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Neutrophils and Idiosyncratic Advese Drug Reactions Resulting from Inflammation-Drug Interaction: Ranitidine and Diclofenac as Examples

By

Xiaomin Deng

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ABSTRACT

NEUTROPHILS AND IDIOSYNRATIC ADVERSE DRUG REACTIONS RESULTING FROM INFLAMMATION-DRUG INTERACTION

By

Xiaomin Deng

Idiosyncratic adverse drug reactions (IADRs) occur in a small fraction of people taking a drug. The liver is a frequent target. For the vast majority of drugs associated with these reactions, including the histamine2 (H2) receptor antagonist ranitidine (RAN) and the nonsteroidal anti-inflammatory drug diclofenac (DCLF), the mechanism of toxicity is unknown. Inflammatory stress might be a susceptibility factor for IADRs, which was supported by the observation that mild inflammation renders nontoxic doses of RAN and DCLF injurious to liver in rats. This dissertation tested the hypothesis that neutrophils (PMNs) are activated in the livers of rats cotreated with LPS and an IADR-producing drug (RAN, DCLF), and these cells contribute to liver injury by enhancing hemostasis and/or releasing neutrophil proteases. In the LPS/RAN model, LPS induces the accumulation of PMNs in the liver, and RAN causes the transmigration and the activation of these PMNs. Activated PMNs release toxic proteases that kill hepatocytes or enhance fibrin deposition in liver, both of which could lead to hepatocellular injury caused by LPS/RAN treatment. PMNs enhance the fibrin deposition through plasminogen activator inhibitor-1 (PAI-1), the negative regulator of the fibrinolytic system. A PAI-1 inhibitor reduced the hepatic fibrin deposition and hepatocellular injury caused by LPS/RAN

cotreatment. The PAI-1 inhibitor also reduced PMN activation independently of neutrophil chemokines, suggesting a direct role for PAI-1 in activating PMNs. The enhanced PAI-1 production by RAN treatment after LPS exposure occured through tumor necrosis factor-alpha (TNFα). RAN augmented TNFα production after LPS treatment, and the prolongation of LPS-induced TNFα production by RAN is crucial for the liver injury caused by LPS/RAN cotreatment, since a TNFα-converting enzyme (TACE) inhibitor, given immediately before RAN, reduced both serum TNFα protein and the hepatocellular injury. The augmentation of TNF-α by RAN occured in a post-transcriptional manner by enhanced p38-dependent TACE activation. In addition to the RAN model, PMNs are also important in the liver injury caused by LPS/DCLF cotreatment. These studies could aid in understanding mechanisms by which inflammation acts as a susceptibility factor for IADRs and biomarker identification for IADRs resulting from inflammation-drug interaction.

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Of all the sections in this dissertation, this is perhaps the most difficult to write. A very large number of people from Michigan State University, from the Department of Biochemistry and Molecular Biology, the Department of Pharmacology and Toxicology and the Center for Integrative Toxicology deserve tremendous thanks. In addition, my interactions with other collaborators and colleagues from Abbott Biosrearch Center, Abbott Laboratory, Bristol-Myers Squibb, Wyeth, University of Gratz and Society of Toxicology have dramatically increased my excitement for toxicology. To paraphrase these thanks would be insufficient.

Let's start with the MSU folks. First and foremost I would like to thank my mentor, Dr. Robert Roth. Deciding to join his laboratory was perhaps the most influential and best decision I have made. I hope I can reflect Bob's enthusiasm and perseverance in toxicological research as I move onward in my career. He not only provided brilliant scientific ideas in my thesis research but also gave advice on the mental development of being a great scientist. Dr. Patricia Ganey also deserves my thanks. Patti played a very significant role in my graduate career as another mentor in the lab. She even managed to teach me lab techniques once after being a faculty for so many years. I perhaps own a big thanks to Dr. James Lueyndyk. Jim is always ready to answer whenver I went to him for questions and suprisingly he always knows the answers. I would like to thank Jim for the significant amount of time he put in to training me in the lab as well as the teamwork on this dissertation work especially in my early graduate study. Another big thank I shall give to is Dr. Jane Maddox. She is my first mentor in lab during my rotation and it is my

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I have had the pleasure of working with so many other incredible people in the laboratory during my time at MSU and all of them deserve my thanks. Several post-docs in the lab have helped me at times including Dr. Francis Tukov, Dr. Sachin Devi, and Dr. Jesus Olivero-Verbel. In addition to these guys, I would like to thank all of the laboratory technicians, graduate students, and undergraduates I have had the opportunity to work with including Sandy Newport, Steve Bezdecny, Colin North, Pat Shaw, Rohan Pradhan, Theresa Eagle, Wei Zou, Erica Sparkenbaugh, Jingtao Lu, Aaron Fullerton. In addition to folks in the lab, I would also like to the administrative staff of the Center for Integrative Toxicology, Department of Biochemistry and Molecular Biology and Department of Pharmacology and Toxicology.

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
ALT	alanine aminotransferase
ALP	alkaline phosphatase
AST	aspartate aminotransferase
COX-2	cyclooxygenase-2
CINC-1	cytokine-induced chemoattractant-1
CS	control serum
DCLF	diclofenac
EU	endotoxin unit
ELISA	enzyme-linked immunosorbent assay
FAM	famotidine
fMLP	f-Met-Leu-Phe
Gal	galactosamine
GGT	gamma-glutamyl transferase
GSH	glutathione
H2	histamine2-
HA	hyaluronic acid
HIF-1α	hypoxia inducible factor-1α
HPC	hepatic parenchymal cell
HPF	high power field
HOCL	hypochlorous acid
IADR	idiosyncratic adverse drug reaction
ICAM-1	intercellular adhesion molecule-1
IL-1	interleukin 1
Iv	intravenous
Ip	intraperitoneal
JNK	c-Jun N-terminal kinase
KC	Kupffer cell
LAL	Limulus amoebocyte lysate
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MIP-2	macrophage inflammatory protein 2
MIP-1α	macrophage inflammatory protein-1α
MK-2	MAPK-activated protein kinase 2
NAS	rabbit anti-rat PMN antiserum
NF-κB	nuclear factor kappaB
CS	control serum
NSAID	nonsteroidal anti-inflammatory drugs
PIM	pimonidazole
PAI-1	plasminogen activator inhibitor-1
PAR	protease activated receptor
PMN	neutrophil

RAN	ranitidine
RECA-1	rat endothelial cell antigen-1
SEC	sinusoidal endothelial cell
TAT	thrombin-antithrombin dimmer
TF	tissue factor
TGZ	troglitazone
TLR4	toll-like receptor 4
TNF	tumor necrosis factor-alpha
TVX	trovafloxacin

CHAPTER ONE

Introduction

1.1 Drug-induced idiosyncratic hepatotoxicity

1.1.1 Overview of drug-induced idiosyncratic hepatotoxicity

Adverse drug reactions (ADRs) remain a challenging human health problem. Although continued efforts are put forward to understanding and prevent these reactions, ADRs still contribute significantly to hospitalization and mortality throughout the world. Furthermore, due to under-reporting, the real incidence might be even higher than reported by current methods (Bagheri et al., 2000;Sgro et al., 2002). An immediate adverse outcome is the withdrawal or limited usage of certain efficacious drugs, leading to deficits in successful treatment of diseases. One example is the anti-epileptic drug felbamate (Pellock, 1999;Dieckhaus et al., 2002), an effective drug in treating severe cases of epilepsy. Unfortunately, its usage was markedly restricted due to the aplastic anemia and hepatotoxicity it caused in patients. In addition, ADRs cause a significant loss of investment, commited effort and future profits and a possible financial loss from lawsuits from the pharmaceutical industry view point.

A variety of drugs with different pharmacological targets are associated with ADRs. One of the common target organs is the liver. Drug-induced liver injury is associated with histopathologic and clinical features of acute hepatocellular necrosis, biliary injury, or a combination of the two (Zimmerman, 1993;Zimmerman, 2000). The main culprits are anti-infectious, central nervous system, musculoskeletal, and gastrointestinal drugs (Andrade et al., 2006;Andrade et al., 2005).

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Adverse drug reactions can be divided into predictable, dose-related reactions (Type A reaction) and idiosyncratic reactions (Type B reaction). Type A reactions are dose-dependent and occur in a relatively defined time frame, and all individuals are suseceptible. A typical example is acetaminophen-induced hepatotoxicity in people (Amar and Schiff, 2007;Larson et al., 2005). In contrast, idiosyncratic adverse drug reactions (IADRs) typically occur only in a small fraction of the patients taking a drug. These reactions are usually unpredictable, not apparently dose-dependent and display a variable onset time after the beginning of drug therapy (Kaplowitz, 2005c;Uetrecht, 2007;Waring and Anderson, 2005). Moreover, the toxicity resulting from most IADRs is not related to the pharmacological target. All of those features of IADRs make these reactions more insidious, more difficult to understand and, unfortunately, more difficult to predict from current preclinical studies or clinical trials.

A well known example of drug-induced idiosyncratic hepatotoxicity is troglitazone-induced liver injury. Troglitazone, which was marketed in 1997, is one of the many thiazolidinediones for treatment of type 2 diabetes. It acts as a peroxisome proliferator-activated receptor-gamma (PPARγ) agonist and improves insulin resistance. However, clinical trials indicated that 1.9% of patients taking the drug experienced elevated serum alanine aminotransferase more that 3 times the upper limit of normal, revealing significant frequency of mild and moderate hepatotoxicity (Watkins and Whitcomb, 1998). Furthermore, it was reported that rare but severe liver injury also occurred, and this serious idiosyncratic hepatotoxicity from troglitazone led to its

withdrawal from the market in 2000 (Graham et al., 2003). Liver biopsy from affected patients revealed predominately hepatic necrosis with occasional bridging fibrosis. Acute and chronic inflammatory infiltrates were cited most often, with rare prominence of eosinophils. The time frame of hepatotoxicity occcurance varied from within a month to several years after initiation of maintenance therapy. IADRs induced by troglitazone are most likely independent of its pharmacological target, since other thiazolidinediones acting as PPARγ agonists, such as rosiglitazone and pioglitazone, raise less concern of hepatotoxicity for diabetic patients (Scheen, 2001a;Scheen, 2001b).

Better prediction of idiosyncratic liver injury will require a better understanding of the mechanisms. It is noted that "no one model fits the characteristics of all idiosyncratic drug reactions" (Seguin and Uetrecht, 2003a). Drug properties, genetic background and environmental factors could all contribute to idiosyncratic liver injury (Boelsterli, 2003a;Kaplowitz, 2001). Two conventional hypotheses have arisen over the years to explain the mechanisms of idiosyncratic drug reactions. One of them is that the reactions are based on drug metabolism polymorphisms among patients, which result in different levels of toxic drug metabolites (Williams and Park, 2003b). The other one argues that the reactions arise from an adaptive immune response to proteins bound to the drug or its metabolites (Park et al., 2001;Ju and Uetrecht, 2002). An extension of this hypothesis of adaptive immune response is the "danger hypothesis" (Seguin and Uetrecht, 2003b;Pirmohamed et al., 2002b). This hypothesis suggests that a second "danger signal," in addition to the presense of antibodies, is necessary to mount a specific immune

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response and the consequent hepatotoxicity. This signal might be any number of factors including some form of cellular stress, underlying disease or other environmental factors. The following sections will use troglitazone, a well known drug associated with IADRs, as an example to review the progress, knowledge gaps and alternative thinking in understanding the mechanisms of idiosyncratic hepatotoxicity.

1.1.2 Example: Troglitazone

Since its withdrawal in 2000, a large amount of effort has been made to elucidate the mechanism of troglitazone (TGZ)-induced hepatotoxicity. A variety of hypotheses were proposed to explain TGZ-induced cell injury including reactive metabolites formation and accumulation, mitochondrial dysfunction and oxidant stress, inhibition of bile salt transporter, and apoptosis (Masubuchi, 2006;Chojkier, 2005b). For example, the major TGZ metabolites (sulfate, glucuronide, quinone) have been identified in both cultured cells, experimental animals and human patients (Loi et al., 1999;Kawai et al., 1997;Yoshigae et al., 2000;Watanabe et al., 2002;Honma et al., 2002). However, TGZ metabolite-protein adducts have only been demonstrated by incubation of liver microsomes from rats with various P450 inducers or "Supersomes" (cDNA-expressed human P450) (He et al., 2004a). Furthermore, although TGZ is cytotoxic to human HepG2 cells and rat and human hepatocytes, inhibitors of drug metabolizing enzymes responsible for TGZ metabolism have not succeeded in protecting against the TGZ-induced cytotoxicity (Yamamoto et al., 2001;Kostrubsky et al., 2000;Tirmenstein et

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al., 2002). In a recent study, HepG2 cells together with microsomes containing cDNA-expressed CYP3A4 or HepG2 cells transfected with CYP3A4 were able to metabolize TGZ, leading to increased cytotoxicity (Vignati et al., 2005). This finding has been questioned since the TGZ quinone metabolite formed by CYP3A4 is less cytotoxic than TGZ, both in rat hepatocytes and HepG2 cells (Tettey et al., 2001a). In addition, in normal human hepatocytes, these CYP3A4-related metabolites are unlikely to be generated and accumulate in these cells enough to exert toxic effects. In summary, studies investigating TGZ-induced cytotoxicty were performed with cultured cell lines using concentrations of TGZ 1–2 orders of magnitude above pharmacological levels. These studies in vitro have not provided a relevant mechanistic explanation for the infrequent troglitazone hepatotoxicity in patients. Thus, the role of the reactive metabolite in TGZ cytoxicity remains a question.

Other evidence supporting the role of metabolic idiosyncrasy in TGZ-induced hepatotoxicity is the finding of single-nucleotide polymorphisms (SNPs) for TGZ-metabolizing enzymes in human populations. SNPs have been found in three genes important to CYP3A activity (Kuehl et al., 2001b). This is relevant because the hepatic gene expression of CYP3A4 varies about 50-fold, and the CYP3A4 overal enzymic activity in vivo varies at least 20-fold among individual people (Eichelbaum and Burk, 2001). SNPs of the CYP3A gene family members affect various ethnic groups , where it is expressed at high levels in a minority of Americans of European descent and Europeans (Kuehl et al., 2001a;Hustert et al., 2001). However, the TGZ has not been

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found to be toxic with doses up to 1200mg/kg after 52 treatment of monkeys, although the TGZ treatment resulted in significant exposure to TGZ quinine (M3) (Rothwell et al., 2002). In human patients, M3-derived reactive intermediates were found to bind covalently to microsomal protein and glutathione (Tettey et al., 2001b;He et al., 2004b), but the importance of these adducts to TGZ- associated hepatoxicity has not been established. In a cohort study of 4,079 patients, combined genetic polymorphisms from several genes were associated with increased susceptibility for TGZ-induced liver injury. They included CYP1A1, NAD(P)H dehydrogenase, quinone 1 (NQO1), glucose transporter type 1 (GLUT-1), PPARγ-892, and PPARγ-1431 (de la Iglesia FA et al., 2003). In another study, a strong correlation was also observed between TGZ-induced liver injury and the combined glutathione S-transferase-theta 1 (GSTT1)- glutathione S-transferase M1 (GSTM1) null genotype (Watanabe et al., 2003). However, none of these studies tested the functional relationship of these SNPs with TGZ-induced liver injury in human patients.

Some of the reported cases had histological evidence for an adaptive immune-mediated reaction (Arioglu et al., 2000;Kohlroser et al., 2000;Murphy et al., 2000) or the responses can be reduced by corticosteroids (Prendergast et al., 2000;Bonkovsky et al., 2002). One of these patients developed a similar cholestatic hepatitis when switched to rosiglitazone after TGZ treatment, consistent with an adaptive immune-mediated reaction and suggesting a class effect related to the drug's pharmacological target. The occurrence of eosinophils or granulomatous inflammatory

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infiltrates in the liver is evidence, albeit inconclusive that hypersensitive immune response plays a role in the injury induced by TGZ in some patients. Unfortunately, there are no studies in experimental animals which could reproduce the liver injury, particularly hypersensitivity reactions, caused by TGZ in human patients.

A recent study by Boelsterli and colleagues showed that TGZ could induce mild hepatocellular injury after 4 weeks of treatment in SOD2+/-- heterozygous mice (Ong et al., 2007). Hepatic mitochondria isolated from TGZ-treated mice exhibited enhanced oxidative stress. Furthermore, in hepatocytes isolated from untreated SOD2+/--, but not wild-type mice, TGZ caused a concentration- dependent increase in superoxide anion production. This was the first demonstration in an animal model that TGZ treatment induced mild liver toxicity. In agreement with previous studies, wild-type animals tolerated the drug without adverse effects, however, mice with a deficiency in mitochondrial antioxidant defense were found to be susceptible. The results support the general concept that subclinical stresses from prolonged drug treatment superimposed on a genetic deficiency in the same organism can lead to cell injury and organ damage. However, a single genetic defect such as SOD in the human population might not be responsible for all the cases of liver injury in patients after TGZ treatment.

The lack of evidence that TGZ is hepatotoxic in healthy experimental animals and the findings of Boelsterli and colleages suggest that some undefined stress and/or differences in certain human patients, either genetic or environmental, make them susceptible to TGZ-induced hapatotoxicity. Stresses such as deficient mitochondrial

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antioxidant defense can be periodic and can go unnoticed. This could explain the erratic temporal and dose relationships that characterize IADRs.

1.1.3 Inflammatory stress and IADRs

Results of numerous studies indicate that a modest inflammatory response can enhance liver sensitivity to a variety of toxic chemicals in rats (Yee et al., 2000;Barton et al., 2000a). Episodes of inflammation are commonplace in people, although most of them cause no obvious injuries. Moreover, they occur irregularly and often go unnoticed. These observations have led us to hypothesize that an episode of inflammation during drug therapy might decrease the threshold for drug toxicity and thereby render an individual susceptible to an adverse drug reaction. This could be an attractive concept to explain the basis for IADRs. During drug therapy, concurrent, perhaps unnoticed inflammatory or other environmental stresses could render an otherwise nontoxic drug dose injurious to certain organs. This could explain the erratic temporal and dose relationships of that characterize idiosyncratic reactions.

Small doses of lipopolysaccharide (LPS), the cell wall component of gram-negative bacteria, precipitate modest inflammatory responses in mammals, resulting in increased susceptibility to toxicitiy from numerous hepatotoxic chemicals (Roth et al., 1997;Ganey and Roth, 2001). For example, exposing rats to a nonhepatotoxic dose of LPS results in a several fold increase in sensitivity to liver injury from allyl alcohol (Sneed et al., 1997). This increase in sensitivity depends on an LPS-stimulated inflammatory response

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involving Kupffer cell-derived eicosanoids (PGD_2), neutrophils and the hemostatic system, the system controls blood clotting in vessels (Kinser et al., 2002;Ganey et al., 2001;Kinser et al., 2004).

In animal studies, evidence is growing that supports the ability of LPS to augment hepatotoxicity induced by several classes of drugs that cause IADRs in people. For example, coadministration to rats of nonhepatotoxic doses of LPS and the antipsychotic drug chlorpromazine (CPZ) results in liver damage and other effects that resemble human CPZ idiosyncrasy (Buchweitz et al., 2002). Trovafloxacin, a fluoroquinolone antibiotic associated with idiosyncratic reactions, also interacts with LPS, resulting in hepatotoxcity both in rats and mice (Waring et al., 2006c;Shaw et al., 2007). In contrast, levofloxacin, another quinolone antibiotic without the tendency for idiosyncratic reactions, does not share this interaction with inflammatory stress. Thus, this model of drug-inflammation interaction is able to distinguish a drug that causes idiosyncratic adverse reactions from one in the same pharmacologic class that does not. A recent study showed that sulindac, a NSAID associated with IADRs, also could induce liver injury at an otherwise nontoxic dose when cotreated with LPS (Zou et al., 2008). These animal models mimicking idiosyncratic reactions in human patients could provide useful tools for mechanistic study, which in turn could lead to novel biomarkers and methods to prevent or cure adverse reactions. The models that will be explored by this thesis are ones in which animals are treated with either ranitidine (RAN) or diclofenac (DCLF). Both of these

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drugs are associated with rare idiosyncratic hepatotoxicity in human patients, which will be discussed in detail in the following sections.

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1.2 Inflammation

1.2.1 Overview of inflammation as a contributor to tissue injury

Inflammation is classically defined as a local protective reaction of tissue to irritation, injury, or infection, characterized as "redness, swelling, pain and heat." However, inflammation is now viewed in terms of activation of cells of the innate immune system, the coordination of the mediators they produce and altered gene expression and cell signaling. Inflammation can both participate in host defense and have the potential to injure tissues. Indeed, it has become clear that inflammation plays a role in the pathogenesis of many diseases, can cause tissue injury by itself and can increase sensitivity of tissues to the toxic effects of some other xenobiotics as mentioned above.

Inflammation comprises not only traditional inflammatory cells (e.g., neutrophils, macrophages) and the mediators they produce (e.g., cytokines/ chemokines, coagulation factors, complement factors) but also endothelial cells and parenchymal cells in the tissue (Ganey et al., 2004). The inflammatory cells can attack and damage the tissues directly by releasing toxic factors such as reactive oxygen species, proteases, etc., or they can release cytokines, eicosanoids or other mediators that lead indirectly to other damaging events. The hemostatic and complement systems are also activated in inflammatory responses and can participate in tissue injury. Changes in gene expression and the transcriptional regulators involved (e.g., nuclear factor kappa B, early growth response gene-1) are integral players in the inflammatory response. The hemostatic and complement systems are also activated in inflammatory responses and can participate in

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tissue injury. Transcriptional regulators not only control the production of inflammatory mediators but also play an important role indirectly in the activation of various cells involved.

