Surg*, **132**, 1342-1347.

Lorant, D.E., Patel, K.D., McIntyre, T.M., McEver, R.P., Prescott, S.M., & Zimmerman, G.A. (1991). Coexpression of GMP-140 and PAF by

endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J Cell Biol*, **115**, 223-234.

Luderitz, O., Freudenberg, M. A., Galanose, C., Lehman, V., Rietschel, E. Th., & Shaw, D. H. (1982). Lipopolysaccarides of Gram-negative bacteria. *Microbial Membrane Lipids*, **17**, 79.

Luderitz, Th., Brandenburg, K., Seydel, U., Roth, A., Galanos, C., & Rietschel, E. Th. (1989). Sturctural and physicochemical requirements of endotoxins for the activation of arachidonic acid metabolism in mouse peritoneal macrophages in vitro. *Eur J Biochem*, **179**, 11.

Lynn, W.A., Raetz, C.R., Qureshi, N., & Golenbock, D.T. (1991). Lipopolysaccharide-induced stimulation of CD11b/CD18 expression on neutrophils. Evidence of specific receptor-based response and inhibition by lipid A-based antagonists. *J Immunol*, **147**, 3072-3079.

Lysz, T.W., Billiar, T.R., Curran, R.D., Simmons, R.L., & Machiedo, G.W. (1990). Kupffer cell-hepatocyte interactions and the changes in 1-^{14}C-arachidonate incorporation in response to endotoxin in vitro. *Prostaglandins*, **39(5)**, 497-514.

Makela, P.H., Hovi, M., Sazen, H., Muotiala, A., & Rhen, M. (1990). Role of LPS in the pathogenesis of salmonellosis. *Cellular and Molecular Aspects of Endotoxin Reactions*, **1**, 537.

Marchant, A., Duchow, J., Delville, J.P., & Goldman, M. (1992). Lipopolysaccharide induces up-regulation of CD14 molecule on monocytes in human whole blood. *Eur J Immunol*, **22**, 1663-1665.

Margaretten, W., McKay, D.G., & Phillips, L.L. (1967). The effect of heparin on endotoxin shock in the rat. *American Journal of Pathology*, **51**, 61-68.

Martin, T.R., Rubenfeld, G.D., Ruzinski, J.T., Goodman, R.B., Steinberg, K.P., Leturcq, D.J., Moriarty, A.M., Raghu, G., Baughman, R.P., & Hudson, L.D. (1997). Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory

response in patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med*, **155**, 937-944.

Matsumoto, M., Tamura, M., Matsubara, S., Matsuno, T., Ono, M., & Yokota, T. (1991). Mechanism of protective effect of recombinant human granulocyte colony-stimulating factor (rG-CSF) on Pseudomonas infection. *Microbiol Immunol*, **35**, 461-474.

Mavier, P., Preaux, A.-M., Guigui, B., Lescs, M.-C., Zafrani, E.S., & Dhumeaux, D. (1988). *In vitro* toxicity of polymorphonuclear neutrophils to rat hepatocytes: evidence for a proteinase-mediated mechanism. *Hepatology*, **8(2)**, 254-258.

Mawet, E., Shiratori, Y., Hikiba, Y., Takada, H., Yoshida, H., Okano, K., Komatsu, Y., Mastumura, M., Niwa, Y., & Omata, M. (1996). Cytokine-induced neutrophil chemoattractant release from hepatocytes is modulated by Kupffer cells. *Hepatology*, **23**, 353-358.

Means, T.K., Golenbock, D.T., & Fenton, M.J. (2000). Structure and function of Toll-like receptor proteins. *Life Sci*, **68**, 241-258.

Means, T.K., Golenbock, D.T., & Fenton, M.J. (2000). The biology of Toll-like receptors. *Cytokine Growth Factor Rev*, **11**, 219-232.

Medzhitov, R. & Janeway, C.J. (2000). The Toll receptor family and microbial recognition. *Trends Microbiol*, **8**, 452-456.

Medzhitov, R., Preston-Hurlburt, P., & Janeway, C.A.J. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, **388**, 394-397.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., & Janeway, C.A.J. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell*, **2**, 253-258.