Inflammation is known to be a feature of numerous diseases and may contribute to disease pathogenesis. Several obvious examples are conditions for which inhibition of inflammatory mediators have been successful in treatment (e.g, arthritis). However, inflammation is now shown to be involved in several other conditions. The list includes Parkinson's disease (Barcia et al., 2003), diabetes (Tracy, 2003), obesity (Cottam et al., 2004), cardiovascular diseases (Willerson and Ridker, 2004) and some types of cancer (De Marzo et al., 2007;Whitcomb, 2004).

Exposure to large amounts of LPS, such as in sepsis, can result in damage to several organs including the liver (Hewett and Roth, 1993c). LPS activates toll-like receptors on macrophages, thus resulting in a cascade of inflammatory events. This renders LPS an effective and commonly used model inflammagen in animal studies. The following section will discuss the roles of some inflammatory mediators in the pathogenesis of liver injury, with LPS-induced liver injury as a model. The involvement of such mediators in liver toxicity from selected xenobiotic agents will also be mentioned.

1.2.2 TNFα

TNFα production is triggered by LPS in several cell types, in the liver especially by Kupffer cells (the liver resident macrophage) (Hewett and Roth, 1993b). This cytokine

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acts at two receptors (TNFR 1 and TNFR 2) present on many cell types to initiate cell death signaling, promote inflammatory mediator release, increase expression of nitric oxide sysnthesis 2, inducible (iNOS), activate the hemostatic system and induce cell proliferation (Hehlgans and Pfeffer, 2005;Vassalli, 1992).

Expression of TNFα mRNA increases shortly after administration of LPS, and the concentration of TNFα protein in blood rises within an hour (Hewett and Roth, 1993a). TNFα-neutralizing antibodies and inhibition of TNFα synthesis significantly attenuate LPS-induced injury in rodents. In contrast, inhibition of TNFα biosynthesis or neutralization of TNFα does not influence hepatic PMN accumulation, suggesting either that TNFα injures the liver after exposure to LPS by affecting activation or function of PMNs (eg, release of cytotoxic proteases) or that it acts in a PMN-independent manner.

TNFα has been identified as the most important pro-inflammatory cytokine of the acute inflammatory response as well as a major component in the pathogenesis of the septic shock syndrome (Tartaglia et al., 1993;Rietschel et al., 1996). Indeed, TNFα infusion into blood circulation leads to a sepsis-like syndrome in rats (Tracey et al., 1986). Administration of anti-TNFα antibodies in baboons protects from lethal bacteraemia triggered by infusion of live Escherichia coli (Tracey et al., 1987). In these models, TNFR 1 is essential in mediating TNF signaling, since TNFR 1-deficient mice are protected from LPS/D-galactasomine (D-GalN) and Staphylococcus aureus superantigen/ D-GalN-induced sepsis shock (Pfeffer et al., 1993). However, in other experimental models using concanavalin A (Con A) or Pseudomonas exotoxin A-induced

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hepatitis, TNFR 2 signalling seems to be important for the host damaging effects (Kusters et al., 1997;Schumann et al., 1998).

In addition to its role in promoting tissue injury, TNFα can also have protective influence. For example, the cecal ligation and puncture (CLP) model of abdominal sepsis generates invasion of gut-derived bacteria into the blood stream and into organs, causing septic-like syndromes. Studies employing this model suggested that TNFα is important for recovery and survival from septic peritonitis independent of its affect on lymphocytes (Echtenacher et al., 1995;Echtenacher et al., 1990). Furthermore, activation of the TNFR II by endogenous TNFα constitutes an important interaction for the development of LPS-induced resistance to bacterial infection (Echtenacher and Mannel, 2002). In addition, a state of immunoparalysis characterized by a reduced production of TNFα develops after CLP, which results in bacterial superinfection and leads to subsequent lethality. Echtenacher and coworkers (Echtenacher et al., 2003) demonstrated in the CLP model that TNFα administration during the phase of immunoparalysis can be beneficial or deleterious, depending on the location of TNFα activity, timing of TNFα administration and the type of infection. From these results, the prevalent concept that TNFα is solely host-damaging needs to be re-evaluated, and additional studies are required to analyze in more detail the role of TNFα in both pathogen defense and host damage.

The signaling pathway for LPS-induced TNFα production has been extensively studied (Kawai and Akira, 2007b;Takeda and Akira, 2005). Macrophages are the primary

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response immune cells to LPS. Toll-like receptor 4 (TLR4) on macrophages serves as a pattern recognition receptor for LPS. After recognizing LPS, TLR4 causes the recruitment of a set of intracellular Toll/IL-1 receptor (TIR) domain-containing adaptors, including myeloid differentiation factor 88 (MyD88), TIR-related adaptor inducing interferon (TRIF) and TRIF-related adaptor molecule (TRAM). Among them, MyD88 is a universal adaptor that activates inflammatory signaling pathways. The association of TLR4 and MyD88 stimulates the recruitment of members of the IL-1 receptor-associated kinase (IRAK) family, especially IRAK4. Once phosphorylated, IRAK dissociates from MyD88 and interacts with TNF receptor-associated factor 6 (TRAF6). TRAF6, an E3 ligase, forms a complex with Ubc 13 to promote the synthesis of polyubiquitin chains, which in turn activate TGF-β-activated kinase 1 (TAK1), a MAPK kinase kinase. TAK1, in combination with TAK1-binding protein (TAB), activates two downstream pathways involving the IkappaB kinase (IKK) complex and the MAPK family (P38, JNK, ERK). The phosphorylation of IκB by the IKK complex is necessary for the degradation of IκB and the subsequent nuclear translocation of the transcription factor NF-κB, which controls the expression of various inflammatory cytokine genes including TNFα. MAPK can phosphorylate and activate the transcription factor AP-1, which has a central role in inflammatory cytokine synthesis. In addition, MAPK (especially P38) can control cytokine production in a post-transcriptional manner by increasing mRNA stability and mRNA translation (Kotlyarov et al., 1999;Mavropoulos et al., 2005;Neininger et al., 2002a;Neininger et al., 2002b).

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1.2.3 Neutrophils (PMNs)

PMNs are involved in producing liver injury induced from large, hepatotoxic doses of LPS (Hewett et al., 1992). After exposure of rodents to LPS, various PMN chemoattractants such as cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2) are elevated in plasma, and adhesion molecules become upregulated on the surface of sinusoidal endothelial cells (SECs), PMNs and hepatocytes (Jaeschke et al., 1996a). The upregulation of these chemokines and adhesion molecules facilitates PMN transmigration across the endothelial cell barrier and promotes subsequent localization of PMNs close to hepatocytes (Springer, 1994a;Butcher, 1991b). This transmigration and activation process involves rolling, binding to and migrating across the endothelium. The migration of neutrophils from the vasculature is regulated by at least three distinct molecular signals: selectin and its receptor (ie., carbohydrate), integrin and its receptors (ie., immunoglobulin family) and chemokines and their receptors. A key feature is that selectin-carbohydrate, integrin-immunoglobulin family and chemoattractant-receptor, interactions act in sequence, not in parallel (Butcher, 1991a;Ley, 1996). Selectins initiate the rolling across the endothelium while integrins causes firm adhesion. Integrins and chemokines comprise the driving force for the transmigration into parenchyma (Kobayashi, 2008). This concept of sequential action has been confirmed by the observation that inhibition of any one of these steps gives essentially complete, rather than partial, inhibition of neutrophil emigration.

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When activated, PMNs release numerous cytotoxic factors, including reactive oxygen species and PMN proteases (Jaeschke et al., 2002;Ganey et al., 1994c). Among the latter, cathespsin G and elastase are important mediators of hepatic parenchymal cell killing by activated PMNs in vitro (Ho et al., 1996a). Release of these factors is involved in several pathological conditions such as acute lung injury, endotoxemia and ischemia-reperfusion injury (Jaeschke et al., 1991;Jaeschke et al., 1990;Kawabata et al., 2002). In addition, PMNs are critical mediators of injury in models of endotoxin-potentiated hepatotoxicity, such as from aflatoxin B1, monocrotaline and trovoflaxacin (Barton et al., 2000b;Yee et al., 2003e;Waring et al., 2006b).

There exists controversy regarding the mechanisms of neutrophil-induced cell injury in the liver. Most coculture experiments in vitro using activated neutrophils and cultured hepatocytes showed that protease inhibitors but not antioxidant enzymes were able to prevent the neutrophil-mediated cell injury, which develops over 15–20 h (Mavier et al., 1988;Harbrecht et al., 1993;Ganey et al., 1994b). This time frame is much longer than that by which hepatocellular injury develops in vivo in LPS-treated rats. Furthermore, neutrophils may be less likely to attack and kill normal hepatocytes in vivo since during inflammatory pathogenesis hepatocytes are exposed to other stress factors (Gujral et al., 2004a) that are not reflected in most in vitro coculture systems. There are studies in vivo showing that PMN protease inhibitors protect the liver from injury caused by ischemia-reperfusion. However, this attenuation of liver injury was accompanied by a decrease in chemokines and hepatic PMN accumulation, so that the protection effect

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might be due to inhibitions of actions other than direct hepatocyte killing by the proteases (Soejima et al., 1999;Yamaguchi et al., 1997;Yamaguchi et al., 1999). Other studies showed that PMNs can kill hepatocytes in vivo within 1 h, which correlates with the appearance of an intracellular oxidant stress and the formation of hypochlorite-mediated chlorotyrosine protein adducts (Hasegawa et al., 2005;Jaeschke et al., 1999a;Gujral et al., 2004b). Furthermore, an inhibitor of PMN NADPH oxidase significantly delayed liver injury in galactosoamine-sensitized mice given LPS (Gujral et al., 2004c). Mice deficient in glutathione peroxidase-1 showed enhanced susceptibility against neutrophil-mediated cytotoxicity (Jaeschke et al., 1999b). These studies underline the crucial role of the PMN-dependent oxidative burst in the pathogenesis of liver injury, but they do not rule out the possibility of PMN protease killing of hepatocytes in vivo. The longer time required for protease mediated killing of hepatocytes in vitro suggests that PMN proteases might act in concert with other inflammatory mediators to damage hepatocytes in vivo. These mediators could be either PMN-dependent or not. Previous work showed that PMNs stimulated with fMLP plus PMA to release proteases and ROS, respectively, kill hepatocytes with a timecourse similar with that of protease alone (Ganey et al., 1994a). This result suggests that an interaction of proteases either with other, non ROS inflammatory mediators from other cell types or stresses not directly related to PMNs that alter hepatocellular homeostasis are necessary for full manifestation of LPS-induced liver injury.

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1.2.4 The hemostatic system and hypoxia

The coagulation and fibrinolytic systems are important controllers of vascular hemostasis (Levi et al., 2003d). Tissue factor and factor VII are important initiating factors in coagulation, and through a complex cascade they activate factor X. Factor X in turn cleaves and activates thrombin. Thrombin is a protease that can cleave fibrinogen into fibrin. Fibrin, upon cross-linking and polymerization, forms clots in blood vessels. This activation of the coagulation system is involved in a variety of disease conditions such as thrombosis and disseminated intravascular coagulation (DIC) (Levi, 2005b;Levi et al., 2003c).

The formation of fibrin clots is tightly regulated by the interplay between the coagulation and fibrinolytic systems. Plasminogen activators (PAs), including urokinase PA and tissue-specific PA, are important activators of the fibrinolytic agent plasmin by cleaving plasminogen into its active form. Plasmin can cleave and dissolve crosslinked fibrin. The activity of plasminogen activator is controlled by endogenous plasminogen activator inhibitor-1 (PAI-1).

PAI-1 is synthesized de novo by a variety of cells in vitro (e.g., hepatocytes, adipocytes, endothelial cells, cardiac myocytes and platelets) (Westrick and Eitzman, 2007;Macfelda et al., 2002), but it is unclear which cell type is the major producer of PAI-1 in vivo. A PAI-1 pool is also found in the granules of platelets, where it is stored and could be released after vessel damage (Hoekstra et al., 2004b). Except in platelets, transcriptional control of PAI-1 is a pivotal mechanism in determining tissue and plasma

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PAI-1 content. Experiments in vitro have demonstrated that secretion of PAI-1 can be induced by a variety of agents, including TNF-α, IL-1, transforming growth factor-β, endotoxin, very-low-density lipoprotein, glucose, glucocorticoid, and insulin. The transcriptional induction is mediated through Sp1 element, hypoxia-responsive element or SMAD 3/4 binding sites in the PAI-1-promoter (Fink et al., 2002;Hou et al., 2004). Upregulation of PAI-1 is associated with DIC and other thrombotic diseases (Padro et al., 1997b;Padro et al., 1995).

PAI-1 is present in 3 forms in the blood circulation: an active, an inactive, and a latent form. The active form converts spontaneously into the latent form with an half-life of 1 h (Hoekstra et al., 2004a). The latent form is more stable and can also be reconverted into the active form. PAI-1 is removed from the circulation by the liver and also is inactivated by endothelium (Hekman and Loskutoff, 1985;Owensby et al., 1991). Endothelium- or platelet-derived PAI-1 is normally complexed to vitronectin, resulting in a 2-4 fold increased half-life of PAI-1 in the circulation.

Inhibition of thrombin activation significantly attenuates LPS-induced liver injury (Hewett and Roth, 1995b;Moulin et al., 2001a), but this protection is independent of circulating fibrinogen (Hewett and Roth, 1995a).This suggests that although thrombin activation is important for liver injury from LPS, the formation of fibrin clots resulting from fibrinogen processing is not required for the injury. Activation by thrombin of protease activated receptor-1 (PAR-1) influences inflammatory cell accumulation and/or activation in the liver. Inhibition of thrombin reduced PMN activation as estimated by

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plasma elastase concentration (Copple et al., 2003b;Pearson et al., 1996). Furthermore, perfusion of livers from LPS-treated rats with thrombin or the PAR-1 agonist TFLLR caused hepatocellular injury (Moulin et al., 2001b;Copple et al., 2003a). This effect was eliminated by prior depletion of PMNs. Thus, these results suggest that thrombin-mediated activation of PAR-1 can be important for PMN activation and that these two events are sufficient for causing liver injury in LPS-treated rats.

Although fibrin deposition does not appear to be important for liver injury caused by LPS treatment, other results suggest that fibrin deposition can to contribute to hepatocellular injury (Luyendyk et al., 2004o). One of the possible consequences of intravascular fibrin deposition is tissue hypoxia caused by blood flow impaired by fibrin clots. Hypoxia can deplete cellular ATP and interfere with intracellular pH and homeostasis of ions such as Na^+ and Ca^{2+}, which could subsequently cause cell death (Carini et al., 1997). In addition, hypoxia can induce the formation of reactive oxygen species (Lluis et al., 2007;Lluis et al., 2005b). It can also activate numerous intracellular signaling pathways related to cell stress, mostly mediated by hypoxia inducible factor-1α, an important transcription factor responsive to hypoxia (Shibayama, 1987b). Interestingly, breathing in a low O_2 atmosphere enhanced the liver lesions in rats caused by large, hepatotoxic dose of LPS (Shibayama, 1987a). This suggests that hypoxia might interact with LPS-induced inflammatory mediators to cause hepatocellular injury in vivo.

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1.3 Mechanisms of liver injury in models of inflammation-drug interaction: ranitidine as example

1.3.1 Ranitidine-induced idiosyncratic hepatotoxicity in human patients

A widely used drug associated with idiosyncratic hepatotoxicity is the histamine 2 (H2)-receptor antagonist, ranitidine (RAN). RAN is available over the counter for oral administration, or by prescription for parenteral administration for treatment of duodenal ulcers, gastric hypersecretory diseases and gastroesophageal reflux disease. Idiosyncratic RAN hepatotoxicity occurs in less than 0.1% of people taking the drug (Ribeiro et al., 2000b). Most liver reactions are mild and reversible; however, extensive liver damage and death have occurred in individuals undergoing RAN therapy (Ribeiro et al., 2000a). Idiosyncratic hepatotoxicity to RAN typically manifests as elevations in serum markers of hepatocellular injury with more modest increases in indicators of cholestatic injury. These reactions are typical of IADRs, as the time of onset of hepatotoxicity relative to initiation of therapy varies greatly. Rechallenge with RAN does not necessarily result in a reoccurrence of toxicity (Halparin, 1984b;Hiesse et al., 1985b). Indeed, in some cases the adverse response resolved despite continued therapy (Chung et al., 2000a). These characteristics do not seem consistent with an adaptive immune response as the cause of RAN IADRs.

RAN is minimally metabolized into three major metabolites in both humans and rats: N-oxide, S-oxide and desmethyl metabolites (Chung et al., 2000b;Carey et al., 1981b). RAN is excreted mostly unchanged in urine in humans as compared to through bile in

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rats (Carey et al., 1981a;Huang et al., 2005). However, no known RAN metabolite toxic to liver has been reported either in human or rodent animals to our knowledge. Accordingly, it seems unlikely that IADRs caused by RAN are due to metabolic bioactivation of this drug.

Although direct evidence implicating inflammation as a contributing factor to these idiosyncratic reactions is lacking, it is interesting that a majority of 34 human case reports of RAN idiosyncratic hepatotoxicity mentioned prodromal signs consistent with endotoxemia (i.e. increased LPS conc. in blood) or inflammation (e.g. diarrhea, fever, nausea/vomiting, and/or abdominal pain) (Luyendyk et al., 2003a). The ability of modest inflammation to potentiate the toxicity of numerous xenobiotic agents, along with observed prodromal inflammatory signs in RAN idiosyncrasy, led us to hypothesize that RAN IADRs can result from episodes of mild inflammation occurring during drug therapy.

1.3.2 Inflammation-ranitidine interaction

Although RAN alone is not hepatotoxic in rats, studies in rats have demonstrated that modest underlying inflammation induced by a nontoxic dose of LPS precipitates IADR-like liver injury from RAN (Luyendyk et al., 2003b). This was not the case with another histamine-2 receptor antagonist, famotidine (FAM), which does not share RAN's propensity to cause idiosyncratic adverse reactions in human patients. This suggested that the pharmacological target is not the toxic target in terms of LPS/RAN-induced liver

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injury. Other studies have shown that histamine attenuates LPS-induced TNFα synthesis by H2-receptor-dependent mechanism in monocyte (Vannier et al., 1991). Furthermore, histamine affords protection against *P.acnes*/LPS-and ethanol-induced liver injury by a H2-receptor dependent mechanism (Yokoyama et al., 2004;Hornyak et al., 2003). This was not inconsistent with our findings since H2-receptor blockade might be a required but insufficient factor for LPS/RAN-induced liver injury,

RAN given to rats 2hr after a nonhepatotoxic dose of LPS caused an acute, midzonal, suppurative, necrotizing hepatitis that resembled lesions in animals treated with a hepatotoxic dose of LPS (Luyendyk et al., 2003c). This result suggested that RAN might increase hepatic parenchymal cell sensitivity to an LPS-like hepatotoxic response. The liver injury in LPS/RAN cotreated rats begins at 2-3 hours after RAN treatment, maximizes at 6hr, and is sustained for at least 24 hours. Hepatic inflammatory infiltrates comprised predominately PMNs, suggesting the possibility of a role for these cells in the liver injury (Luyendyk et al., 2005e). In addition, a gene array study showed that expression of several genes involved in the hemostatic system and hypoxia were greatly enhanced in LPS/RAN-cotreated rats compared to either LPS or RAN treatment alone. One of these genes was PAI-1 (Luyendyk et al., 2004p). Since PAI-1 is an important negative regulator of the fibrinolytic system, its selectively enhance expression suggested the possibility of hemostatic system involvement. Furthermore, the crucial role of TNFα and other cytokines in large-dose LPS-induced liver toxicity raised the possibility of the importance of inflammatory cytokines in the LPS/RAN model. The following sections

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will review the evidence that supports the roles of these inflammatory factors and will discuss how they interact with each other to contribute to the liver injury caused by LPS/RAN cotreatment.

1.3.3 Involvement of hemostasis, PMNs and TNFα

In rats, LPS alone caused a mild and transient increase in plasma thrombin-antithrombin dimer (TAT) and PAI-1 concentration, suggesting activation of the hemostatic system. RAN cotreatment augmented and sustained the increases in plasma TAT and PAI-1 (Luyendyk et al., 2004n). Consistent with this, significant sinusoidal fibrin deposition occurred only in livers of LPS/RAN-treated rats relative to rats treated with LPS or RAN alone. The anticoagulant agent heparin and the fibrinolytic agent streptokinase, both of which reduced hepatic fibrin deposition, prevented the liver injury induced by LPS/RAN, suggesting a role for the hemostatic system in this model of idiosyncrasy-like liver injury (Luyendyk et al., 2004m). Interestingly, the activation of the coagulation system in LPS/RAN cotreated rats is more pronounced than that in LPS/FAM cotreated rats (Luyendyk et al., 2006a), suggesting the possibility of a role for the coagulation system in the idiosyncratic liver injury caused by RAN. It appears that RAN selectively augments the coagulation system activation triggered by LPS, and this in turn causes subsequent fibrin deposition, tissue hypoxia and hepatocyte death. Indeed, hepatic hypoxia occurs in LPS/RAN treated rats and heparin

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reduced the tissue hypoxia as well as fibrin deposition (Luyendyk et al., 2005d). The latter observation suggested that fibrin deposition leads to liver hypoxia in this model.

One possible contributor to coagulation system activation ad PAI-1 production in LPS/RAN-treated rats could be endothelial cell activation. Previous results suggested the occurrence of an early, transient effect of RAN and a prolonged LPS effect on sinusoidal endothelial (SEC) function, reflected as an increase in serum hyaluronic acid (HA) concentration (Luyendyk et al., 2004l). The effects of the two agents appeared to be additive at earlier times in LPS/RAN-cotreated rats. The early changes in serum HA concentration were not accompanied by decreased rat endothelial cell immunostaining of liver sections, suggesting the absence of overt SEC destruction. Overall, these results suggested that SEC dysfunction in LPS/RAN-treated rats occurred to a greater degree than in LPS/Veh-treated rats at a time prior to liver injury. Although its contribution is not fully understood, a perturbation of SEC function might be important for the coagulation system activation, PAI-1 production and subsequent liver injury after LPS/RAN treatment. For example, endothelial cells activated after LPS exposure could express tissue factor and thereby initiate the coagulation cascade (Camerer et al., 1996). In addition, HA has been reported to enhance expression of PAI-1, thus causing sustained fibrin deposition (Horton et al., 1999).

Data presented in this thesis will confirm and explore an essential role for PMNs in liver injury caused by LPS/RAN. In the LPS/RAN model, PMN accumulation in liver is caused largely by LPS, evidenced by the same degree of PMN accumulation in the livers

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of rats treated with LPS/RAN and as in those treated only with LPS (Luyendyk et al., 2005c). The mechanism of initial recruitment of neutrophils into liver is unknown, but it might occur through cytokines released from Kupffer cells activated by LPS, from interaction of adhesion molecules (e.g., selectins) or from sinusoidal contraction. The lack of liver injury after LPS treatment suggests that PMNs are not extravasated and activated in liver after exposure to the noninjurious LPS dose used in these studies. The fact that PMNs accumulate in the liver to about the same degree in rats treated only with LPS compared to LPS/RAN indicates that PMNs need a secondary signal provided by RAN treatment to be activated and cause their damaging effects. RAN itself does not activate PMNs directly; in fact, it inhibits PMN activation both in vitro and in vivo (Okajima et al., 2000d;Okajima et al., 2002d). This suggests that RAN acts indirectly through other inflammatory mediators.These mediators might be PMN chemokines (i.e., MIP-2) and/or hemostatic factors like PAI-1 since both have been shown to be involved in PMN activation (Lentsch et al., 1998;Li et al., 2004;Maher et al., 1997). In addition, PAI-1 can potentiate LPS-induced PMN activation in vitro (Kwak et al., 2006c). Interestingly, mRNA and serum protein concentration of both MIP-2 and PAI-1 are more upregulated after treatment with LPS/RAN than after LPS only at a time before liver injury onset (Luyendyk et al., 2006h), and this increase is unique to RAN compared to FAM, raising the possibility of MIP-2 and/or PAI-1 as signals to activate PMNs in this model.

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Since TNFα is a critical cytokine involved in large-dose, LPS-induced liver injury, the effects of LPS/RAN treatment on TNFα production was examined. At a nontoxic dose, LPS rapidly induced serum TNFα production but did not cause liver injury (Tukov et al., 2007s). The serum TNFα concentration peaked at about 1.5 hours and rapidly decreased after that, returning almost to normal by 8 hours. This indicates that an LPS-induced increase in TNFα of this magnitude and duration is not sufficient to cause hepatocellular damage. Despite not being damaging by itself, this large increase in TNFα concentration could be critical for the pathogenesis of liver injury when combined with other effects of RAN cotreatment. Indeed, RAN-cotreatment caused the serum TNFα increase to last longer than in rats given LPS alone, and these RAN-cotreated rats developed hepatotoxicity. In contrast, FAM neither enhanced TNFα production nor produced liver injury when administered with LPS. Thus, the prolongation of LPS-stimulated TNFα production distinguished a drug that causes human IADRs from one that does not. Interestingly, some TNFα -dependent cytokines/chemokines, such as IL-6, IL-1β and MIP-2 also had the same pattern of prolonged increase after RAN cotreatment (Tukov et al., 2007r).

Kupffer cells (KCs) are a primary response cell to endotoxin challenge in liver. They are also the major sources of many inflammatory cytokines/chemokines. Some of the inflammatory mediator expression induced by xenobiotic/LPS coexposures in rats could be recapitulated using a KC/HPC coculture system (Tukov et al., 2007q). Indeed, RAN enhanced TNFα release in the presence of LPS in KC/HPC cocultures, but only at a large

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RAN concentration (0.5mM) that is unlikely to be achieved in the LPS/RAN animal model. FAM had a similar effect on TNFα release in KC/HPC coculture (Tukov et al., 2007p). However, RAN but not FAM caused a prolonged increase in serum TNFα after drug treatment in vivo. Thus, this cell-based system *in vitro* failed to distinguish a drug that causes IADRs in humans from one that does not. RAN had no effect on LPS-induced MIP-2 production in KC/HPC coculture. This also differed from the response in vivo, where LPS/RAN treatment caused a selective upregulation of serum MIP-2 compared to LPS/FAM treatment (Luyendyk et al., 2006). The disparity between these responses in vitro and in vivo suggests that the RAN-induced enhancement of TNFα and MIP-2 production seen in vivo likely occurs by mechanisms involving cell types other than or in addition to KCs and the inflammatory mediators those cells produce. The exact mechanism of RAN augmented TNFα production after LPS treatment in vivo remains unknown.

To determine the role of TNFα in LPS/RAN-induced hepatotoxicity, both pentoxyfylline (PTX) and etanercept (Etan) were used to reduce TNFα. PTX is a methylxanthine that inhibits the synthesis of TNFα (Dezube et al., 1993;Barton et al., 2001;Yee et al., 2003d). However, PTX also has several other pharmacological effects. Accordingly, Etan was also used. It is a dimeric fusion protein that contains a soluble TNFα receptor capable of selectively neutralizing serum TNFα. PTX and Etan significantly reduced serum TNFα concentration and activity, respectively, and both were effective in reducing hepatocellular injury in LPS/RAN cotreated rats (Tukov et al.,

2007o). These results indicate that TNFα is critically involved in LPS/RAN-induced liver injury. However, since PTX and Etan were given before the LPS challenge, those results could not differentiate whether LPS-induced TNFα elevation or the RAN-induced prolongation of the TNFα response was crucial for the pathogenesis.

1.3.4 Interaction of TNFα, hemostasis and PMNs

In LPS/RAN-cotreated rats, TNFα inhibition leads to decreases in PMN chemokines such as MIP-2 and in plasma markers of hemostasis, such as TAT and PAI-1 (Tukov et al., 2007n). This suggests that TNFα is a more proximal inflammatory mediator in the pathogeneic cascade relative to the hemostatic system and PMNs. Indeed, TNFα inhibition reduced hepatic fibrin deposition, indicating that TNFα acts upstream of the hemostatic system. How exactly TNFα contributes to hemostatic system activation remains unknown in this model. One possibility is that TNFα could act on vascular endothelial cells or on macrophages to induce tissue factor expression (Parry and Mackman, 1995;Bierhaus et al., 1995;Schwager and Jungi, 1994). In addition, in the presence of PMNs, TNFα exposure can cause sinusoidal endothelial cell (SEC) damage in vitro (Smedly et al., 1986;Takei et al., 1995), which can activate the coagulation system. TNFα and IL-1 can also stimulate the expression and release of PAI-1 by endothelial cells in vitro (Fan et al., 2000b). The contribution of TNFα to tissue factor production and inhibition of fibrinolysis has also been demonstrated in vivo in a model of lung hemorrhagic shock (Fan et al., 2000a). Thus, TNFα could cause the disruption of the

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hemostatic system and subsequent fibrin deposition by inducing either tissue factor or PAI-1 expression or both.

TNFα can prompt the accumulation of PMNs in tissues by activating endothelial cells (Bradham et al., 1998;Vassalli et al., 1992) and can also prime PMNs for activation (Schleiffenbaum and Fehr, 1990;Kushimoto et al., 1996;Nagaki et al., 1991;Vassalli, 1992). In LPS/RAN-cotreated rats, neutralization of TNFα did not impact hepatic PMN accumulation but did reduce serum MIP-2 and PAI-1 concentration (Tukov et al., 2007m). These results suggest that the signal for PMN extravasation and activation might be dependent on TNFα, whereas PMN accumulation is not. Interestingly, anticoagulant heparin similarly reduced serum MIP-2 and PAI-1 concentration while having no effect on hepatic PMN accumulation (Luyendyk et al., 2006b). This suggests that TNFα activation of coagulation induces the expression of MIP-2 and PAI-1, which could activate PMNs accumulated in the liver.

Inhibition of coagulation did not reduce serum TNFα concentration (unpublished results), further supporting the idea that coagulation activation lies downstream of TNFα production. Coagulation activation is not the cause of the initial neutrophil accumulation, since heparin treatment did not impact hepatic PMN accumulation, as mentioned above. However, coagulation activation might elicit PMN activation in the LPS/RAN model, a possibility suggested by the finding that heparin treatment reduced serum MIP-2 concentration. This could happen in several ways. One possibility is that coagulation activation causes activation of PAR-1, the receptor for thrombin on Kupffer cells,

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endothelial cells and hepatic stellate cells (Copple et al., 2003c). PAR-1 has been shown to contribute to PMN activation in an indirect manner through releasing PMN chemokines (Copple et al., 2003d). Another possibility is that hypoxia caused by occlusive fibrin clots in liver sinusoids promotes PMN activation by altering expression of chemokines and adhesion molecules. As mentioned above, hypoxia occurs in LPS/RAN treated rats before the onset of liver injury (Luyendyk et al., 2004k). Hypoxia can induce chemokines such as MIP-2 or adhesion molecules such as ICAM-1 and P-selectin in endothelial cells and/or hepatocytes (Pinsky et al., 1996;Laurens et al., 2005;Shreeniwas et al., 1992;Xu et al., 1999). These actions would favor the adhesion, transmigration and activation of PMNs. Indeed, PMNs isolated from humans after acute exposure to a hypoxic atmosphere showed increased activation compared to normoxia exposure as evidenced by superoxide anion production and elastase release (Harada et al., 1999a).

How PMNs contribute to the hepatotoxicity in this model remains unknown. It seems likely that PMNs are involved in TNFα production since Kupffer cells are the major source of TNFα in liver. Both coagulation system activation and PMNs are required for producing liver injury in this LPS/RAN model. In other models of liver injury, PMN activation can enhance activation of the coagulation system and vice versa (Harada et al., 1999b;Yee et al., 2003c). This raised the possibility of interplay between the hemostatic system and PMNs in LPS/RAN-induced liver injury. In fact, PMNs in other models are involved in coagulation system activation, fibrinolysis and platelet

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activation (Kimura and Yokoi-Hayashi, 1996a;Wu et al., 1995a;Goel and Diamond, 2003d;Pintucci et al., 1992a). For instance, the PMN protease cathepsin G can cleave plasma factor V and factor X to induce thrombin activation, and it also can promote prothrombinase and fibrin formation by activating platelets (Goel and Diamond, 2003c;Allen and Tracy, 1995;Sambrano et al., 2000). In addition, PMN proteases can cleave and release active PAI-1 from endothelial matrix to inhibit fibrinlysis (Pintucci et al., 1992b;Kimura and Yokoi-Hayashi, 1996b).

In addition to their role in hemostasis, PMNs upon activation release toxic products such as reactive oxygen species and proteases, which can directly damage hepatocytes. Interestingly, killing of hepatocytes in vitro by neutrophil elastase is enhanced by a hypoxic environment (Luyendyk et al., 2005b). The time course over which hepatocyte killing occurred during hypoxia in vitro (ie, 2hr) is consistent with the onset of liver injury in LPS/RAN-treated rats. Taken together with the evidence that fibrin deposition and hypoxia are critical to liver injury in LPS/RAN-treated rats, it seems possible that PMNs contribute to the pathogenesis by releasing proteases that kill hepatocytes in an environment made hypoxic by fibrin deposition or other events.

As summarized in Fig 1.1, LPS causes an early and transient increase in serum TNFα, which is prolonged by RAN treatment. TNFα causes coagulation system activation and PAI-1 production, leading to subsequent fibrin deposition and hypoxia (Tukov et al., 2007l;Luyendyk et al., 2004j). LPS also causes PMN accumulation in liver, and RAN might indirectly activate these cells, which can release toxic ROS and

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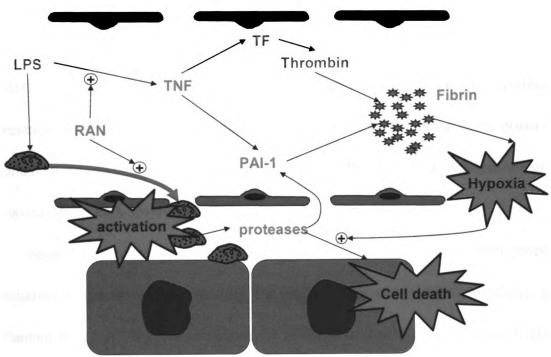
proteases. TNFα dependent production of MIP-2 and PAI-1 might contribute to PMN activation. Hypoxia might act synergistically with PMN proteases to kill hepatocytes (Luyendyk et al., 2005a). In addition to its direct killing of hepatocytes, PMNs might contribute to fibrin deposition and hypoxia.

Figure 1.1 Possible pathogenic mechanism of LPS/RAN-induced liver injury. LPS causes an early and transient increase in serum TNFα, which is prolonged by RAN treatment. TNFα causes coagulation system activation and PAI-1 production, leading to subsequent fibrin deposition and hypoxia (Tukov et al., 2007k;Luyendyk et al., 2004i). LPS also causes PMN accumulation in liver, and RAN might indirectly activate these cells, which can release toxic ROS and proteases. TNFα dependent production of MIP-2 and PAI-1 might contribute to PMN activation. Hypoxia might act synergistically with PMN proteases to kill hepatocytes (Luyendyk et al., 2005f). In addition to its direct killing of hepatocytes, PMNs might contribute to fibrin deposition and hypoxia.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1.4 Diclofenac-induced idiosyncratic hepatotoxicity

Non steroidal anti-inflammatory drugs (NSAIDs) comprise another class of drugs, many of which cause hepatic IADRs. For example, diclofenac (DCLF) has caused rare but sometimes serious hepatotoxicity in humans (Boelsterli, 2003f). Although the apparent incidence of severe DCLF-induced hepatic adverse reactions is quite low (from 1 to 2 cases per million prescriptions or 6 to 18 cases/100,000 person-year.), the large number of patients treated with DCLF makes the absolute number of cases impressive (Walker, 1997b). In addition, cases of severe injury leading to liver transplantation occur in a large proportion of the reported cases of DCLF-induced hepatotoxicity (Lewis et al., 2003). The scarceness of liver biopsies from patients and the diverse histopathlogical presentations in available samples make it difficult to draw clues about mechanisms from human liver pathology alone (Zimmerman et al., 1999). Thus, the pathogenesis of this low-incidence/ high-severity DCLF hepatotoxicity is largely unknown.

Several mechanisms for diclofenac-induced hepatotoxicity have been proposed, including formation of reactive drug metabolites (Lim et al., 2006), oxidative stress (Cantoni et al., 2003a) and mitochondrial injury (Masubuchi et al., 2002a). However, cytotoxocity of DCLF to hepatocytes is only observed in vitro with large concentrations that are not achieved in vivo. In human patients developing hepatotoxicity after DCLF, polymorphisms in enzymes that metabolize DCLF, such as UGT2B7 and CYP2C8, and in the DCLF glucuronide transporter ABCC2 have been discovered (Daly et al., 2007). These could lead to increased formation of hydroxylated and/or glucuronidated DCLF

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metabolites. However, no direct evidence has been found to support the a causal relationship between these DCLF metabolites and hepatotoxicity in vivo, although there are reports that DCLF acyl glucuronide is directly involved in small intestinal injury in rats (Seitz and Boelsterli, 1998d).

There is some evidence suggesting immune-modulated hypersensitivity in diclofenac-induced hepatotoxicity. For example, a 53-year-old female developed fulminant hepatic failure caused by inadvertent rechallenge with diclofenac (Greaves et al., 2001a). DCLF-conjugated mouse serum albumin or keyhole limpet hemocyanin (KLH) is immunogenic in mice (Kretz-Rommel and Boelsterli, 1995). Furthermore, T cells isolated from DCLF-KLH conjugate-treated mice were able to kill hepatocytes previously exposed in vitro to noncytotoxic concentrations of DCLF. However, there are no animal models which have reproduced toxic DCLF-induced immunoallergic reactions in the liver. In fact, the T cell popliteal lymph node reaction was attenuated if animals were treated orally with diclofenac as compared to direct injection into the footpad (Gutting et al., 2003;Gutting et al., 2002). Since DCLF is normally given orally to human patients, this raises a question about the likelihood that an immunoallergic reaction contributes to DCLF-induced IADRs. DCLF autoantibodies have been found in human patients, but the relevance of those autoantibodies to idiosyncratic hepatotoxicity is unknown (Aithal et al., 2004a). Thus, the lack of a demonstrable connection between an immunoallergic response to DCLF and hepatotoxicity raises a question as to whether DCLF IADRs occur by this mechanism.

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Based on the observation that a number of drugs that have the ability to cause IADRs in human patients also cause liver injury in LPS-treated rats, we hypothesized that a modest inflammatory episode increases the sensitivity of the liver to hepatotoxic effects of DCLF. This merits special attention since most selective cyclooxygenase (COX)-1 and mixed COX-1/COX-2 inhibitors, including diclofenac, have been associated with gastrointestinal injury, including increased permeability of the mucosa, ulceration, and, in extreme cases, perforation of the small intestinal wall. This holds true for laboratory rodents, in which a single, small dose of diclofenac (e.g., 1.5 mg/kg) can induce small intestinal ulceration (Atchison et al., 2000;Seitz and Boelsterli, 1998c) and thereby promote LPS and or bacterial translocation from the intestine into the circulation. Moreover, DCLF is normally prescribed to patients with inflammatory diseases such as arthritis, in which inflammatory mediators (ie.TNF, PMNs) are usually present. The role of inflammatory stress in DCLF hepatotoxicity is further suggested by the clinical findings that IL-4 and IL-10 polymorphisms have been found in human patients who developed hepatotoxicity after DCLF treatment (Aithal et al., 2004b).

The potentiation of DCLF induced hepatotoxicity by LPS might appear paradoxical, since DCLF acts as a NSAID to dampen inflammatory responses. COX catalyzes production of prostaglandins (PGs) and thromboxanes (Tx) from arachidonic acid. PGs and Txs have diverse biological actions, either proinflammatory or anti-inflammatory. However, as a nonselective COX inhibitor (i.e., inhibits both isoforms of the enzyme, COX-1 and COX-2), DCLF inhibits the synthesis of cytoprotective and

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anti-inflammatory PGs produced by constitutive COX activity as well as products of inducible COX-2, which are mostly proinflammatory. Moreover, the toxic interaction of DCLF with LPS might arise from inflammatory mediators (eg., cytokines) that are not products of the COX pathway and are therefore not inhibited by NSAIDs.

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1.5 Overview of the thesis

1.5.1 Knowledge gaps

As mentioned above, LPS/RAN treatment caused an idiosyncrasy-like liver injury in rats (Luyendyk et al., 2003d). Both PMNs and the hemostatic system have been shown to be important in LPS/RAN-induced liver injury (Luyenkyk et al., 2004; (Luyendyk et al., 2005g). However, how PMNs participate in the liver injury induced by LPS/RAN and their interactions with the hemostatic system have not been elucidated. Moreover, how PMNs are recruited and whether they become activated in the LPS/RAN model remains unknown. Since fibrin deposition and hypoxia appear to be important in the LPS/RAN model, PMNs might contribute to liver injury by interacting with the hemostatic system to cause fibrin deposition. On the other hand, PMN-derived proteases might directly kill hepatocytes in a hypoxic environment. The data presented in this thesis will explore these possibilities. The results will contribute to the understanding of mechanisms by which inflammation acts as a susceptibility factor for toxicity due to drugs and other xenobiotic agents. In addition, the findings could lead to improved strategies for preventing or treating conditions involving PMN-dependent tissue injury.

TNF-α has proved to be important in LPS/RAN-induced hepatotoxicity (Tukov et al., 2007j). Activation of p38 and MAPKAPK-2 can be involved in the production of this and other proinflammatory cytokines. Thus, p38 activation could be a potential upstream signal that leads to production of cytokines/chemokines and to subsequent cascades contributing to LPS/RAN-induced liver injury (Luyendyk et al., 2006g). Hence,

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we investigated the role of P38 activation in LPS/RAN-induced hepatotoxicity. These studies will begin to explore the intracellular signaling crucial to the initiation of LPS/RAN induced-liver injury.

There are no animal models that reproduce DCLF-induced idiosyncratic hepatotoxicity in vivo. Based on the observation that a number of drugs that have the ability to cause IADRs in human patients also cause liver injury in LPS-treated rats, we tested the hypothesis that a modest inflammatory episode in rats increases the sensitivity of the liver to hepatotoxic effects of DCLF. DCLF is often given to patients with inflammatory disease such as arthritis, in which PMNs comprise at least some of the inflammatory infiltrates. Furthermore, PMNs are involved in other idiosyncratic hepatotoxicity models based on LPS-drug interactions. This leads us to hypothesize that PMNs are important in the hepatotoxicity induced by LPS-DCLF interaction.

1.5.2 Hypothesis and specific aims

Overall Hypothesis: Neutrophils are activated in part by p38 dependent TNF production in the livers of rats cotreated with LPS and an IADR-producing drug, and these cells contribute to liver injury by enhancing hemostasis and/or releasing neutrophil proteases. This general hypothesis will be addressed by specific hypotheses represented in 3 specific aims:

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Aim 1 Hypothesis: Neutrophils are activated in the livers of LPS/RAN treated rats and contribute to liver injury by enhancing hemostasis and/or releasing proteases. (Chapter 2)

Aim 2 Hypothesis: p38 contributes to neutrophil activation and subsequent liver injury caused by LPS/RAN cotreatment by inducing TNFα. (Chapter 3)

Aim 3 Hypothesis: LPS cotreament with a nontoxic dose of DCLF causes hepatocellular injury in rats and this LPS/DCLF interaction is PMN dependent. (Chapter 4)

CHAPTER TWO

Xiaomin Deng, James P. Luyendyk, Wei Zou, Jingtao Lu, Ernst Malle, Patricia E. Ganey, Robert A. Roth.(2007) Neutrophil interaction with the hemostatic system contributes to liver injury in rats cotreated with lipopolysaccharide and ranitidine. J Pharmacol Exp Ther. 322 (2): 852-61.