Miccadei, S., Nakae, D., Kyle, M.E., Gilfor, D., & Farber, J.L. (1988). Oxidative cell injury in the killing of cultured hepatocytes by allyl alcohol. *Archives of Biochemistry and Biophysics*, **265(2)**, 302-310.

Michael, S.L., Pumford, N.R., Mayeux, P.R., Niesman, M.R., & Hinson, J.A. (1999). Pretreatment of mice with macrophage inactivators decreases acetaminophen hepatotoxicity and the formation of reactive oxygen and nitrogen species. *Hepatology*, **30**, 186-195.

Morgan, E.T. (2001). Regulation of cytochrome p450 by inflammatory mediators: why and how? *Drug Metab Dispos*, **29**, 207-212.

Moriya, S., Yokoyama, H., Fukuda, M., Okamura, Y., Kamegaya, Y., Mizukami, T., Ohgo, H., & Ishii, H. (2000). Glutathione depletion enhances the formation of superoxide anion released into hepatic sinusoids after lipopolysaccharide challenge. *Alcohol Clin Exp Res*, **24**, 59S-63S.

Morrison, D.C., Vukajlovich, S.W., Goodman, S.A., & Wollenweber, H.W. (1985). Regulation of lipopolysaccharide biologic activity by polysaccaride. *The Pathogenesis of Bacterial Infections (Bayer Symp. VIII)*, 68.

Morrison, D.C., Lei, M.G., Chen, T.Y., Flebbe, L.M., Halling, J., & Field, S. (1992). Identification and characterization of mammalian cell membrane receptors for LPS-endotoxin. *Adv Exp Med Biol*, **319**, 23-29.

Moulin, F., Pearson, J.M., Schultze, A.E., Scott, M.A., Schwartz, K.A., Davis, J.M., Ganey, P.E., & Roth, R.A. (1996). Thrombin is a distal mediator of lipopolysaccharide-induced liver injury in the rat. *J Surg Res*, **65**, 149-158.

Mulford, C.A. & Osborn, M.J. (1983). An intermediate step in translocation of lipopolysaccharide to the outer membrane of Salmonella typhimurium. *Proc Natl Acad Sci*, **80**, 1159.

Munford, R.S., Hall, C.L., Lipton, J.M., & Dietschy, J.M. (1982). Biological activity, lipoprotein-binding behavior, and in vivo disposition of

extracted and native forms of Salmonella typhimurium lipopolysaccharides. *J Clin Invest*, **70**, 877-888.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., & Mantovani, A. (1998). The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J Exp Med*, **187**, 2097-2101.

Myc, A., Buck, J., Gonin, J., Reynolds, B., Hammerling, U., & Emanuel, D. (1997). The level of lipopolysaccharide-binding protein is significantly increased in plasma in patients with the systemic inflammatory response syndrome. *Clin Diagn Lab Immunol*, **4**, 113-116.

Nikaido, H. & Vaara, M. (1987). Outer membrane. *Escherichia coli and salmonella typhimurium Cellular and Molecular Biology*, 7-22.

Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., & Matsumoto, K. (1999). The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature*, **398**, 252-256.

Nolan, J.P. & Leibowitz, A.I. (1978). Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by polymyxin b--an antiendotoxin. *Gastroenterology*, **75**, 445-449.

Osborn, M.J. (1979). Biosynthesis and assembly of lipopolysaccharides of the outer membrane. *Bacterial Outer Membranes, Biogenesis and Functions*, 15.

Palmer, K.R., Duerden, B.I., & Holdsworth, C.D. (1980). Bacteriological and endotoxin studies in cases of ulcerative colitis submitted to surgery. *Gut*, **21**, 851-854.

Patel, J.M., Wood, J.C., & Leibman, K.C. (1980). The biotransformation of allyl alcohol and acrolein in rat liver and lung perparations. *Drug Metabolism and Disposition*, **8(5)**, 305-308.

Paul, A., Wilson, S., Belham, C.M., Robinson, C.J., Scott, P.H., Gould, G.W., & Plevin, R. (1997). Stress-activated protein kinases: activation, regulation and function. *Cell Signal.*, **9**, 403-410.

Pearson, J.M., Schultze, A.E., Jean, P.A., & Roth, R.A. (1995). Platelet participation in liver injury from gram-negative bacterial lipopolysaccharide in the rat. *Shock*, **4**, 178-186.

Pearson, J.M., Schultze, A.E., Schwartz, K.A., Scott, M.A., Davis, J.M., & Roth, R.A. (1996). The thrombin inhibitor, hirudin, attenuates lipopolysaccharide-induced liver injury in the rat. *J Pharmacol Exp Ther*, **278**, 378-383.

Peavy, D.L. & Fairchild, E.J. (1987). Toxicologic interactions between ozone and bacterial endotoxin. *Environ Res*, **42**, 63-71.

Pedron, T., Girard, R., & Chaby, R. (1995). Variation of LPS-binding capacity, epitope expression, and shedding of membrane-bound CD14 during differentiation of human monocytes. *J Immunol*, **155**, 1460-1471.

Penttila, K.E. (1988). Allyl alcohol cytotoxicity and glutathione depletion in isolated periportal and perivenous rat hepatocytes. *Chemico-Biological Interactions*, **65**, 107-121.

Pereira, F.E., Motta, L., & Cardoso, A.A. (1997). Kupffer cell activation with BCG. Corynebacterium parvum or zymosan protects against acute liver injury induced by carbon tetrachloride in rats. *Arq Gastroenterol*, **34**, 157-162.

Pernerstorfer, T., Hollenstein, U., Hansen, J., Knechtelsdorfer, M., Stohlawetz, P., Graninger, W., Eichler, H.G., Speiser, W., & Jilma, B. (1999). Heparin blunts endotoxin-induced coagulation activation. *Circulation*, **100**, 2485-2490.

Piccardoni, P., Evangelista, V., Piccoli, A., De Gaetano, G., Walz, A., & Cerletti, C. (1996). Thrombin-activated human platelets release two NAP-2 variants that stimulate polymorphonuclear leukocytes. *Thromb Haemost*, **76**, 780-785.

Pinzani, M., Carloni, V., Marra, F., Riccardi, D., Laffi, G., & Gentilini, P. (1994). Biosynthesis of platelet-activating factor and its 1O-acyl analogue by liver fat-storing cells. *Gastroenterology*, **106**, 1301-1311.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Huffel, C.V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., & Beutler, B. (1998). Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, **282**, 2085-2088.

Pompella, A., Romani, A., Benedetti, A., & Comporti, M. (1991). Loss of membrane protein thiols and lipid peroxidation in allyl alcohol hepatotoxicity. *Biochem.Pharmacol.*, **41**, 1255-1259.

Prager, R.L., Dunn, E.L., Kirsh, M.M., & Penner, J.A. (1979). Endotoxin-induced intravascular coagulation (DIC) and its therapy. *Advances in Shock Research*, **2**, 277-287.

Przybocki, J.M., Reuhl, K.R., Thurman, R.G., & Kauffman, F.C. (1992). Involvement of nonparenchymal cells in oxygen-dependent hepatic injury by allyl alcohol. *Toxicology and Applied Pharmacology*, **115**, 57-63.

Qi, M. & Jones, S.B. (1990). Contribution of platelet activating factor to hemodynamic and sympathetic responses to bacterial endotoxin in conscious rats. *Circulatory Shock*, **32**, 153-163.

Raetz, C.R.H., Brozek, K.A., Clementz, T., Coleman, J.D., Galloway, S.M., Golenbock, D.T., & Hampton, R.Y. (1988). Gram-negative endotoxin: A biologically active lipid. *Cold Spring Harbor Symposia on Quantitative Biol.*, **LIII**, 973-982.

Rahman, M.M., Suzuki, M., Unno, M., Endo, K., Takeuchi, H., Kakita, T., & Matsuno, S. (1999). Hepatic phosphatidylcholine hydroperoxide content in noncirrhotic, cirrhotic, and antioxidant-treated rats with endotoxemia. *Surg Today*, **29**, 1047-1052.

Reid, W.D. (1972). Mechanism of allyl alcohol-induced hepatic necrosis. *Experientia*, **28**, 1058-1061.