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2.1 Abstract

Cotreatment of rats with nontoxic doses of ranitidine (RAN) and lipopolysaccharide (LPS) causes liver injury, and this drug-inflammation interaction might be a model for idiosyncratic adverse drug responses (IADRs) in humans. Both neutrophils (PMNs) and the hemostatic system have been shown to be important in the injury. We tested the hypothesis that PMNs cause liver injury by interacting with the hemostatic system and producing subsequent hypoxia. In rats cotreated with LPS/RAN, PMN depletion by anti-PMN serum reduced fibrin deposition and hypoxia in the liver. PMN depletion also reduced the plasma concentration of active plasminogen activator inhibitor-1 (PAI-1), a major downregulator of the fibrinolytic system. This suggests that PMNs promote fibrin deposition by increasing PAI-1 concentration. PMNs were activated in the livers of LPS/RAN-cotreated rats as evidenced by increased staining for hypochlorous acid (HOCl)-modified proteins generated by the myeloperoxidase-hydrogen peroxide-chloride system of activated phagocytes. Antiserum against the PMN adhesion molecule CD18 protected against LPS/RAN-induced liver injury. Since CD18 is important for PMN transmigration and activation, these results suggest that PMN activation is required for the liver injury. Furthermore, anti-CD18 serum reduced biomarkers of hemostasis and hypoxia, suggesting the necessity for PMN activation in the interaction between PMNs and the hemostatic system/hypoxia. Liver injury, liver fibrin and plasma PAI-1 concentration were also reduced by eglin C, an inhibitor of proteases released by activated PMNs. In summary, PMNs are activated in LPS/RAN-cotreated rats and

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participate in the liver injury in part by contributing to hemostasis and hypoxia.

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2.2 Introduction

In rats, cotreatment with lipopolysaccharide (LPS) and ranitidine (RAN) causes liver injury resembling hepatotoxic idiosyncratic adverse drug responses (IADRs) that RAN causes in humans (Luyendyk et al., 2003e). Both polymorphonuclear neutrophils (PMNs) and the hemostatic system are important in LPS/RAN-induced liver injury (Luyendyk et al., 2005h;Luyendyk et al., 2004h). Hemostasis-associated fibrin deposition likely contributes to injury in this model by causing liver hypoxia (Luyendyk et al., 2005i).

The hemostatic system is tightly regulated by the interplay between the coagulation and fibrinolytic systems (Lasne et al., 2006). Tissue factor is the principal initiator of the coagulation system, a complex cascade that ultimately generates active thrombin. Thrombin cleaves circulating fibrinogen into fibrin monomers, which upon cross-linking and polymerization can form obstructive clots in blood vessels. Plasminogen activators (PAs), including urokinase and tissue-specific PA, are important proteolytic activators of plasmin, which cleaves and dissolves crosslinked fibrin. The activity of plasminogen activators is inhibited by plasminogen activator inhibitor-1 (PAI-1) (Keller et al., 2006;Padro et al., 1997a).

PMNs usually require transmigration across the endothelial barrier and subsequent activation to kill pathogens or injure tissues (Springer, 1995). These cytotoxic effects are mediated in part by release of reactive oxygen species and/or granular proteases (Jaeschke et al., 1996b). PMN derived-proteases such as elastase and cathepsin G kill hepatocytes directly in vitro (Hill and Roth, 1998;Ho et al., 1996b). Moreover, the killing

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of hepatocytes by PMN-derived proteases is potentiated by hypoxia (Luyendyk et al., 2005j).

In the LPS/RAN model of hepatotoxic drug-inflammation interaction, PMNs accumulate in liver, but how they participate in the pathogenesis and their relationship to the hemostatic system are unknown. Here we tested the hypothesis that hepatic PMNs are activated in the livers of LPS/RAN-cotreated rats and contribute to liver injury by releasing proteases and interacting with the hemostatic system.

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2.3 Methods

2.3.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Two lots of LPS derived from Escherichia Coli serotype O55:B5 (catalog number L-2880) with activities of 6.6 x 106 EU/mg (lot number 51K4115) and 13 x 106 EU/mg (lot number 43K4112) were used for these studies. These activities were determined using a QCL Chromogenic LAL endpoint assay purchased from Cambrex Bio Science, Inc. (Baltimore, MD).

2.3.2 Animals

Male, Sprague-Dawley rats [Crl:CD (SD)IGS BR; Charles River Breeding Laboratories, Portage, MI] weighing 250 to 350 g were fed standard chow (rodent chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum. They were allowed to acclimate for 1 week in a 12h light/dark cycle prior to use.

2.3.3. Experimental Protocol

Rats fasted for 24hr were given 2.5 x 106 EU/kg LPS (lot 43K4112) or its saline vehicle (Veh) i.v. at 5ml/kg, and food was then returned. For the 6hr CD18 antiserum study, 44.4 x 106 EU/kg LPS (lot 51K4115) or its vehicle at 2ml/kg was given. These two LPS doses from different lots render approximately the same degree of hepatocellular injury in terms of serum alanine aminotransferase (ALT) activity. Two

hours later, 30 mg/kg RAN or its vehicle [sterile phosphate-buffered saline (PBS)] was administered (i.v.). RAN solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. Two, three or six hr after RAN administration, rats were anesthetized with sodium pentobarbital (75 mg/kg i.p.). Plasma was collected by drawing 2 ml of blood from the vena cava into a syringe containing sodium citrate (final concentration, 0.38%). Another portion of blood was collected and allowed to clot at room temperature; serum was prepared from this fraction and stored at -20°C until use. Representative slices (3–4-mm thick) of the ventral portion of the left lateral liver lobe were collected and fixed in 10% (v/v) neutral buffered formalin. For immunohistochemical analysis, a portion of the left medial lobe of the liver was flash-frozen in isopentane cooled by liquid nitrogen.

2.3.4 PMN Depletion, CD18 Neutralization and Eglin C Treatment

PMN depletion was accomplished by administration of a PMN antiserum before treatment with LPS/RAN. Rats were given 0.25 ml of either normal rabbit serum (CS) or rabbit anti-rat PMN serum (NAS) (Intercell Technologies, Jupiter, FL) diluted 1:1 in sterile saline (0.5 ml per rat, i.v.) 18 hr before administration of LPS. A previous study in which anti-PMN serum was administered to rats demonstrated a selective and complete depletion of PMNs (Snipes et al., 1995). Rats were killed 2 and 6 hr after treatment with RAN, and citrated plasma was collected. Total blood leukocytes were quantified using a Unopette white blood cell determination kit (BD Biosciences, San Jose, CA) and a

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hemocytometer. Slides were prepared from whole blood and stained using the Hema 3 staining system (Fisher Diagnostics, Middletown, VA), and differential counting was performed.

For the CD18 neutralization study, rabbit anti-CD18 serum raised against rat CD18 specific peptide (Ac-CLKFDKGPFQKN-amide) was obtained from New England Peptide (Gardner, MA). The anti-CD18 serum or normal rabbit serum was diluted 1:1 (v/v) in sterile saline and administered (0.5ml per rat, i.v.) immediately after LPS injection. Rats were killed 6hr after RAN treatment for evaluation of serum ALT activity, plasma PAI-1 concentration, liver PMN accumulation and fibrin. Rats were killed 3hr after RAN for hypoxia and PMN activation evaluation. For the eglin C study, eglin C (provided by Novartis Pharm AG, Basel, Switzerland) was given at 8mg/kg (i.v.) immediately after RAN, and 2hr and 4hr later. Rats were killed 6hr after RAN treatment.

2.3.5 Hepatotoxicity Assessment

Hepatic parenchymal cell injury was estimated as an increase in serum ALT activity. ALT activity was determined spectrophotometrically using Infinity-ALT reagent from Thermo Electron Corporation (Louisville, CO). Previous studies in this LPS/RAN model have shown that serum ALT activity reflects histopathologic evidence of hepatocellular necrosis (Luyendyk et al., 2003f).

2.3.6 Fibrin Immunohistochemistry and Quantification

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Fibrin immunohistochemistry and quantification were performed as described previously (Copple et al., 2002a). This protocol solubilizes all fibrinogen and fibrin except for cross-linked fibrin (Schnitt et al., 1993a). Therefore, only cross-linked fibrin stains in sections of liver. The positive area fraction refers to the percentage of area with positive staining in the total microscopic field.

2.3.7 Evaluation of Coagulation System Activation and Plasma PAI-1

Plasma fibrinogen was determined from thrombin clotting time of diluted samples using a fibrometer and a commercially available kit (B4233) from Dade Behring Inc. (Deerfield, IL). Plasma thrombin-antithrombin (TAT) concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using a kit from Dade Behring Inc. (catalog number OWMG15). Total plasma PAI-1 concentration was evaluated using a commercially available ELISA purchased from American Diagnostica Inc. (Greenwich, CT). This ELISA measures PAI-1 in active, inactive, and plasminogen activator/PAI-1 complexed forms. The concentration of functionally active PAI-1 in plasma was assessed using a commercially available ELISA kit purchased from Molecular Innovations Inc. (Southfield, MI).

2.3.8 Evaluation of Liver Hypoxia

Liver hypoxia was evaluated by immunohistochemical staining for pimonidazole (PIM) adducts. PIM is a 2-nitroimidazole marker of hypoxia and has been used to

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identify regions of hypoxia in liver (Arteel et al., 1995;Arteel et al., 1998b). Unless otherwise noted, rats were given 120 mg/kg Hypoxyprobe-1 (PIM hydrochloride; Chemicon International, Temecula, CA) i.p. 2 hr before they were killed. PIM-adduct immunostaining was performed as described previously (Copple et al., 2004). Quantification of immunostaining was performed using Scion Image Beta 4.0.2 software (Scion Corporation, Frederick, MD). Background was set to be the average pixel intensity in periportal regions of Veh/Veh-treated livers (i.e., an area where no hypoxia occurs) (Arteel et al., 1998a). An increase in positive immunostaining for PIM-modified proteins indicates hypoxia in the liver tissue.

2.3.9 Evaluation of Circulating Leukocytes, PMNs, Liver PMNs and PMN Activation.

Total blood leukocyte count was quantified using a Unopette white blood cell determination kit (BD Biosciences, San Jose, CA) and a hemocytometer. Slides were prepared from whole blood and stained using the Hema 3 staining system (Fisher Diagnostics, Middletown, VA), and differential counting was performed. PMNs accumulated in liver were visualized by immunohistochemical staining and quantified as described previously (Yee et al., 2003b). PMN activation was measured by staining of hypochlorous acid (HOCl)-protein adducts in liver. The monoclonal antibody (clone 2D10G9, subtype IgG2bk) is specific for hypochlorous acid (HOCl)-modified epitopes generated in vivo (Malle et al., 1997a) and in vitro (Malle et al., 1995a) and does not

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crossreact with other oxidative protein modifications. Frozen sections of liver were fixed in 4% (v/v) formalin for 10 minutes at room temperature with gentle rocking. The slides were washed 3 times, 5 minutes each, with PBS (phosphate-buffered saline), then blocked for 1 hour at room temperature with 3% goat serum in PBS. Antibody (diluted 1:1 in 3% goat serum) was added and incubated for 2 hr at room temperature with gentle rocking. The slides were washed 3 times, 5 minutes each, with PBS. Alexa Fluro 488-labeled goat anti-mouse secondary antibody (diluted 1:500 in 3% goat serum, Molecular Probes, Carlsbad, California) was applied, and the slides were incubated for 3 hr at room temperature. After washing 3 times, 5 minutes each, with PBS, they were examined microscopically. Staining was quantified as for fibrin staining described above and presented as positive area fraction. The integrated density within areas of positive staining was calculated as the sum of the products of pixel gray intensity and pixel numbers with that intensity.

2.3.10 Evaluation of PMN Protease Effects on Fibrinogen and PAI-1 in vitro

Normal rat plasma was incubated with a mixture of elastase and cathepsin G or vehicle (PBS) at two different conditions: elastase (0.04U/mL)/cathepsin G (4ug/ml) and elastase (8U/ml)/ cathepsinG (4ug/ml). Elastase activity was determined using the colorometric PMN elastase substrate, MeOSuc-Ala-Ala-Pro-Val-pNA (Calbiochem, San Diego, CA). One unit of PMN elastase activity was defined as the amount of enzyme required to cause a change of absorbance of 1.0 at 410 nm in 10 min at 37°C. After 2hr

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and 6hr incubation, fibrinogen was measured by fibrometer as described above. Purified PAI-1 purchased from American Diagnostica Inc. (Greenwich, CT) was incubated with elastase and cathepsin G under the same conditions, and the reaction was stopped by adding anti-trypsin (40nM) after 2 or 6hr. The total PAI-1 level was measured by PAI-1 ELISA as described above. For the SDS-PAGE analysis, purified fibrinogen (250ug/ml) was incubated with PBS, thrombin (0.1U/ml), elastase (8U/ml)/cathepsin G (4ug/ml) or elastase (0.04U/ml)/cathepsin G (4ug/ml). The reaction was stopped by adding1% SDS and 2% 2-mercaptoethanol after 2 or 6hr. The resulting protein products were separated on 4-12% gradient SDS-PAGE and visualized with silver staining (Biorad, Hercules, CA).

2.3.11 Statistical Analysis

For the PMN depletion study and in vitro study, two way ANOVA was applied with time and treatment as the two factors. For the CD18 neutralization and eglin C study, two way ANOVA was used with LPS/RAN and drug (anti-CD18 serum or eglin C) as the two factors. For the ALT data from the eglin C study, a student's t-test was applied. Tukey test was used as post-hoc analysis for ANOVA. The criterion for statistical significance for all studies was $p<0.05$.

Time after RAN	Treatment	Leukocytes(#/ul)	PMNs(#/ul)
2hr	CS/Veh/Veh	4012+457	776+277
	CS/LPS/RAN	4640+628	2482+661*
	NAS/LPS/RAN	1560+181* #	75+17* #
6hr	CS/Veh/Veh	4138+819	497+153
	CS/LPS/RAN	4236+591	2594+559
	NAS/LPS/RAN	1812+368* #	344+121#

Table 2.1. Circulating leukocytes after PMN antiserum treatment in LPS/RAN-treated rats. PMN depletion of LPS/RAN-treated rats was accomplished as in Methods. Circulating total leukocytes and PMNs were measured at 2hr and 6hr after LPS/RAN treatment as described in Methods. *significantly different from CS/Veh/Veh group at the same time. #significantly different from CS/LPS/RAN group at the same time. $P<0.05$, n=4-7.

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2.4 Results

2.4.1 PMN depletion studies in the LPS/RAN model.

To assess the role of PMNs in hemostasis and hepatocellular injury, PMN numbers were reduced prior to LPS/RAN-cotreatment. Rabbit anti-rat PMN antiserum (NAS) or its control normal rabbit serum (CS) was administered 16 h before LPS treatment. Circulating total leukocytes and PMN and liver PMNs were evaluated at 2hr and 6hr after RAN treatment. As shown previously (Luyendyk et al., 2005k), the antiserum markedly reduced blood PMNs but did not significantly affect other blood leukocytes at either 2hr or 6hr (Table. 2.1). It also significantly reduced liver PMN accumulation caused by LPS/RAN treatment at both 2hr and 6hr (data not shown). In addition, the antiserum produced a qualitative change in liver PMNs, causing a shift toward the hepatic accumulation of immature PMNs (i.e. band cells). Antiserum alone did not cause serum ALT activity to increase either at 2hr or 6hr. Serum ALT activity was unaffected by LPS/RAN treatment at 2hr after RAN administration but was significantly elevated by 6hr (Fig. 2.1). Treatment with the antiserum significantly decreased ALT activity in the serum of LPS/RAN-treated rats at 6 hr after RAN, confirming previous results (Luyendyk et al., 2005l).

CS/LPS/RAN treatment caused a significant increase in liver fibrin compared to CS/Veh/Veh control both at 2hr and 6hr (Fig. 2.2). NAS pretreatment did not affect fibrin deposition at 2hr after RAN but significantly reduced it at 6hr. Activation of the coagulation system leads to a decrease in plasma fibrinogen, as this protein is cleaved to

Figure 2.1. Effects of PMN depletion on hepatotoxicity induced by LPS/RAN. PMN antiserum (NAS) or control normal serum (CS) was administered 16 hr before LPS or its vehicle. Two hours after LPS treatment, RAN or its vehicle was administered. Serum ALT activity was measured 2hr and 6hr after administration of RAN or its vehicle. * significantly different from the respective group without LPS/RAN at the same time. # significantly different from the respective group without CS at the same time. a, significantly different from the same treatment group at 2hr. $P < 0.05$, n= 4-7.

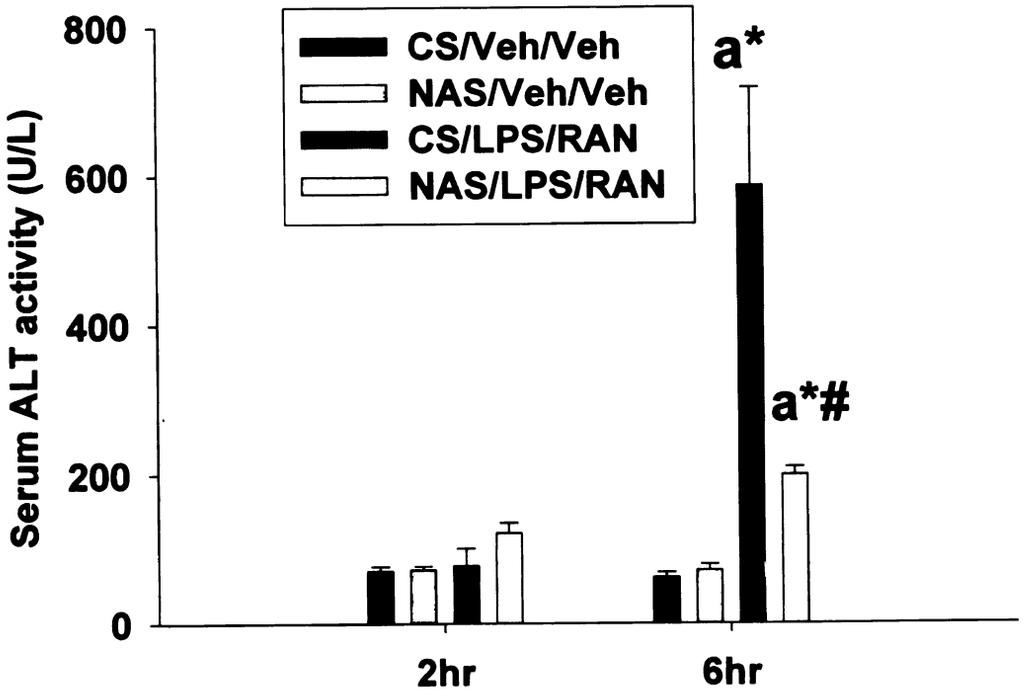

800
600
400
200
0
Serum ALT activity (U/L)
CS/Veh/Veh
NAS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN
a*
a*#
2hr
6hr

Figure 2.2. Effects of PMN depletion on liver fibrin after LPS/RAN treatment. PMN depletion of LPS/RAN-treated rats was accomplished as in Fig 2.1. Fibrin staining was performed 2hr and 6hr after treatment with RAN or its vehicle treatment. * significantly different from CS/Veh/Veh group at the same time. # significantly different from CS/LPS/RAN group at the same time. $P< 0.05$, n= 4-7.

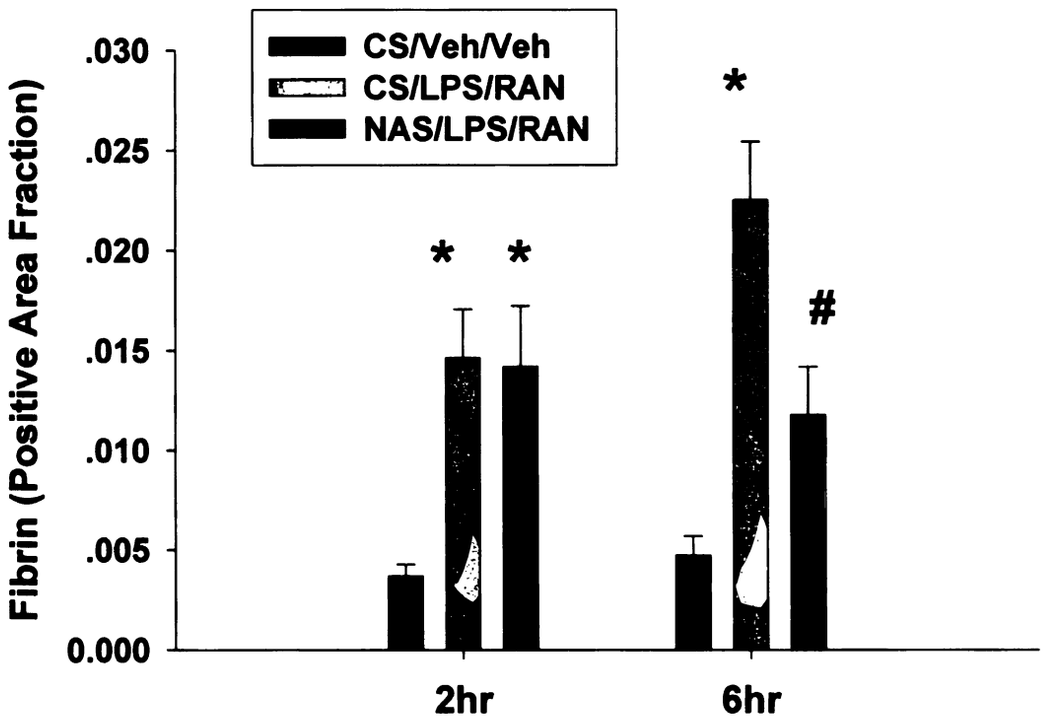

CS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN
Fibrin (Positive Area Fraction)
.030
.025
.020
.015
.010
.005
0.000
*
*
*
#
2hr
6hr

Figure 2.3. Effects of PMN depletion on coagulation system activation after LPS/RAN treatment. PMN depletion of LPS/RAN-treated rats was accomplished as in Fig 2.1. Plasma fibrinogen and TAT concentration were measured 2hr and 6hr after RAN or vehicle treatment. * significantly different from CS/Veh/Veh group at the same time. # significantly different from CS/LPS/RAN group at the same time. $P < 0.05$, n= 4-7.