Riesenberg, K., Schlaeffer, F., Katz, A., & Levy, R. (1995). Inhibition of superoxide production in human neutrophils by combinations of heparin and thrombolytic agents. *Br Heart J*, **73**, 14-19.

Rietschel, E.T., Kirikae, T., Schade, F.U., Mamat, U., Schmidt, G., Loppnow, H., Ulmar, A.J., Zahringer, U., Seydel, U., di Padova, F., Schreier, M., & Brade, H. (1994). Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB Journal*, **8**, 217-225.

Rikans, L.E. (1987). The oxidation of acrolein by rat liver aldehyde dehydrogenases. Relation to allyl alcohol hepatotoxicity. *Drug Metabolism and Disposition*, **15**, 356-362.

Rikans, L.E. & Cai, Y. (1994). Dithiothreitol reversal of allyl alcohol cytotoxicity in isolated rat hepatocytes. *Toxicology*, **86**, 147-161.

Rikans, L.E., Cai, Y., & Hornbrook, K.R. (1995). Allyl alcohol cytotoxicity in isolated rat hepatocytes: effects of azide, fasting and fructose. *J.Tox.Env.Health*, **44**, 1-11.

Ritchie, H., Jamieson, A., & Booth, N.A. (1995). Thrombin modulates synthesis of plasminogen activator inhibitor type 2 by human peripheral blood monocytes. *Blood*, **86**, 3428-3435.

Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., & Bazan, J.F. (1998). A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A*, **95**, 588-593.

Roth, R.A., Harkema, J.R., Pestka, J.P., & Ganey, P.E. (1997). Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? *Toxicol Appl Pharmacol*, **147**, 300-311.

Ruetten, H. & Thiemermann, C. (1997). Effects of tyrphostins and genistein on the circulatory failure and organ dysfunction caused by endotoxin in the rat: a possible role for protein tyrosine kinase. *Br J Pharmacol*, **122**, 59-70.

Ruttinger, D., Vollmar, B., Wanner, G.A., & Messmer, K. (1996). In vivo assessment of hepatic alterations following gadolinium chloride-induced Kupffer cell blockade. *J Hepatol*, **25**, 960-967.

Rylander, R. & Nordstrand, A. (1974). Pulmonary cell reactions after exposure to cotton dust extract. *British Journal of Industrial Medicine*, **31**, 220-223.

Salmon, M., Koto, H., Lynch, O.T., Haddad, E.B., Lamb, N.J., Quinlan, G.J., Barnes, P.J., & Chung, K.F. (1998). Proliferation of airway epithelium after ozone exposure: effect of apocynin and dexamethasone. *Am J Respir Crit Care Med*, **157**, 970-977.

Sarphie, T.G., D'Souza, N.B., & Deaciuc, I.V. (1996). Kupffer cell inactivation prevents lippolysaccharide-induced structural changes in the rat liver sinusoid: and electron-microscopic study. *Hepatology*, **23**, 788-796.

Sarphie, T.G., D'Souza, N.B., Spitzer, J.J., & Deaciuc, I.V. (1996). Chronic alcohol feeding in liquid diet or in drinking water has similar effects on electron microscopic appearance of the hepatic sinusoid in the rat. *Alcohol Clin Exp Res*, **20**, 973-979.

Sasse, D. & Maly, I.P. (1991). Studies on the periportal hepatotoxicity of allyl alcohol. *Progress in Histo- and Cytochemistry* , **23**, 146-149.

Sato, T., Shinzawa, H., Abe, Y., Takahashi, T., & Arai, S. (1993). Inhbition of *corynebacterium parvum*-primed and lipopolysaccharide-induced hepatic necrosis in rats by selective depletion of neutrophils using a monoclonal antibody. *Journal of Leukocyte Biology*, **53**, 144-150.

Schreck, R., Rieber, P., & Baeuerle, P.A. (1991). Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J*, **10**, 2247-2258.

Schwandner, R., Dziarski, R., Wesche, H., Rothe, M., & Kirschning, C.J. (1999). Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem*, **274**, 17406-17409.