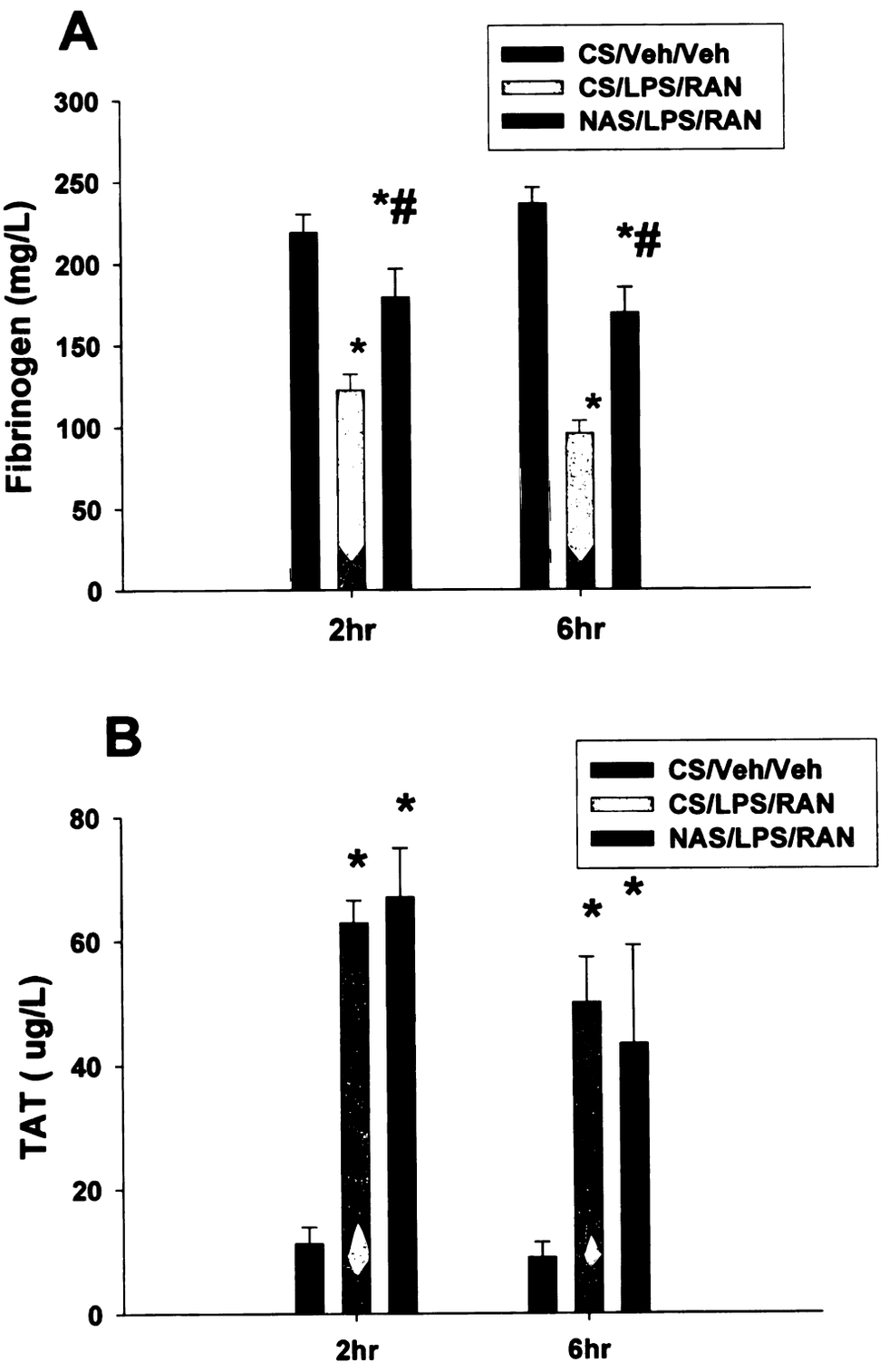
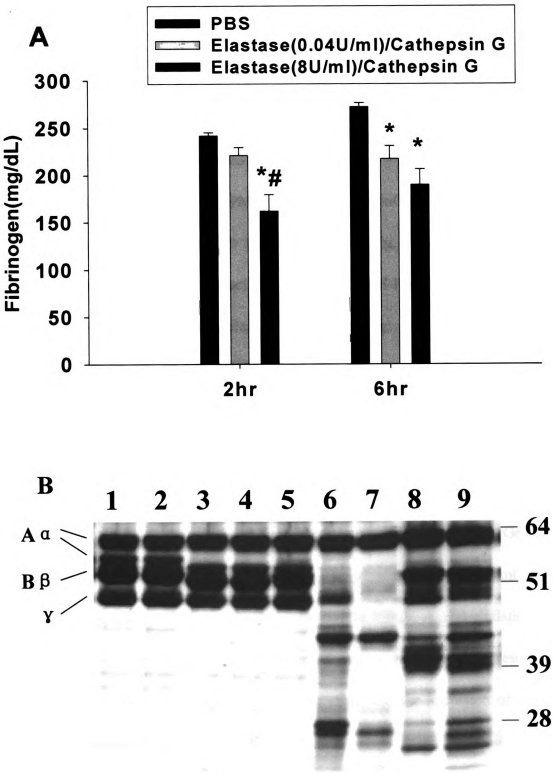

A
CS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN
Fibrinogen (mg/L)
300
250
200
150
100
50
0
*#
*
*#
*
2hr
6hr
B
CS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN
TAT (ug/L)
80
60
40
20
0
*
*
*
*
2hr
6hr

Figure 2.4. Effects of PMN proteases on plasma fibrinogen in vitro. A: elastase and cathepsin G or their vehicle (PBS) was incubated with plasma fibrinogen as described in Methods. Fibrinogen was measured using a fibrometer 2hr and 6hr later. *, significantly different from PBS group. #, significantly different from elastase (0.04U/ml)/cathepsin G treatment. $P< 0.05$, $n= 4$. B: thrombin, elastase/cathepsin G or their vehicle (PBS) was incubated with purified fibrinogen, and the protein products were analyzed by SDS-PAGE. Lane 1,2: fibrinogen+PBS, 2hr and 6hr respectively; lane 3,4,5: fibrinogen + thrombin, 2hr; lane 6,7: fibrinogen + elastase(8U/ml) /cathepsin G(4ug/ml), 2hr and 6hr; lane 8,9: fibrinogen + elastase(0.04U/ml)/cathepsin G(4ug/ml), 2hr and 6hr. Molecular weight was marked at the right margin (kDa).


A
PBS
Elastase(0.04U/ml)/Cathepsin G
Elastase(8U/ml)/Cathepsin G
Fibrinogen(mg/dL)
300
250
200
150
100
50
0
*#
*
*
2hr
6hr
B
1 2 3 4 5 6 7 8 9
A α
B β
γ
64
51
39
28

form insoluble fibrin clots. At both 2hr and 6hr, LPS/RAN treatment decreased plasma fibrinogen and increased plasma TAT, a biomarker of coagulation system activation. This suggests early and persistent activation of the coagulation system (Fig. 2.3). In PMN-depleted rats, there was a more modest decrease in plasma fibrinogen (Fig. 3A), but the elevation in TAT was unaffected (Fig. 2.3B).

In vitro, incubation of PMN elastase/cathepsinG with fibrinogen reduced fibrinogen concentration after 2 or 6hr of incubation (Fig. 2.4A). These results suggest that PMN proteases cleave and inactivate fibrinogen. SDS-PAGE of fibrinogen produced three predominant bands identified by silver staining, with molecular weights of approximately 62 kD, 53 kD, 47 kD (Fig. 2.4B). These are likely the Aα (62Kd), Bβ (53Kd) and γ chains (47Kd) of fibrinogen as described by Gorkun et al (Gorkun et al., 1997). There was also a minor band, which is likely the smaller Aα chain lacking a C-terminal fragment. Thrombin treatment reduced the size of the smaller Aα and Bβ chains, probably due to the release of fibrin peptides A and B, respectively (Gorkun et al., 1994;Weisel et al., 1993). Elastase (0.04U/ml) and cathepsin G (4ug/ml) treatment caused the degradation of the smaller Aα chain and the formation of degradation products of smaller size. Increasing the concentration of elastase in the mixture resulted in the degradation of Bβ, γ and the smaller size Aα chain and the formation of degradation products of smaller size.

PAI-1 is the major enodogenous downregulator of the fibrinolytic system, which lyses insoluble fibrin clots and thereby controls the degree of fibrin deposition. Total

PAI-1 includes active PAI-1, inactive PAI-1 and PAI-1 complexed with plasminogen activator. LPS/RAN treatment caused a significant increase in the plasma concentrations of both total and active PAI-1 at 2hr and 6hr after RAN (Fig. 2.5). PMN depletion did not affect total plasma PAI-1 concentration at 6hr but increased it at 2hr (Fig. 2.5A). The increase in total plasma PAI-1 concentration with PMN depletion suggested that PMN-derived proteases might degrade PAI-1. Indeed, incubation of purified PAI-1 with elastase/cathepsinG reduced total PAI-1 concentration (Fig. 2.6). On the other hand, PMN depletion did not significantly affect active PAI-1 concentration at 2hr but decreased it at 6hr (Fig. 2.5B).

Since fibrin deposition and hemostasis can lead to tissue hypoxia, immunohistochemistry of pimonidazole (PIM)-bound protein was used as a marker for liver hypoxia. LPS/RAN treatment caused a significant increase in PIM-protein adduct staining at 2hr after RAN, and this was prevented by NAS treatment (Fig. 2.7). Hypoxia staining in LPS/RAN treated livers tended to be panlobular, as compared to the necrotic foci which were predominately midzonal.

2.4.2 HOCl-protein adduct staining.

Activation of PMNs is required for many of their damaging effects on host tissue. The potent oxidant HOCl, generated in vivo by the myeloperoxidase-hydrogen peroxide-chloride system of activated phagocytes, reacts with proteins to form chloramines that may be used as a biomarker for PMN activation. Neither LPS alone nor

Figure 2.5 Effects of PMN depletion on plasma PAI-1 concentration after LPS/RAN treatment. PMN depletion of LPS/RAN-treated rats was accomplished as in Fig 2.1. Plasma active PAI-1 (A) and total PAI-1 (B) was measured 2hr and 6hr after LPS/RAN treatment as described in Methods. * significantly different from CS/Veh/Veh group at the same time. # significantly different from CS/LPS/RAN group at the same time. a, significantly different from the same treatment group at 2hr. $P< 0.05$, n= 4-7.

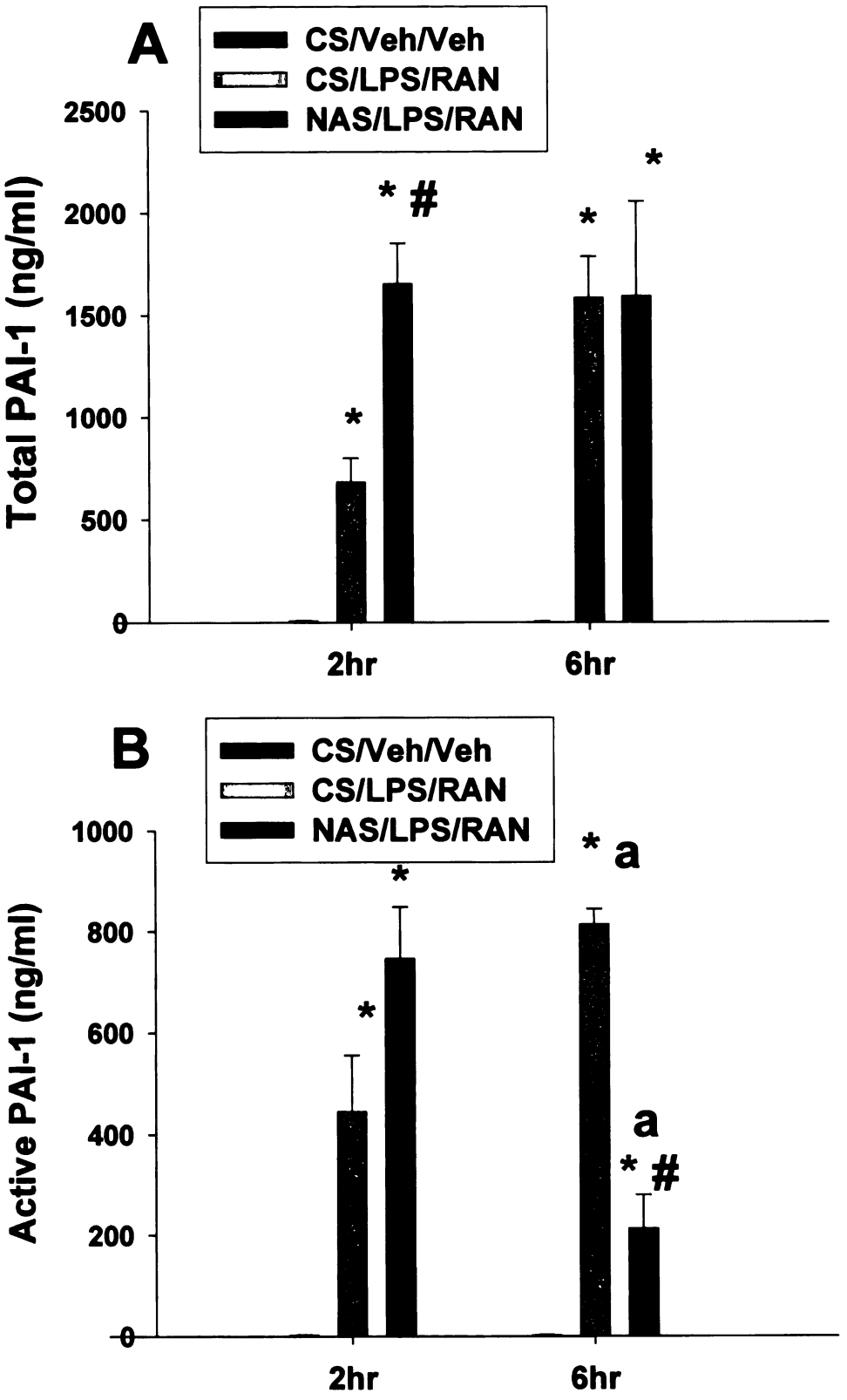

A
CS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN
Total PAI-1 (ng/ml)
2500
2000
1500
1000
500
0
2hr
6hr
B
CS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN
Active PAI-1 (ng/ml)
1000
800
600
400
200
0
2hr
6hr

Figure 2.6. Effects of PMN proteases on PAI-1 concentration in vitro. Elastase and cathepsin G or their vehicle was incubated with purified PAI-1 as described in Methods. Total PAI-1 concentration was measured by ELISA 2hr and 6hr later. * significantly different from vehicle group. # significantly different from elastase (0.04U/ml)/Cathepsin G treatment. $P< 0.05$, $n= 4$.

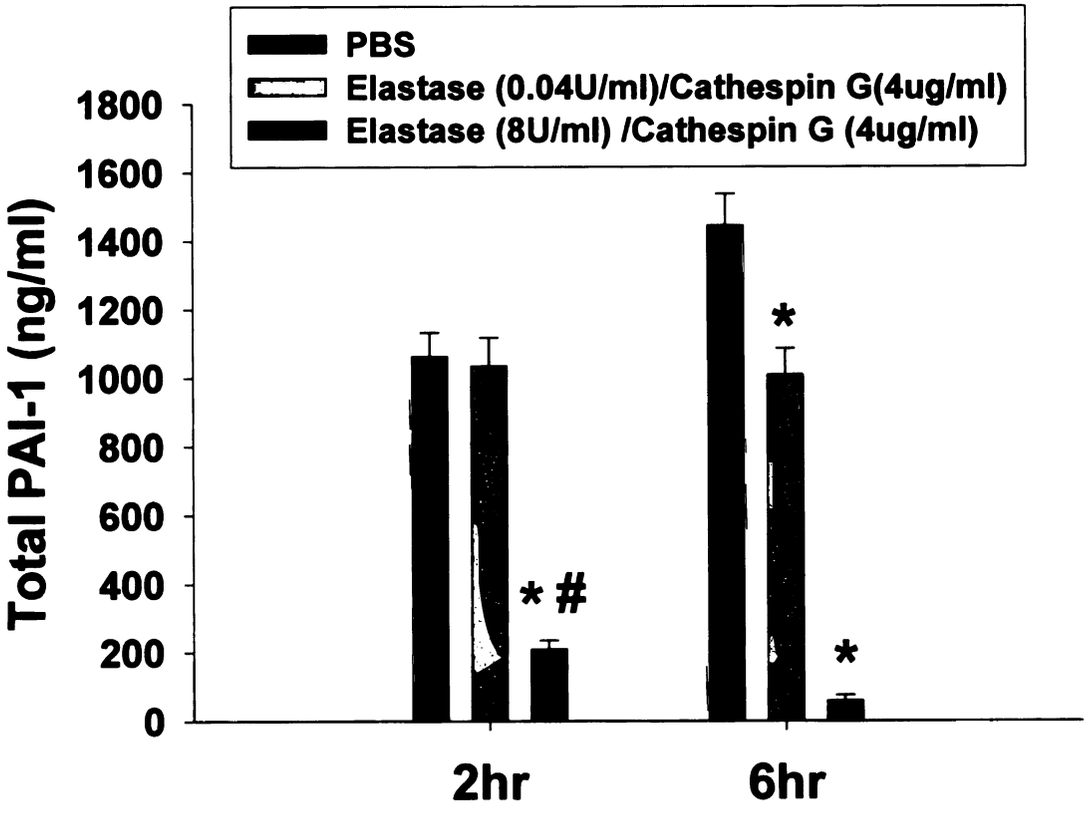
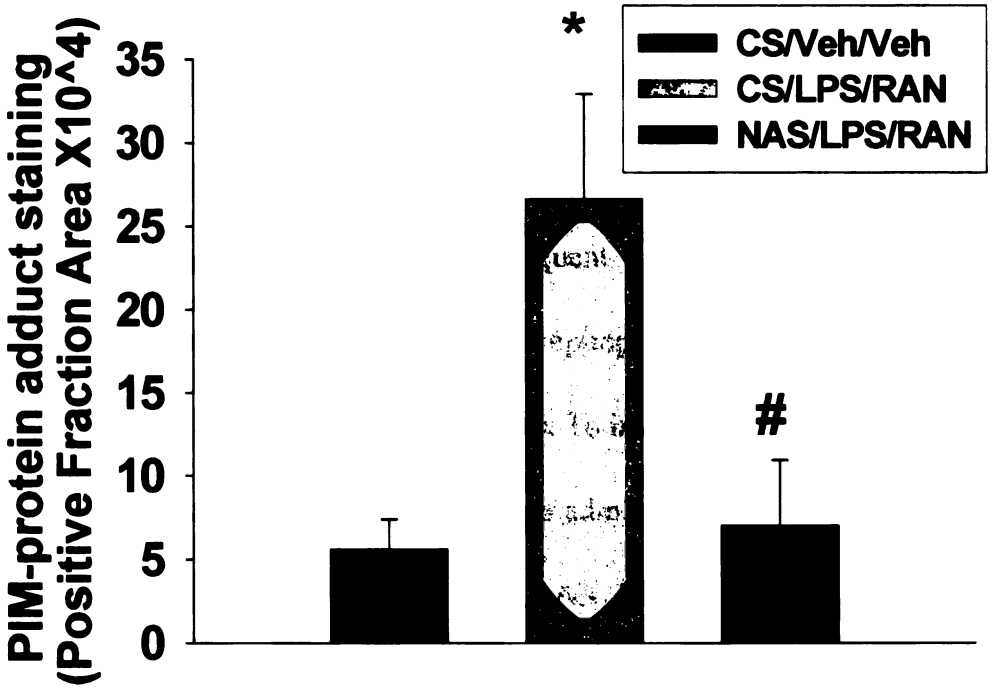

PBS
Elastase (0.04U/ml)/Cathespin G(4ug/ml)
Elastase (8U/ml) /Cathespin G (4ug/ml)
Total PAI-1 (ng/ml)
1800
1600
1400
1200
1000
800
600
400
200
0
* #
*
*
2hr
6hr

Figure 2.7. Effect of PMN depletion on liver hypoxia after LPS/RAN treatment. PMN depletion of LPS/RAN-treated rats was accomplished as in Fig 2.1. PIM was administered to rats 1hr after RAN, and the rats were killed at 2hr after RAN. * significantly different from CS/Veh/Veh group. # significantly different from CS/LPS/RAN group. $P< 0.05$, n= 4-7.

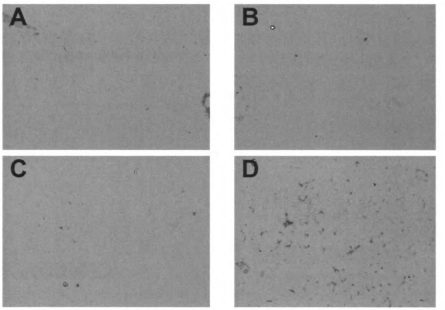

PIM-protein adduct staining
(Positive Fraction Area X10^4)
35
30
25
20
15
10
5
0
*
#
CS/Veh/Veh
CS/LPS/RAN
NAS/LPS/RAN

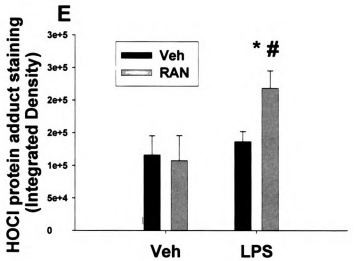
RAN alone affected HOCl-protein adduct staining (Fig. 2.8B,C,E). LPS/RAN cotreatment increased the staining, indicating the activation of PMNs in LPS/RAN-treated livers (Fig. 2.8D,E). The HOCl-protein epitopes were predominatly within the midzonal areas of liver lobules, which was where the necrotic foci occurred in livers of LPS/RAN-cotreated rats. This increase was apparent 3-6hr after RAN but not at 2hr (Fig. 2.8F).