Seglen, P.O. (1973). Preparation of rat liver cells. 3. Enzymatic requirements for tissue dispersion. *Exp Cell Res*, **82**, 391-398.

Serafini-Cessi, F. (1972). Conversion of allyl alcohol into acrolein by rat liver. *Biochem.J.*, **128**, 1103-1107.

Shapiro, R.A., Cunningham, M.D., Ratcliffe, K., Seachord, C., Blake, J., Bajorath, J., Aruffo, A., & Darveau, R.P. (1997). Identification of CD14 residues involved in specific lipopolysaccharide recognition. *Infect Immun*, **65**, 293-297.

Shibayama, Y. (1987). Sinusoidal circulatory disturbance by microthrombosis as a cause of endotoxin-induced hepatic injury. *Journal of Pathology*, **151**, 315-321.

Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., & Kimoto, M. (1999). MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J Exp Med*, **189**, 1777-1782.

Shin, C.S., Han, J.U., Kim, J.L., Schenarts, P.J., Traber, L.D., Hawkins, H., & Traber, D.L. (1997). Heparin attenuated neutrophil infiltration but did not affect renal injury induced by ischemia reperfusion. *Yonsei Med J*, **38**, 133-141.

Silva, J.M. & O'Brien, P.J. (1989). Allyl alcohol-and acrolein-induced toxicity in isolated rat hepatocytes. *Archives of Biochemistry and Biophysics*, **275(2)**, 551-558.

Sneed, R.A., Buchweitz, J.P., Jean, P., & Ganey, P.E. (2000). Pentoxifylline attenuates bacterial lipopolysaccharide-induced enhancement of allyl alcohol hepatotoxicity. *Toxicol Sci*, **56**, 203-210.

Sneed, R.A., Grimes, S.D., & Ganey, P.E. (1995). Lipopolysaccharide enhances the hepatotoxicity of allyl alcohol in sprague-dawley rats. *Fundamental and Applied Toxicology*, **15**, 219(Abstract)

Sneed, R.A., Grimes, S.D., Schultze, A.E., Brown, A.P., & Ganey, P.E. (1997). Bacterial endotoxin enhances the hepatotoxicty of allyl alcohol. *Toxicology and Applied Pharmacology*, **144**, 77-87.

Spitzer, J.A. & Mayer, M.S. (1993). Hepatic neutrophil influx: eicosanoid and superoxide formation in endotoxemia. *J.Surg.Res.* , **55**, 60-67.

Stachlewitz, R.F., Seabra, V., Bradford, B., Bradham, C.A., Rusyn, I., Germolec, D., & Thurman, R.G. (1999). Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. *Hepatology*, **29**, 737-745.

Steinemann, S., Ulevitch, R.J., & Mackman, N. (1994). Role of the lipopolysaccharide (LPS)-binding protein/CD14 pathway in LPS induction of tissue factor expression in monocytic cells. *Arterioscler Thromb*, **14**, 1202-1209.

Stolk, J., Hiltermann, T.J., Dijkman, J.H., & Verhoeven, A.J. (1994). Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol*, **11**, 95-102.

Sugatani, J., Igarashi, T., Munakata, M., Komiyama, Y., Takahashi, H., Komiyama, N., Maeda, T., Takeda, T., & Miwa, M. (2000). Activation of coagulation in C57BL/6 mice given verotoxin 2 (VT2) and the effect of co-administration of LPS with VT2. *Thromb Res*, **100**, 61-72.

Supinski, G., Stofan, D., Nethery, D., Szweda, L., & DiMarco, A. (1999). Apocynin improves diaphragmatic function after endotoxin administration. *J Appl Physiol*, **87**, 776-782.

Suzuki, Y., Tanigaki, T., Heimer, D., Wang, W.Z., Ross, W.G., Sussman, H.H., & Raffin, T.A. (1992). Polyethylene glycol-conjugated superoxide dismutase attenuates septic lung injury in guinea pigs. *Am Rev Respir Dis*, **145**, 388-393.

Takada, H. &Kotani, S. (1992). Structure function relations of lipid A. *Bacterial Endotoxic Lipopolysaccharides, Molecular Biochemistry and Cellular Biology*, 107-130.