2.4.3 Effects of CD18 neutralization on hepatotoxicity, PMNs and hemostasis.

CD18 is an adhesion molecule expressed on PMN plasma membranes that is important for the transmigration and subsequent activation of these cells (Springer, 1994b). Based on staining for HOCl-modified epitopes (Fig. 2.8), it was clear that PMNs are activated in livers of LPS/RAN-treated rats. To investigate the role of PMN activation in the LPS/RAN model, anti-CD18 serum was administered immediately after LPS, and various biomarkers were evaluated 6hr after RAN. The anti-CD18 serum did not reduce the number of circulating PMNs after LPS/RAN treatment (data not shown). LPS/RAN caused an increase in PMNs accumulated in liver (Fig. 2.9A). Anti-CD18 serum alone caused an increase in the number of PMNs in livers of Veh/Veh-treated rats but did not affect hepatic PMN accumulation after LPS/RAN treatment. Anti-CD18 serum reduced staining for HOCl-modified epitopes at 3hr after RAN (Fig. 2.9B) and protected from LPS/RAN-induced hepatotoxicity, as reflected by reduction of serum ALT activity (Fig.

Figure 2.8. PMN activation after LPS/RAN treatment. Rats were treated with LPS or vehicle 2hr before RAN or its vehicle treatment. HOCl-protein protein staining was performed 3hrs after treatment with RAN or vehicle. The representative photomigraphs for individual treatment group are presented in gray scale. A: Veh/Veh; B: Veh/RAN; C: LPS/Veh; D: LPS/RAN; E: Quantification of staining for all four treatment groups. * significantly different from the respective group without LPS. # significantly different from the respective group without RAN. F: Rats were treated with LPS/RAN or Veh/Veh as described in Fig 2.1. HOCl-modifed protein staining was performed 2hr, 3hrs, 4hrs and 6hr after LPS/RAN treatment. The Veh/Veh-treated animals were killed at 2hr after RAN for HOCl-protein adduct staining. * significantly different from Veh/Veh group. $P<0.05$, n=5-7.


A
B
C
D


E
HOCl protein adduct staining
(Integrated Density)
3e+5
3e+5
2e+5
2e+5
1e+5
5e+4
0
Veh
RAN
* #
Veh
LPS

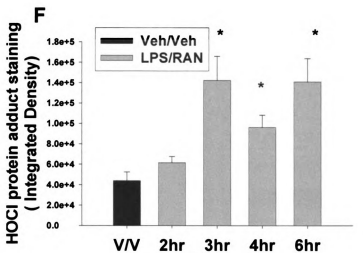

F
HOCl protein adduct staining
(Integrated Density)
1.8e+5
1.6e+5
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
Veh/Veh
LPS/RAN
*
*
*
V/V
2hr
3hr
4hr
6hr

Figure 2.9. Effects of CD18 neutralization on biomarkers of hemostasis, PMNs and hepatotoxicity in LPS/RAN-cotreated rats. Rabbit anti-CD18 serum or normal serum was administered at the same time as LPS or its vehicle. RAN or its vehicle was administered 2hr after LPS treatment. A: Liver PMN accumulation; B: HOCl-protein adduct staining; C: Serum ALT activity; D: Liver fibrin; E: Plasma total PAI-1 and F: Plasma active PAI-1 were evaluated 6hr after RAN or its vehicle treatment except the HOCl-protein adduct staining was evaluated 3hrs after RAN treatment. * significantly different from the respective group without LPS/RAN. # significantly different from the respective group without anti-CD18 serum. $P<0.05$, n=4-7.

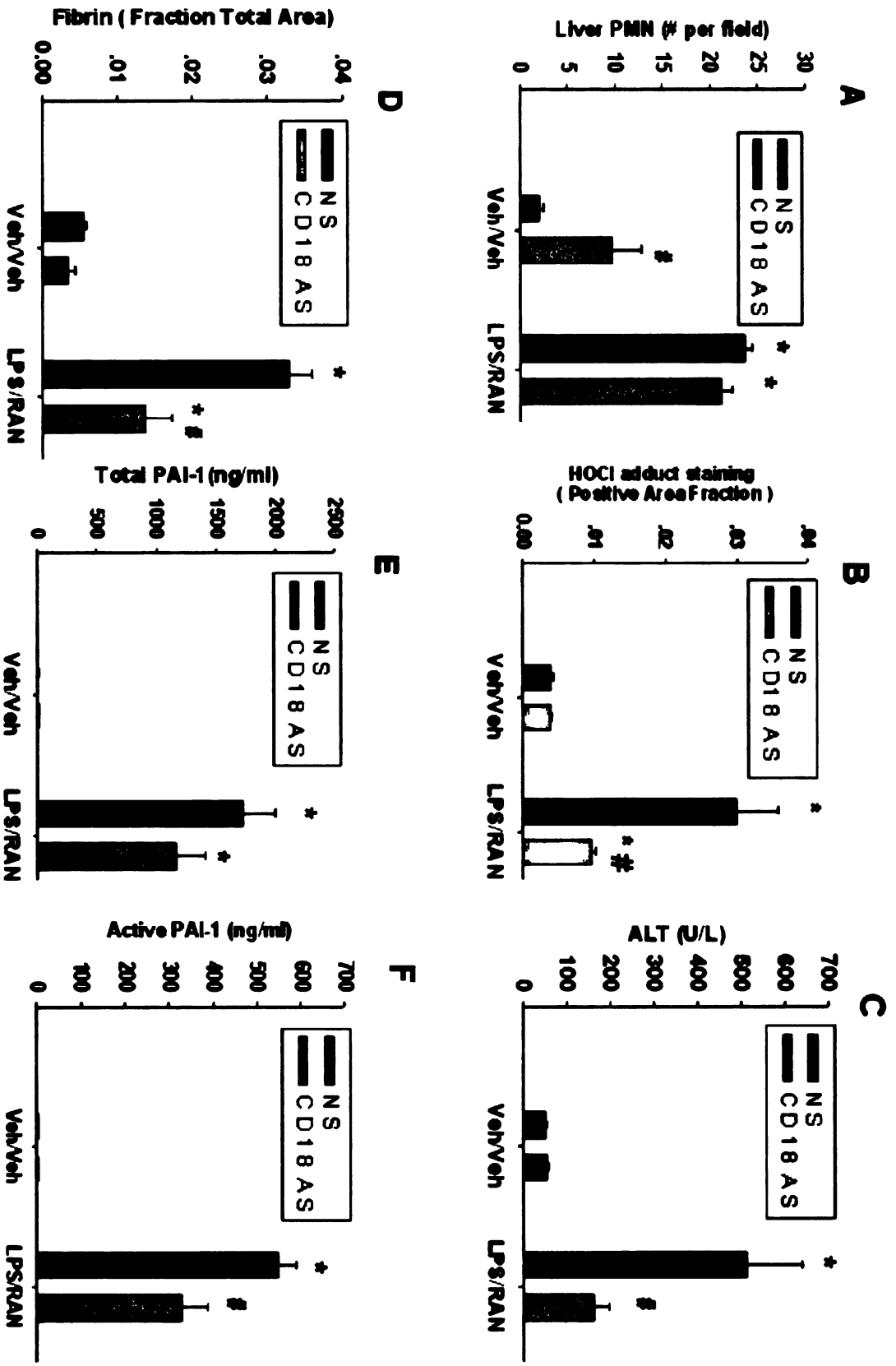
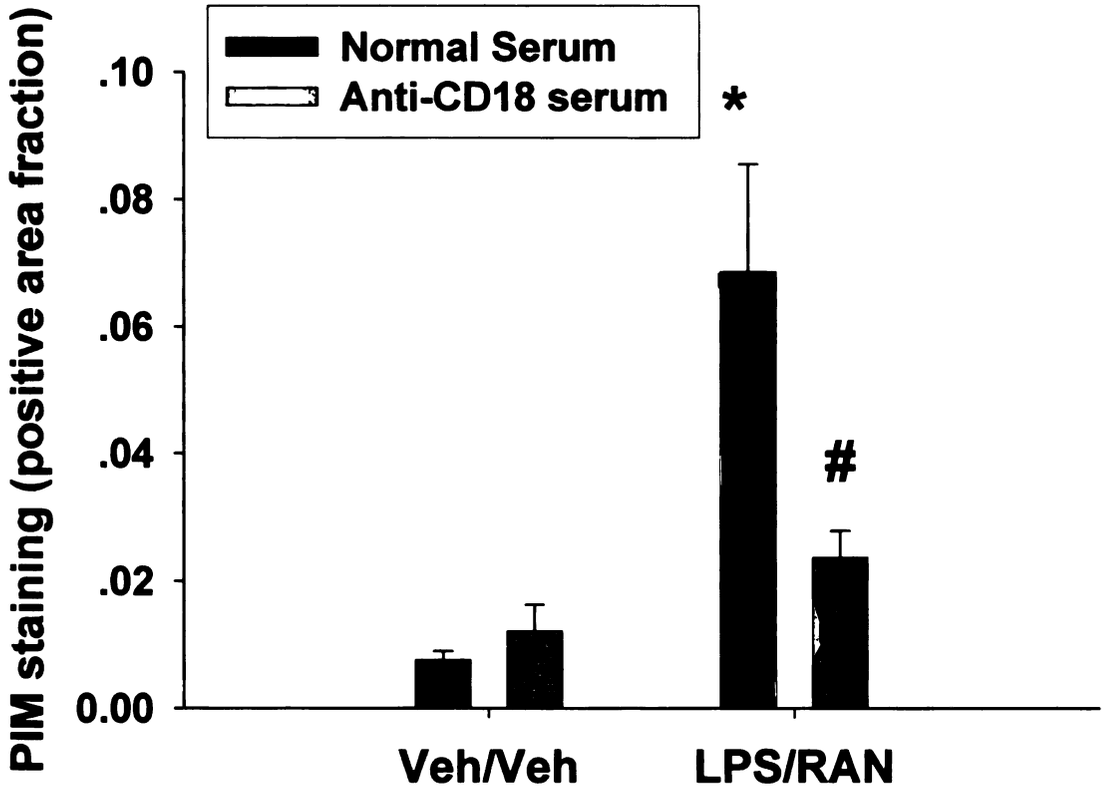

A
Liver PMN (# per field)
0
5
10
15
20
25
30
NS
CD18 AS
Veh/Veh
LPS/RAN
B
HOCl adduct staining
(Positive Area Fraction)
0.00
.01
.02
.03
.04
C
ALT (U/L)
0
100
200
300
400
500
600
700
D
Fibrin (Fraction Total Area)
E
Total PAI-1 (ng/ml)
0
500
1000
1500
2000
2500
F
Active PAI-1 (ng/ml)

Figure 2.10. Effects of CD18 neutralization on liver hypoxia after LPS/RAN treatment. CD18 neutralization in LPS/RAN-treated rats was accomplished as described in Fig 2.9. PIM probe was administered 1hr after RAN treatment, and PIM-protein adduct staining was performed 3hrs after RAN treatment. * significantly different from the respective group without LPS/RAN. # significantly different from the respective group without anti-CD18 serum. $P<0.05$, n=4-7.


Normal Serum
Anti-CD18 serum
*
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PIM staining (positive area fraction)
.10
.08
.06
.04
.02
0.00
Veh/Veh
LPS/RAN

2.9C). In addition, anti-CD18 serum decreased liver fibrin at 6hr after RAN treatment (Fig. 2.9D).

As reported above (Fig. 2.9E and 9F), LPS/RAN treatment increased both total PAI-1 and active PAI-1 concentration 6hr after RAN. Anti-CD18 serum did not affect the increase in total PAI-1 in plasma but reduced active PAI-1 concentration. As reported previously, liver hypoxia occurred 3hr after LPS/RAN treatment, as marked by increased PIM protein adduct staining (Luyendyk et al., 2005 and Fig. 2.10). Cotreatment with anti-CD18 serum prevented the liver hypoxia.

2.4.4 Effects of eglin C on serum ALT activity and hemostasis

Eglin C is a peptide inhibitor of both PMN elastase and cathepsin G (Braun et al., 1987). It was used to investigate the contribution of PMN proteases to LPS/RAN-induced hepatotoxicity and biomarkers of hemostasis. Eglin C administration significantly reduced serum ALT activity, indicating attenuation of hepatocellular injury (Fig. 2.11). Eglin C also reduced plasma active PAI-1 concentration and liver fibrin after LPS/RAN treatment (Fig. 2.12 A and B). In addition, it partially restored the fibrinogen consumption caused by LPS/RAN treatment but did not affect plasma TAT concentration (Fig. 2.12 C and D).

Figure 2.11. Effects of PMN-protease inhibitor on hepatotoxicity after LPS/RAN treatment. Rats were treated with LPS/RAN as described in Fig 2.1. Eglin C or its vehicle was administered 0hr, 2hr and 4hrs after RAN treatment. Serum ALT activity was evaluated 6hr after RAN treatment. # significantly different from Veh group. $P<0.05$, n=11-12.

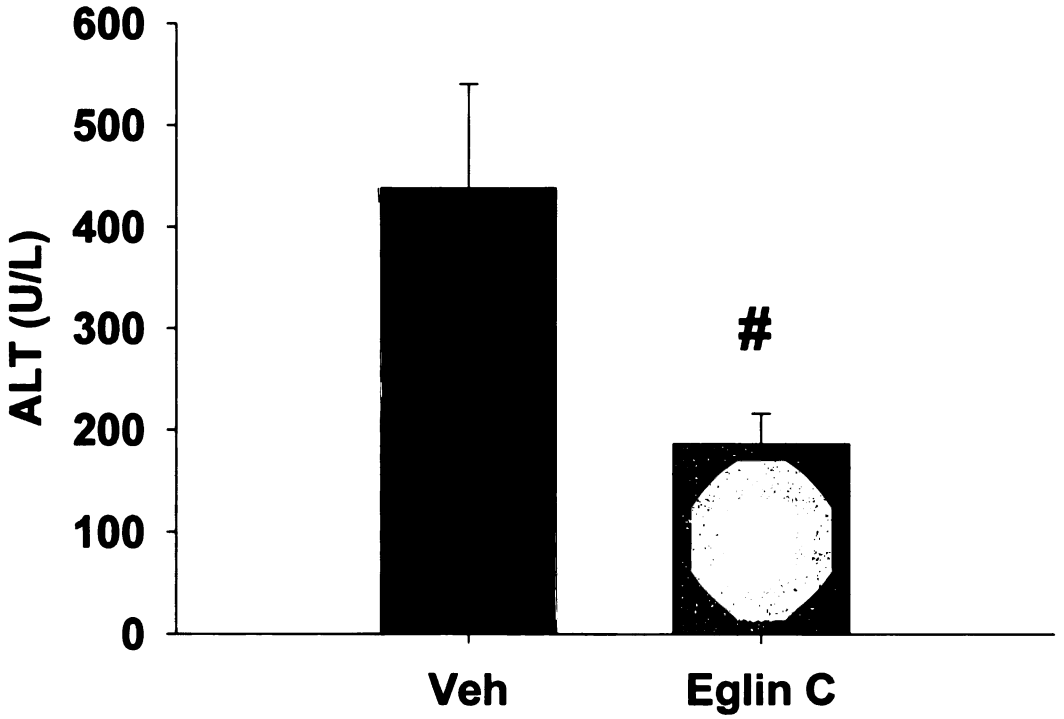
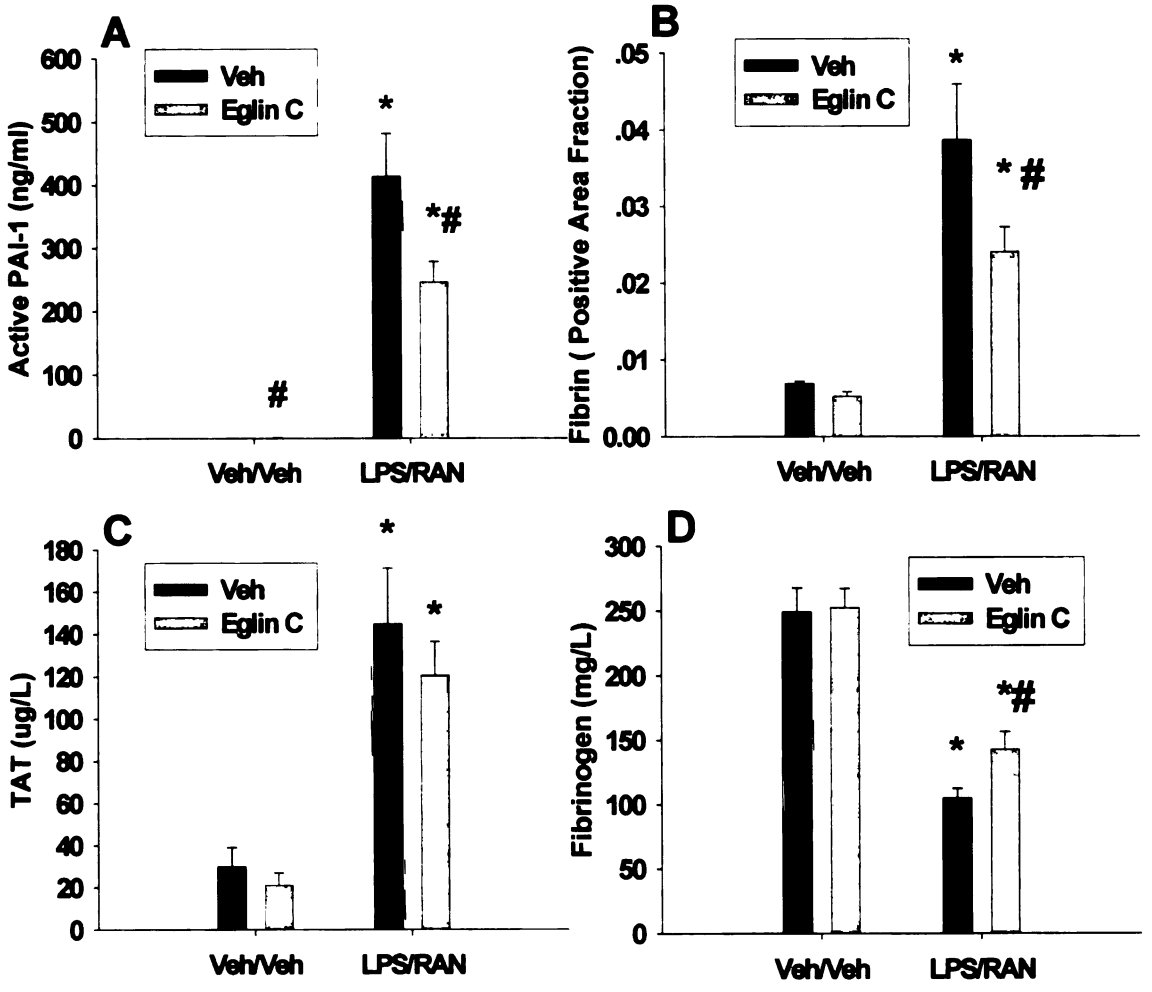

600
500
400
300
200
100
0
ALT (U/L)
#
Veh
Eglin C

Figure 2.12. Effects of PMN protease inhibitor on markers for hemostasis and liver fibrin after LPS/RAN treatment. Rats were treated with LPS/RAN or Veh/Veh as described in Fig. 2.1. Eglin C or its vehicle was administered 0hr, 2hr, 4hr after RAN treatment. A: Plasma active PAI-1, B: liver fibrin, C: plasma TAT and D: fibrinogen were evaluated 6hr after RAN treatment. * significantly different from the respective group without LPS/RAN. # significantly different from the respective group without eglin c. $P<0.05$, n=5-12.


A
Active PAI-1 (ng/ml)
600
500
400
300
200
100
0
Veh
Eglin C
*
*#
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Veh/Veh
LPS/RAN
B
Fibrin (Positive Area Fraction)
.05
.04
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.02
.01
0.00
Veh
Eglin C
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* #
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140
120
100
80
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20
0
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*
*
Veh/Veh
LPS/RAN
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Fibrinogen (mg/L)
300
250
200
150
100
50
0
Veh
Eglin C
*
*#
Veh/Veh
LPS/RAN

Discussion

In LPS/RAN-treated rats, hepatocellular injury begins 2-3hr after RAN administration and progresses through 6hr, at which time serum ALT activity is maximal (Luyendyk et al., 2003g). Confirming previous results (Luyendyk et al., 2005m), PMN depletion with NAS dramatically reduced serum ALT activity 6hr after RAN, suggesting an important role for PMNs in the liver injury. PMNs could contribute to this response by a variety of mechanisms. One possibility is that PMNs promote coagulation and fibrin deposition in liver, leading to hypoxia. Fibrin deposition occurs prior to injury (2-3 hr) in liver sinusoids of LPS/RAN-cotreated rats (Luyendyk et al., 2004g). PMN depletion decreased fibrin deposition at 6 hr after LPS/RAN treatment (Fig. 2.2) but not at 2 hr. This result suggests that PMN-dependent fibrin deposition contributes to the progression but not to the initiation of hepatocellular injury in LPS/RAN-treated rats. To investigate how PMNs contribute to fibrin deposition in the LPS/RAN model, coagulation system activation and PAI-1 were evaluated. Interestingly, PMN depletion attenuated the decrease in plasma fibrinogen caused by LPS/RAN (Fig. 2.3A) but did not influence the elevation in plasma TAT concentration (Fig. 2.3B). Since TAT concentration reflects generation of active thrombin, this result suggests that PMNs do not participate in activation of the coagulation system but are capable of thrombin-independent cleavage of fibrinogen. The latter might be due to the action of PMN-derived proteases such as elastase/cathepsin G. Indeed, a combination of PMN elastase and cathepsin G cleaved fibrinogen in vitro in a manner different from thrombin (Fig. 2.4). Furthermore, eglin C

reduced the consumption of fibrinogen caused by LPS/RAN treatment but did not affect plasma TAT concentration (Fig. 2.12 C and D). Taken together, these results suggest that PMNs contribute to fibrin deposition but not via activating the coagulation cascade.