Tamura, Y., Chi, L.G., Driscoll, E.M.J., Hoff, P.T., Freeman, B.A., Gallagher, K.P., & Lucchesi, B.R. (1988). Superoxide dismutase conjugated to polyethylene glycol provides sustained protection against myocardial ischemia/reperfusion injury in canine heart. *Circ Res*, **63**, 944-959.

Terashita, Z.-I., Kawamura, M., Takatani, M., Tsushima, S., Imura, Y., & Nishikawa, K. (1992). Beneficial effects of TCV-309, a novel potent and selective platelet activating factor antagonist in endotoxin and anaphylactic shock in rodents. *J.of Pharmacology and Experimental Theraputics*, 748-755.

Thomson, P.D., Till, G.O., Woolliscroft, J.O., Smith, D.J., & Prasad, J.K. (1990). Superoxide dismutase prevents lipid peroxidation in burned patients. *Burns*, **16**, 406-408.

Thurman, R.G. (1998). II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. *Am J Physiol*, **275**, G605-G611

Tobias, P.S., Mathison, J.C., & Ulevitch, R.J. (1988). A family of lipopolysaccharide binding proteins involved in responses to gram-negative sepsis. *J Biol Chem*, **263**, 13479-13481.

Tobias, P.S., Soldau, K., Iovine, N.M., Elsbach, P., & Weiss, J. (1997). Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. *J Biol Chem*, **272**, 18682-18685.

Tobias, P.S., Tapping, R.I., & Gegner, J.A. (1999). Endotoxin interactions with lipopolysaccharide-responsive cells. *Clin Infect Dis*, **28**, 476-481.

Todoroki, H., Nakamura, S., Higure, A., Okamoto, K., Takeda, S., Nagata, N., Itoh, H., & Ohsato, K. (2000). Neutrophils express tissue factor in a monkey model of sepsis. *Surgery*, **127**, 209-216.

Troelstra, A., Giepmans, B.N., Van Kessel, K.P., Lichenstein, H.S., Verhoef, J., & Van Strijp, J.A. (1997). Dual effects of soluble CD14 on LPS priming of neutrophils. *J Leukoc Biol*, **61**, 173-178.

Ulevitch, R.J. (2000). Molecular mechanisms of innate immunity. *Immunol Res*, **21**, 49-54.

Ulevitch, R.J. & Tobias, P.S. (1999). Recognition of gram-negative bacteria and endotoxin by the innate immune system. *Curr Opin Immunol*, **11**, 19-22.

Underhill, D.M., Ozinsky, A., Hajjar, A.M., Stevens, A., Wilson, C.B., Bassetti, M., & Aderem, A. (1999). The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature*, **401**, 811-815.

Vaara, M. & Nikaido, H. (1984). Molecular organization of bacterial outer membrane. *Chemistry of Endotoxin*, 1.

Vergnolle, N., Wallace, J.L., Bunnett, N.W., & Hollenberg, M.D. (2001). Protease-activated receptors in inflammation, neuronal signaling and pain. *Trends Pharmacol Sci*, **22**, 146-152.

Villa, P., Sartor, G., Angelini, M., Sironi, M., Conni, M., Gnocchi, P., Isetta, A.M., Grau, G., Buurman, W., & van Tits, L.J. (1995). Pattern of cytokines and pharmacomodulation in sepsis induced by cecal ligation and puncture compared with that induced by endotoxin. *Clin Diagn.Lab Immunol*, **2**, 549-553.

Viriyakosol, S. & Kirkland, T.N. (1996). The N-terminal half of membrane CD14 is a functional cellular lipopolysaccharide receptor. *Infect Immun*, **64**, 653-656.

Vollmar, B., Ruttinger, D., Wanner, G.A., Leiderer, R., & Menger, M.D. (1996). Modulation of kupffer cell activity by gadolinium chloride in endotoxemic rats. *Shock*, **6**, 434-441.

Wang, W., Suzuki, Y., Tanigaki, T., Rank, D.R., & Raffin, T.A. (1994). Effect of the NADPH oxidase inhibitor apocynin on septic lung injury in guinea pigs. *Am J Respir Crit Care Med*, **150**, 1449-1452.

Wang, Y. & Hollingsworth, R.I. (1996). An NMR spectroscopy and molecular mechanics study of the molecular basis for the supramolecular structure of lipopolysaccharides. *Biochemistry*, **35**, 5647-5654.

Wesche, H., Henzel, W.J., Shillinglaw, W., Li, S., & Cao, Z. (1997). MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*, **7**, 837-847.

Wolpert, J.S. & Albersheim, P. (1976). Host-symbiont interactions. I. The lectins of legumes interact with the o-antigen-containing lipopolysaccharides of their symbiont Rhizobia. *Biochem Biophys Res Commun* , **70**, 729-737.

Woodman, R.C., Teoh, D., Payne, D., & Kubes, P. (2000). Thrombin and leukocyte recruitment in endotoxemia. *Am J Physiol Heart Circ Physiol*, **279**, H1338-H1345

Wright, S.D., Ramos, R.A., Hermanowski-Vosatka, A., Rockwell, P., & Detmers, P.A. (1991a). Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. *J Exp Med*, **173**, 1281-1286.

Wright, S.D., Ramos, R.A., Hermanowski-Vosatka, A., Rockwell, P., & Detmers, P.A. (1991b). Activation of the adhesive capacity of CR3 on neutrophils by endotoxin: dependence on lipopolysaccharide binding protein and CD14. *J Exp Med*, **173**, 1281-1286.

Wright, S.D., Ramos, R.A., Tobias, P.S., Ulevitch, R.J., & Mathison, J.C. (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science*, **249**, 1431-1433.

Wueweera, J.B., Gandolfi, A.J., Badger, D.A., Sipes, I.G., & Brendel, K. (1996). Vitamin A potentiation of vinylidene chloride hepatotoxicity in rats and precision-cut rat liver slices. *Fundam Appl Toxicol*, **34**, 73-83.

Wurfel, M.M., Kunitake, S.T., Lichenstein, H., Kane, J.P., & Wright, S.D. (1994). Lipopolysaccharide (LPS)-binding protein is carried on lipoproteins and acts as a cofactor in the neutralization of LPS. *J Exp Med*, **180**, 1025-1035.

Wurfel, M.M., Monks, B.G., Ingalls, R.R., Dedrick, R.L., Delude, R., Zhou, D., Lamping, N., Schumann, R.R., Thieringer, R., Fenton, M.J., Wright, S.D., & Golenbock, D. (1997). Targeted deletion of the lipopolysaccharide (LPS)-binding protein gene leads to profound suppression of LPS responses ex vivo, whereas in vivo responses remain intact. *J Exp Med*, **186**, 2051-2056.

Yamanaka, H., Nukina, S., Handler, J.A., Currin, R.T., Lemasters, J.J., & Thurman, R.G. (1992). Transient activation of hepatic glycogenolysis by thrombin in perfused rat livers. *Eur.J Biochem*, **208**, 753-759.

Yang, R.B., Mark, M.R., Gray, A., Huang, A., Xie, M.H., Zhang, M., Goddard, A., Wood, W.I., Gurney, A.L., & Godowski, P.J. (1998). Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature*, **395**, 284-288.

Yee, S.B., Kinser, S., Hill, D.A., Barton, C.C., Hotchkiss, J.A., Harkema, J.R., Ganey, P.E., & Roth, R.A. (2000). Synergistic Hepatotoxicity from Coexposure to Bacterial Endotoxin and the Pyrrolizidine Alkaloid Monocrotaline. *Toxicology and Applied Pharmacology*, **166**, 173-185.

Zhang, P., Xie, M., Zagorski, J., & Spitzer, J.A. (1995). Attenuation of hepatic neutrophil sequestration by anti-CINC antibody in endotoxic rats. *Shock*, **4**, 262-268.

Zimmerman, B.J., Paulson, J.C., Arrhenius, T.S., Gaeta, F.C.A., & Granger, D.N. (1994). Thrombin receptor peptide-mediated leukocyte rolling in rat mesenteric venules: roles of P-selectin and sialyl Lewis X. *Am.J.Physiol.*, **267**, H1049-H1053