An alternative mechanism by which PMNs could contribute to deposition of fibrin is through inhibiting fibrinolysis by increasing active PAI-1. PMN lysosomal proteases cathepsin B, D and G increased PAI-1 activity in the medium of human umbilical vein endothelial cells by cleaving PAI-1 from extracellular matrix (Kimura and Yokoi-Hayashi, 1996c;Pintucci et al., 1993a;Pintucci et al., 1992c). Consistent with this, PMN depletion reduced active PAI-1 at 6hr after RAN, although it did not affect it at 2hr (Fig. 2.5B). This suggests that PMNs contribute to fibrin deposition after 2hr by activating PAI-1 and thereby inhibiting fibrinolysis. In contrast, PMN depletion did not affect total plasma PAI-1 concentration at 6hr, but it did increase total plasma PAI-1 concentration at 2hr. The mechanism by which PMN depletion increases total PAI-1 concentration is unknown. PMN derived proteases such as elastase can cleave and inactivate PAI-1 (Wu et al., 1995b), which has also been shown here (Fig. 2.6). However, this is unlikely in vivo at 2hr in the LPS/RAN model since PMNs are not activated to release granular products at that time (Fig. 2.8). Interestingly, internalization of uPA-PAI-1 complexes by immune cells can occur through uPA receptors (Conese et al., 1995;Nagatomi et al., 1997a), this might explain why PMN depletion increased total PAI-1 but had no effect on active PAI-1 at the earlier time (ie., 2hr).

One explanation for the dual effects of PMNs on active and total PAI-1 is that PMNs are not activated to release proteases during earlier times but begin to degranulate thereafter. In this regard, the shedding of PAI-1 from endothelial matrix by PMNs proteases might play a more dominate role at later times (ie. 6hr). This is consistent with the results showing that formation of HOCl-modified epitopes, a marker of PMN degranulation/activation, did not increase until 3hr after LPS/RAN treatment (Fig. 2.8), suggesting that PMNs did not start the transmigration and activation process until after 2hr.

In the LPS/RAN model, PMN accumulation in liver is caused largely by LPS . However, LPS exposure alone did not lead to activation of the accumulated PMNs (Fig. 2.8A). RAN did cause activation of the PMNs that accumulated in liver after LPS treatment, as reflected by an increase in HOCl-protein adduct formation. Anti-CD18 serum did not alter PMN accumulation but reduced PMN activation in liver (Fig. 2.9A, 9B), suggesting that the early, LPS-induced sinusoidal accumulation of PMNs does not require CD18 whereas their activation is CD18-dependent. In addition, anti-CD18 serum markedly reduced hepatocellular injury (Fig. 2.9A), suggesting that PMN transmigration/activation is required for LPS/RAN-induced liver injury.

The anti-CD18 serum and NAS had similar effects on the hemostatic system. Anti-CD18 serum also reduced fibrin deposition 6hr after LPS/RAN treatment (Fig. 2.9D), suggesting that activation of PMNs is required for their effect on hemostasis. Moreover, PMN proteases are important in this regard since eglin C also reduced the

deposition of fibrin (Fig. 2.12B). This promotion of fibrin deposition by activated PMNs is most likely mediated by PAI-1 since both anti-CD18 serum and eglin C reduced the elevation in active PAI-1. In addition, the reduction of active PAI-1 by eglin C treatment supports the findings of others (Kimura and Yokoi-Hayashi, 1996;Pintucci et al., 1993) that PMN proteases can cleave PAI-1 from endothelial matrix and increase the concentration of active PAI-1 in plasma.

There are several reports showing that hypoxia can cause hepatocyte death in vitro (Nagatomi et al., 1997b;Lluis et al., 2005a). In the LPS/RAN model, agents that reduce hypoxia also reduce hepatocellular injury. These include heparin (Luyendyk et al., 2005n), PMN antiserum (Fig. 2.7) and CD18 antiserum (Fig. 2.8). Accordingly, it seems likely that hypoxia caused in part by sinusoidal fibrin deposition that reduces blood flow plays a role in inducing hepatocellular injury in this model. PMNs could also contribute to tissue hypoxia. In LPS/RAN-treated rats, PMN depletion reduced PIM staining at 2hr after LPS/RAN treatment, suggesting that PMNs contribute to early development of liver hypoxia. This could occur by several mechanisms. One possibility is that accumulated PMNs could clog sinusoidal vessels and impair microvascular blood flow. Furthermore, PMN-derived factors could impair the balance between vasodilators and vasoconstrictors, causing constriction of sinusoids and subsequent hypoxia. For example, PMN-derived proteases reduce vasodilatory prostacyclin production both in vitro and in vivo (Harada et al., 1997;Okajima et al., 2004b;Weksler et al., 1989). Myeloperoxidase, abundantly present in PMNs (5% of total cell protein content) and released by PMNs during the

oxidative burst, could also oxidize NO and thereby impair vascular relaxation (Eiserich et al., 2002). This early contribution of PMNs to hypoxia did not depend on sinusoidal fibrin deposition, since PMN deletion in LPS/RAN-treated rats did not affect liver fibrin at 2hr after RAN (Fig. 2.2). Furthermore, PMNs accumulated in livers are not activated at 2hr (Fig. 8), suggesting that enhancement of hypoxia by PMNs at this time does not require their activation and might be mediated directly by plugging sinusoids.

LPS-RAN cotreatment caused a greater degree of tissue hypoxia than LPS given alone (Luyendyk et al., 2004f), although these two treatments had a similar effect on PMN accumulation (Luyendyk et al., 2005o). It is possible that RAN cotreatment causes PMNs to adhere more firmly to sinusoidal endothelium and/or to undergo a shape change that results in reduced sinusoidal blood flow and consequent hypoxia. Interestingly, the early tissue hypoxia and PMN accumulation were both panlobular in distribution, whereas the hepatocellular necrosis that followed was midzonal. The HOCl adduct staining also tended to be midzonal. These results suggest that the early hypoxia might be necessary but insufficient to cause the liver injury without additional factors such as PMN activation.

The contribution of PMNs to liver hypoxia at later times might occur by a different mechanism. Anti-CD18 serum reduced liver hypoxia at 3hr, suggesting that the contribution of PMNs to liver hypoxia after 2hr requires PMN transmigration and/or activation. Since sinusoidal fibrin deposition appears to be important for liver hypoxia

occurring at 3hr after LPS/RAN treatment (Luyendyk et al., 2005p), PMNs might contribute to liver hypoxia after 2hr by promoting sinusoidal fibrin deposition.

Among the products released by activated PMNs, cathepsin G and elastase are important mediators of hepatic parenchymal cell killing caused by PMNs in vitro (Ho et al., 1996c). These proteases also have been implicated in models of liver injury in vivo such as ischemia-reperfusion (Okajima et al., 2004a) and LPS-induced liver dysfunction (Luyendyk et al., 2005q). Interestingly, killing of hepatocytes in vitro by neutrophil elastase is enhanced by a hypoxic environment (Luyendyk et al., 2005r). The time course over which hepatocyte killing occurred during hypoxia in vitro is consistent with the development of liver injury in LPS/RAN-treated rats. Taken together with the evidence that fibrin deposition and hypoxia are critical to liver injury in LPS/RAN-treated rats, it is likely that PMNs contribute to the pathogenesis by releasing proteases that kill hepatocytes in an environment made hypoxic by fibrin deposition. Indeed, eglin C attenuated the hepatocellular injury caused by LPS/RAN treatment. Thus, PMN proteases could contribute to liver injury by direct killing hepatocytes and indirectly by promoting fibrin deposition and hypoxia, thereby increasing hepatocellular susceptibility to injury.

In conclusion, PMNs and the hemostatic system are key players in the pathogenesis of liver injury in LPS/RAN-cotreated rats. PMN accumulation in liver in this model is mediated by LPS and is insufficient to damage hepatocytes. Activation of hepatic PMNs by RAN occurs around the time liver injury begins and is critical for the progression of hepatocellular injury. Morever, the protection afforded by eglin C suggests that proteases

released during PMN activation are important. These proteases may injure hepatocytes directly, but they also participate in liver injury progression by promoting hemostasis, in part by activating antifibrinolytic PAI-1. PMNs also contribute to the liver hypoxia at a time before PMN activation is evident. In addition, during injury progression, PMNs likely enhance tissue hypoxia by promoting hemostasis in this model of idiosyncratic adverse drug reaction.

Figure 2.13. Interaction of PMNs with the hemostatic system in the LPS/RAN model of liver injury: working hypothesis. LPS causes modest coagulation system activation and PAI-1 production, leading to fibrin deposition and hypoxia (Luyendyk et al., 2004e). RAN augments fibrin deposition by enhancing coagulation system activation and PAI-1. LPS also causes PMN accumulation in liver, and RAN activates these cells, which release toxic PMN proteases (Fig 2.8). The early hypoxia (2hr) is PMN-dependent (Fig 2.7), whereas later hypoxia is enhanced by hemostasis. Hypoxia acts synergistically with PMN proteases to kill hepatocytes (Luyendyk et al., 2005s). In addition to its direct killing of hepatocytes, PMN proteases increase active PAI-1 production and subsequent fibrin deposition (Fig. 2.12).

TNF
LPS
Altered SEC Homeostasis (procoagulant)
Coagulation System Activation
Sinusoidal Fibrin Deposition
PMN Accumulation
PAI-1
Decreased Fibrinolysis
PMN Activation
PMN Proteases
Hypoxia
?
Hepatocellular Injury

CHAPTER THREE

Xiaomin Deng, Jingtao Lu, Lois D.Lehman-McKeeman, Ernst Malle, David Crandall, Patricia E.Ganey, Robert A. Roth. (2008) p38-dependent TNF-α converting enzyme is important for liver injury in hepatotoxic interaction between lipopolysaccharide and ranitidine. *J Pharmacol Exp Ther*. Submitted

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3.1 Abstract

Idiosyncratic adverse drug reactions (IADRs) remain an important issue in human health and drug development. One of the drugs associated with IADRs in human patients is ranitidine (RAN). In rats, cotreatment with nontoxic doses of lipopolysaccharide (LPS) and RAN causes liver injury. This is a potential animal model for RAN-induced IADRs in humans. Previous studies showed that RAN augmented serum tumor necrosis factor-alpha (TNF-α) production and hepatic neutrophil activation after LPS treatment and that both TNF-α and neutrophils are crucial for the liver pathogenesis. We tested the hypothesis that p38 is necessary for TNF-α production, neutrophil activation and subsequent liver injury caused by LPS/RAN cotreatment. LPS/RAN cotreatment caused more p38 activation compared with treatment only with LPS. SB239063, a p38 kinase inhibitor, reduced liver injury in rats cotreated with LPS/RAN. This inhibitor also reduced neutrophil activation in livers and attenuated the hemostatic system. SB239063 decreased serum TNF-α concentration after LPS/RAN treatment to the same level as LPS treatment. However, the inhibitor did not reduce TNF-α mRNA in liver, suggesting a post-transcriptional mode of action. This might occur through TNF-α converting enzyme (TACE), which cleaves pro-TNF-α into its active form. Indeed, a TACE inhibitor administered just before RAN treatment reduced serum TNF-α protein. The TACE inhibitor also reduced liver injury and active plasminogen activator inhibitor-1 (PAI-1). Furthermore, a PAI-1 inhibitor reduced neutrophil activation and subsequent liver injury after LPS/RAN treatment. In summary, RAN enhanced TNF-α production after LPS

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treatment through augmented p38 activation, and this appears to occur through the activation of TACE. The prolonged TNF-α production enhanced PAI-1 production after RAN cotreatment and this is important for the hepatotoxicity.

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3.2 Introduction

Idiosyncratic adverse drug reactions (IADRs) occur during treatment with numerous drugs, typically in a small fraction of people taking the drug. These responses are seemingly unrelated to dose, and the time of onset relative to beginning of drug therapy is often variable (Kaplowitz, 2005b;Uetrecht, 2007). A widely used drug associated with rare idiosyncratic hepatotoxicity is the histamine 2 (H2)-receptor antagonist, ranitidine (RAN). RAN is available over the counter for oral administration or by prescription for parenteral administration for treatment of duodenal ulcers, gastric hypersecretory diseases and gastroesophageal reflux disease. Idiosyncratic RAN hepatotoxicity occurs in less than 0.1% of people taking the drug (Fisher and Le Couteur, 2001a;Fisher and Le Couteur, 2001b). Most liver reactions are mild and reversible; however, extensive liver damage and death have occurred in individuals undergoing RAN therapy (Cherqui et al., 1989;Ribeiro et al., 2000c). Rechallenge with RAN does not necessarily result in a reoccurrence of toxicity (Halparin, 1984a;Hiesse et al., 1985a).

In rats, cotreatment with nontoxic doses of lipopolysaccharide (LPS) and ranitidine (RAN) causes liver injury. This was not the case with another histamine-2 receptor antagonist, famotidine (FAM), which is not associated with IADRs in human patients (Fisher and Le Couteur, 2001c). Thus, this LPS-drug interaction model in rodents could differentiate a drug that causes IADRs from a drug that does not. Previous mechanistic studies showed that RAN augmented serum tumor necrosis factor-alpha (TNF-α) production and hepatic neutrophil activation after LPS treatment, and both TNF-α and

neutrophils are crucial for the liver pathogenesis (Deng et al., 2007g;Tukov et al., 2007i). Moreover, TNF-α is likely to be a proximal signal in the pathogenic cascade (Tukov et al., 2007h). The mechanism behind RAN augmentation of TNF-α production and neutrophil activation is unknown.

TNF-α production involves gene expression of proTNF-α mRNA, translation of proTNF-α protein and its cleavage and release of active TNF-α. LPS-induced TNF-α transcriptional activation has been well studied (Kawai and Akira, 2007a). However, TNF-α production can also been regulated at a post-transcriptional level. For example, TNF-α mRNA stabilization and translation are regulated by p38 mitogen activated protein kinase (MAPK) (Hitti et al., 2006d;Neininger et al., 2002c). Furthermore, TNF-α converting enzyme (TACE) cleaves the 26 kDa membrane-bound proTNF-α protein to generate secreted 17 kDa mature TNF-α (Aggarwal et al., 1985b;Mullberg et al., 2000a). This cleavage occurs at the Ala76-Val77 bond. The release of TNF-α from cells in vitro and in vivo can be selectively blocked by hydroxamate-based metalloprotease inhibitors that inhibit TACE activity (Mohler et al., 1994b;Gearing et al., 1994;McGeehan et al., 1994). These TACE inhibitors protect against endotoxin-mediated lethality, in which TNF-α plays a critical role (Mohler et al., 1994a).

p38 and its downstream MAPK Activated Protein Kinase 2 (MK-2) have been shown to be involved in the production of several cytokines and chemokines (ie. TNF-α, macrophage inflammatory protein-2 [MIP-2], interleukin 6 [IL-6]) (Hitti et al., 2006c;Neininger et al., 2002d;Numahata et al., 2003b) and in neutrophil activation (Nick

et al., 1997;Mocsai et al., 2000). Thus, p38 activation is a potential upstream signal that leads to production of cytokines/chemokines and subsequently to downstream cascades that contribute to LPS/RAN-induced liver injury (Luyendyk et al., 2006f). Here we tested the hypothesis that p38 is necessary for TNF-α production, neutrophil activation and subsequent liver injury caused by LPS/RAN cotreatment. These studies elucidated intracellular signaling events that are crucial to the initiation of LPS/RAN induced-liver injury.

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3.3 Methods

3.3.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). LPS derived from *Escherichia Coli* serotype O55:B5 (catalog number L-2880) with activity of 13 x 10^6 EU/mg (lot number 43K4112) was used for these studies. This activity was determined using a QCL Chromogenic LAL endpoint assay purchased from Cambrex Bio Science, Inc. (Baltimore, MD).

3.3.2 Animals

Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR; Charles River Breeding Laboratories, Portage, MI) weighing 250 to 350 g were fed standard chow (rodent chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad *libitum*. They were allowed to acclimate for 1 week in a 12h light/dark cycle prior to use.

3.3.3 Experimental Protocol

Rats fasted for 24hr were given 2.5 x 10^6 EU/kg LPS or its saline vehicle (Veh) i.v. at 5ml/kg, and food was then returned. Two hours later, 30 mg/kg RAN, 6mg/kg FAM or their vehicle (sterile phosphate-buffered saline (PBS)) was administered (i.v.). RAN solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. The FAM dose was selected as a pharmacologically equi-efficacious dose based on relative potencies of RAN and FAM in antagonizing H2-receptors (Luyendyk et al., 2006e). At

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the time of sacrifice, rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.). Plasma was collected by drawing 2 ml of blood from the vena cava into a syringe containing sodium citrate (final concentration, 0.38%). Another portion of blood was collected and allowed to clot at room temperature; serum was prepared from this fraction and stored at -20°C until use. Representative slices (3–4-mm thick) of the ventral portion of the left lateral liver lobe were collected and fixed in 10% (v/v) neutral buffered formalin. For immunohistochemical analysis, a portion of the left medial lobe of the liver was flash-frozen in isopentane cooled by liquid nitrogen.

3.3.4 Treatment with Inhibitors of p38, TACE or Plasminogen Activator Inhibitor-1 (PAI-1)

A p38 inhibitor, SB230963, or its vehicle (acidfied PBS, pH=6) was administered (2.5mg/kg,iv) at the same time as RAN and again 2hr after RAN. Two administrations of SB230963 were given because of its short half life (Barone et al., 2001). A TACE inhibitor, BMS-561392 (60mg/kg, also named DPC-333, provided by Bristol-Myers Squibb, Princeton, NJ) (Grootveld and McDermott, 2003;Qian et al., 2007), or its vehicle (1% Tween 80 + 0.5% methylcellulose in PBS), was administered 15min before RAN orally. This dose was shown in a preliminary study to reduce LPS (2.5X10^6 EU/kg,iv)-induced serum TNF-a increase in rats when given 30min before LPS. A PAI-1 inhibitor, WAY-140312 (10mg/kg, provided by Wyeth Research, Philadelphia, PA)

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(Crandall et al., 2004) or its vehicle (2.0% Tween 80+ 0.5% methylcellulose in PBS) was administered orally 1hr before RAN and again 1hr and 3hr after RAN.

3.3.5 Hepatotoxicity Assessment

Hepatic parenchymal cell injury was estimated as an increase in serum ALT activity. ALT activity was determined spectrophotometrically using Infinity-ALT reagent from Thermo Electron Corporation (Louisville, CO). Previous studies in this LPS/RAN model have shown that serum ALT activity reflects histopathologic evidence of hepatocellular necrosis (Luyendyk et al., 2003h).

3.3.6 Evaluation of Hepatic Phospho-p38 and Phospho-MK-2

A liver section of 5mm length from the right lateral lobe was homogenized in 1ml lysis buffer (1mM EDTA, 0.5% Triton X-100, 5mM NaF, 6M urea, 10ug/ml leupeptin, 10ug/ml pepstatin, 100uM PMSF, 3ug/ml aproptinin, 2.5 mM sodium pyrophosphate, 1mM activated sodium orthovanadate). After sitting on ice for 15min, the tissue homogenates were sonicated for 10 sec. After centrifugation at 14,000 RPM for 10min, the supernatants were collected. Phospho-p38 and total p38 in the tissue homogenates were evaluated using a commercial ELISA kit (R&D systems, Minneapolis, MN) and p38 activation was expressed as ratio of phospho-p38 to total-p38.

The protein concentration was determined by a Bradford assay (Bio-rad laboratories, Hercules, CA). 10ug protein was loaded and separated on SDS-PAGE gel. Phospho-MK-2 and total-MK-2 were detected by western analysis using rabbit

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excep

polyclonal anti phospho-MK-2 (Thr222) and anti total-MK-2 antibody (Cell Signalling, Danvers, MA) (both at 1:1000 dilution, overnight at 4 ^{0}C). MK-2 activation was expressed as the density ratio of phospho-MK-2 to total MK-2.

3.3.7 Evaluation of Coagulation System Activation and Plasma PAI-1

Plasma thrombin-antithrombin (TAT) concentration was used as a marker for coagulation activation. Plasma TAT concentration was determined by an enzyme-linked immunosorbent assay (ELISA) using a kit from Dade Behring Inc. (catalog number OWMG15). The concentration of functionally active PAI-1 in plasma was assessed using a commercially available ELISA kit purchased from Molecular Innovations Inc. (Southfield, MI).

3.3.8 Serum TNF-α and MIP-2 Evaluation

Serum TNF-α concentration was evaluated using a commercial ELISA kit (BD Biosciences, San Diego, CA). Serum MIP-2 concentration was also evaluated by commercial ELISA kit (Biosources, Camarillo, CA).

3.3.9 Fibrin Immunohistochemistry and Quantification

Fibrin immunohistochemistry and quantification were performed as described previously (Copple et al., 2002b). This protocol solubilizes all fibrinogen and fibrin except for cross-linked fibrin (Schnitt et al., 1993b). Therefore, only cross-linked fibrin

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stains in sections of liver. The positive area fraction refers to the percentage of area with positive staining in the total microscopic field.

3.3.10 Evaluation of Liver PMNs and PMN Activation

PMNs accumulated in liver were visualized by immunohistochemical staining and quantified as described previously (Yee et al., 2003a). PMN activation was measured by staining of hypochlorous acid (HOCl)-protein adducts in liver. The monoclonal antibody (clone 2D10G9, subtype IgG2bk) is specific for hypochlorous acid (HOCl)-modified epitopes generated in vivo (Malle et al., 1997b) and in vitro (Malle et al., 1995b) and does not crossreact with other oxidative protein modifications. Frozen sections of liver were fixed in 4% (v/v) formalin for 10 minutes at room temperature with gentle rocking. The slides were washed 3 times, 5 minutes each, with PBS (phosphate-buffered saline), then blocked for 1 hour at room temperature with 3% goat serum in PBS. Antibody (diluted 1:1 in 3% goat serum) was added and incubated for 2 hr at room temperature with gentle rocking. The slides were washed 3 times, 5 minutes each, with PBS. Alexa Fluro 488-labeled goat anti-mouse secondary antibody (diluted 1:500 in 3% goat serum, Molecular Probes, Carlsbad, California) was applied, and the slides were incubated for 3 hr at room temperature. After washing 3 times, 5 minutes each with PBS they were examined microscopically. Staining was quantified as for fibrin staining described above and presented as positive area fraction.

3.3.11 Evaluation of Hepatic TNF-α mRNA and TACE activity

Total RNA was extracted from frozen liver tissues using the MELT Total Nucleic Acid isolation system (Ambion, Austin, TX) according to the manufacturer's instructions. First-strand cDNA was synthesized from isolated RNA using oligo(dT)12–18 primer and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). In the real time PCR step, cDNA was amplified using the TaqMan universal PCR master mix and TaqMan pre-developed gene expression assay reagents for rat TNF-α (Applied Biosystems, Foster City,CA). β -actin was measured as an endogenous control, using TaqMan endogenous control assay Rat ACTB (VIC® / MGB Probe, Primer Limited, Pismo Beach, CA). Quantification was conducted on the Applied Biosystems StepOne Real-Time PCR System, according to the manufacturer's protocols. A standard curve for each gene was made of 4-fold serial dilutions of total RNA from one sample. The curve was then used to calculate the relative amounts of target mRNA in the samples. The ratio between TNF-α mRNA and β -actin was used as an indicator for TNF-α mRNA abundance. The TNF-α mRNA level in each liver sample was expressed as ratio vs one Veh/Veh-treated liver for Fig 6 and vs one LPS/Veh/Veh-treated liver for Fig 7B.

Protein extraction for TACE activity measurement was conducted as follows: Protein was extracted from liver tissue by homogenization in RIPA buffer (50 mM Tris pH 7.2, 150mM NaCl, 1% deoxycholic acid, 1% Triton X-100, and 0.1% SDS) containing 10mM 4-nitrophenyl phosphate, 20 mM β-glycerophosphate, 500uM Pefabloc, 2 ug/mL aprotinin, 50uM sodium orthovanadate and 0.5ug/mL leupeptin.

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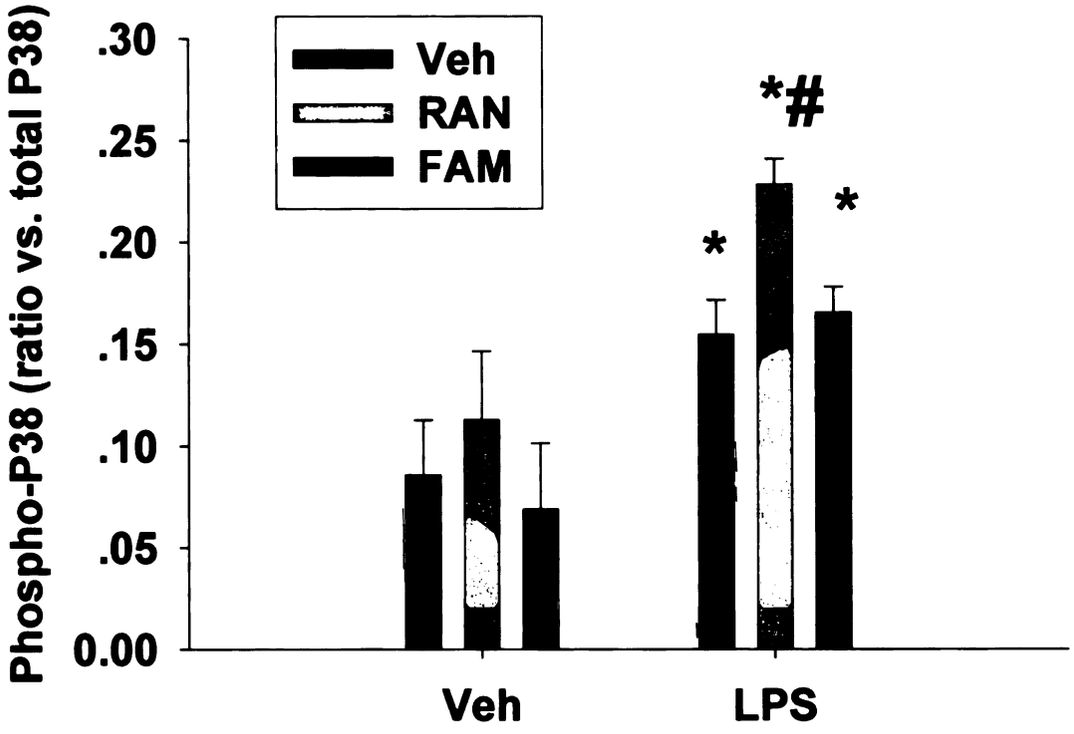
Homogenization was followed by a 30-minute tumbling period and a 10- minute centrifugation at 14,000g (both at 4 ° C). Supernatant was collected and protein concentration determined by Bradford assay. The TACE activity was measured using a commercial TACE activity kit (Calbiochem, San Diego, CA).

3.3.12 Statistical Analysis

A two way analysis of variance (ANOVA) with Tukey's post hoc test was used for all data analyzed, except where only two groups were present, in which case student's t-test was applied. The criterion for statistical significance for all studies was $p<0.05$.

Figure 3.1 p38 activation after LPS/RAN treatment. Rats were given either LPS or its vehicle and cotreated with RAN, FAM (at a dose equiefficacious to RAN) or vehicle 2 hrs later. Hepatic p38 activation was evaluated 1 hr after drug treatment. * significantly different from the respective treatment without LPS. # significantly different from the respective treatment without the drug. n=5-9.



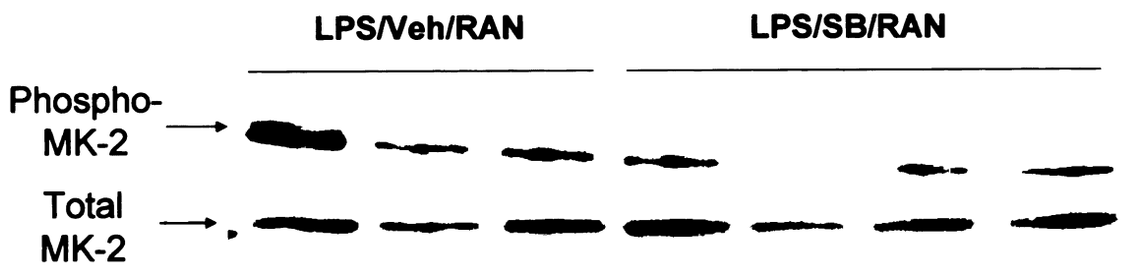
ither LPS or its

or vehicle 2

significantly

nt from the

Figure 3. 2 Effects of a p38 inhibitor on MK-2 phosphorylation. Rats were given LPS and RAN as in Fig. 3.1. A p38 inhibitor, SB 239063, was administered at the same time as RAN and again 2hr later. Hepatic phospho-MK-2 protein and total MK-2 were measured 2hr after RAN treatment as an indicator of p38 activation. # significantly different from the respective treatment without SB 239063. n=3-4.



given LPS

same time

K-2 were

nificantly

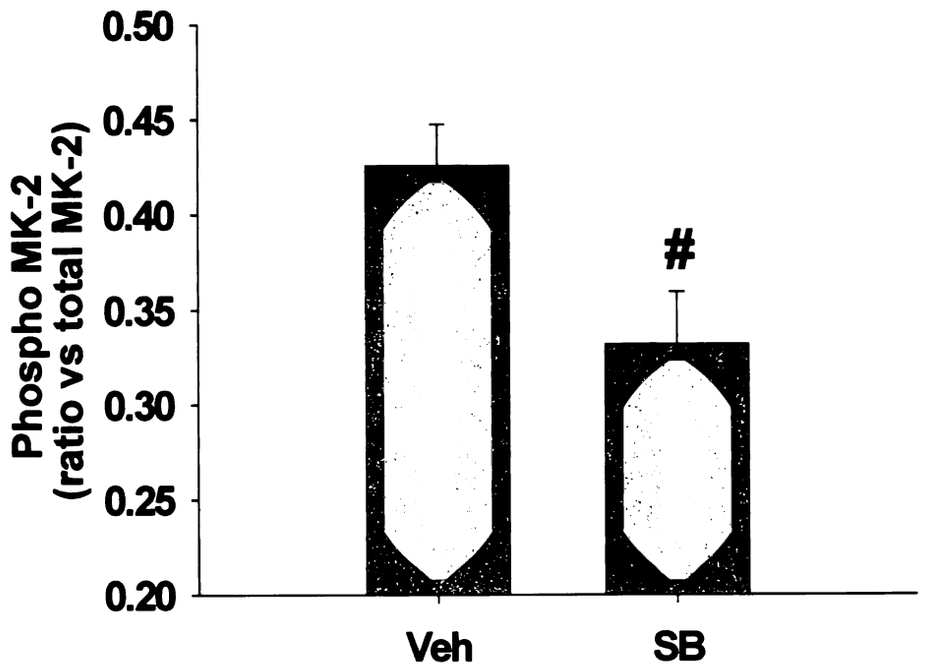


Figure 3.3 Effects of a p38 inhibitor on serum ALT activity. Rats were given LPS and RAN as in Fig. 3.1. A p38 inhibitor, SB 239063, was administered at the same time as RAN and again 2hr later. Serum ALT activity was evaluated 6hr after RAN. **#** significantly different from the respective treatment without SB 239063. n=5-7.

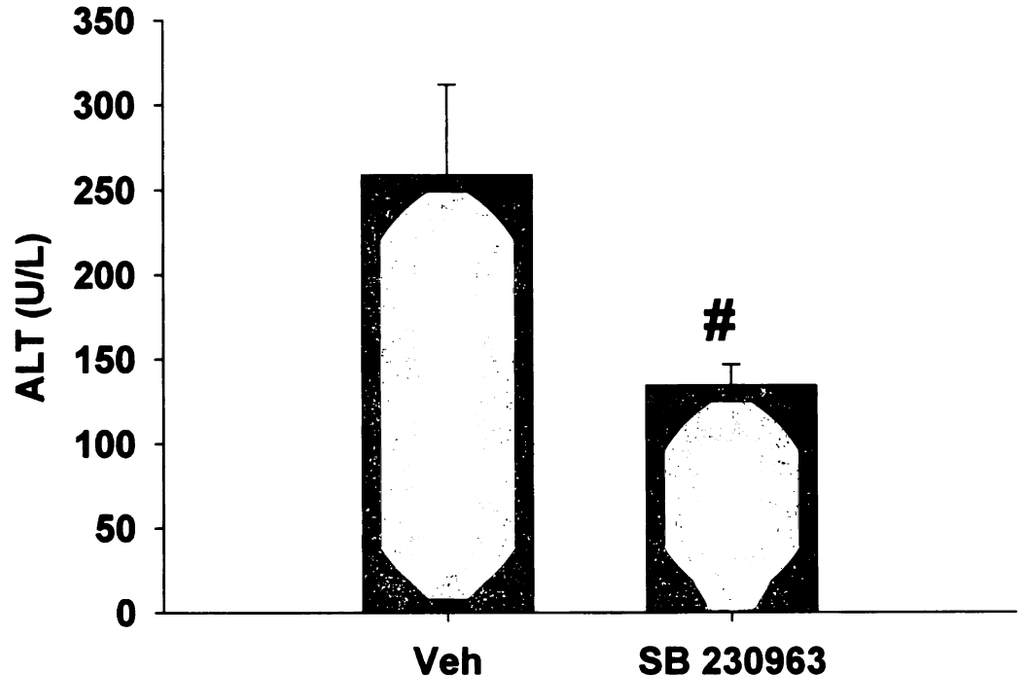
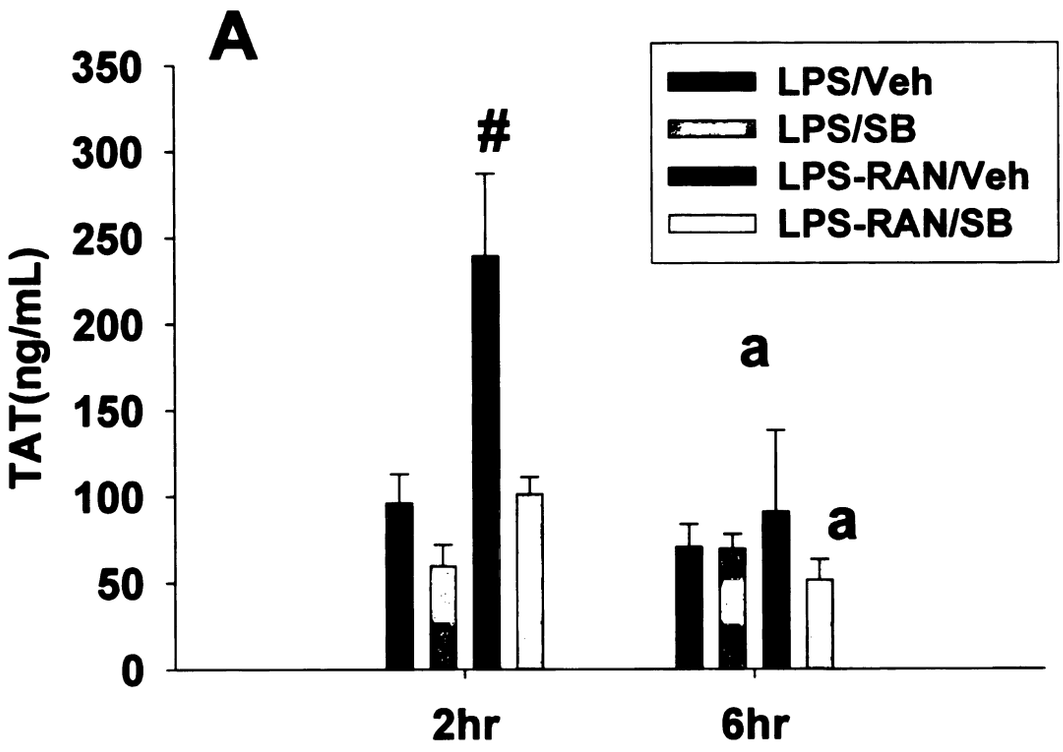
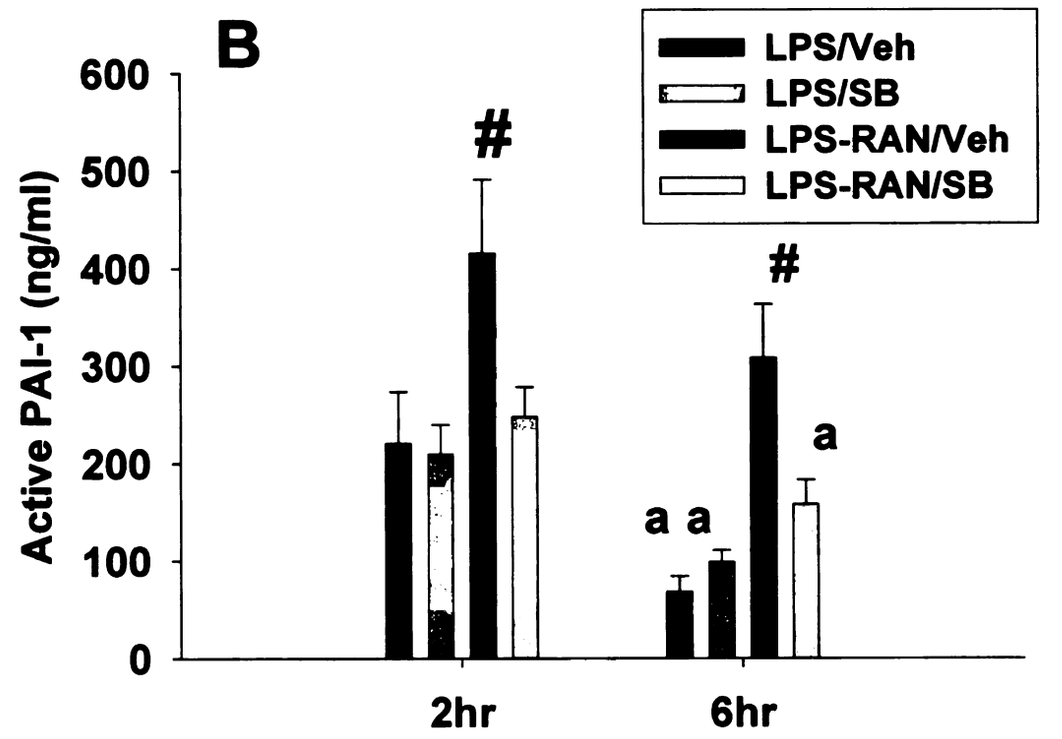

350
300
250
200
150
100
50
0
ALT (U/L)
#
Veh
SB 230963

Figure 3.4 Effects of a p38 inhibitor on biomarkers of hemostasis. Rats were treated with LPS, and 2hrs later they were treated with RAN or its vehicle. SB 239063 or its vehicle was administered as in Fig 3.3. TAT and active PAI-1 were measured 2hrs and 6hrs after RAN or its vehicle. **#** significantly different from all the groups at the same time. **a** significantly different from the respective treatment at 2hr. n=4-6.


A
350
300
250
200
150
100
50
0
TAT(ng/mL)
#
a
a
LPS/Veh
LPS/SB
LPS-RAN/Veh
LPS-RAN/SB
2hr
6hr


B
600
500
400
300
200
100
0
Active PAI-1 (ng/ml)
#
#
a
a a
a
LPS/Veh
LPS/SB
LPS-RAN/Veh
LPS-RAN/SB
2hr
6hr

3.4 Results

3.4.1 p38 activation after LPS/RAN treatment

Rats were given either LPS or its vehicle and cotreated 2 hrs later with RAN, FAM (at a dose equiefficacious to RAN) or vehicle. Hepatic p38 activation was evaluated 1 hr after drug treatment. Neither RAN nor FAM alone caused p38 activation as reflected by the ratio of phospho-p38 to total p38 (Fig 3.1). LPS alone increased p38 activation. Treatment of LPS-exposed rats with RAN caused greater p38 activation compared to LPS alone, whereas FAM cotreatment had no additional effect (Fig 3.1).

3.4.2 Effects of p38 inhibitor on hepatotoxicity

Rats were given LPS and RAN as described above and a p38 inhibitor, SB 239063, was administered at the same time as RAN and again 2hr after RAN. Hepatic phospho-MK-2 protein was measured 2hrs after RAN treatment as an indicator of p38 activation, and serum ALT activity was evaluated at 6hrs as a marker of hepatotoxicity. The p38 inhibitor reduced phospho-MK-2 protein (Fig 3.2), indicating attenuation of p38 activation. The inhibitor also significantly reduced the increase in serum ALT activity (Fig 3.3).

3.4.3 Effects of p38 inhibitor on biomarkers of hemostasis and liver PMNs

Since previous findings showed that the hemostatic system and PMN activation are crucial for liver injury caused by LPS/RAN (Luyendyk et al., 2004d;Deng et al., 2007f),

the effect of p38 inhibition on these events was evaluated. Two hours and six hours after RAN or its vehicle, TAT and active PAI-1 were measured as biomarkers of coagulation and fibrinolysis, respectively (Levi et al., 2003b). At 2hr, LPS/RAN treatment caused a greater increase in plasma TAT and active PAI-1 compared to LPS/Veh treatment 2hrs after RAN (Fig 3.4), confirming previous results (Luyendyk et al., 2004c). SB 239063 reduced both plasma TAT and active PAI-1 to the same levels as LPS/Veh treatment. At 6hrs, active PAI-1 remained elevated after LPS/RAN treatment, and SB 239063 eliminated this increase. SB 239063 had no effect on plasma TAT or active PAI-1 after LPS/Veh treatment at either 2hrs or 6hrs (Fig 3.4).

In inflammatory liver injury, PMNs accumulate in sinusoids then transmigrate into parenchyma in response to stimuli and become activated to injure hepatocytes (Springer, 1994c). LPS/RAN treatment did not change hepatic PMN accumulation compared to LPS/Veh treatment at either 2hrs or 6hrs, and SB 239063 had no effect on hepatic PMN accumulation (Fig 3.5A). Serum MIP-2, a PMN chemokine, was elevated at 2hr by RAN in LPS treated rats, and this increase was prevented by SB 239063 (Fig 3.5B). Upon activation, PMNs release myoperoxidase to form HOCl, which forms protein adducts in liver. Thus, HOCl adduct staining was measured as a marker for PMN activation 6hrs after RAN (Fig 3.5C), since PMNs are not activated until 3hrs after RAN (Deng et al., 2007e). SB 239063 reduced HOCl adduct staining after LPS/RAN treatment, suggesting attenuation of PMN activation.

3.4.4 Hepatic TNF-α mRNA after LPS/RAN treatment

Rats were treated with LPS or Veh and cotreated with RAN or Veh as in Fig 3.1. Hepatic TNF-α mRNA was evaluated 1, 2 and 3hr after the drug treatment. RAN alone did not affect TNF-α mRNA at any of the times examined (Fig 3.6). By contrast, LPS alone increased TNF-α mRNA at all the times examined. LPS/RAN treatment decreased TNF-α mRNA compared to LPS/Veh treatment at 1hr after RAN but had no significant effect at 2hr or 3hr (Fig 3.6).

3.4.5 Effects of p38 inhibitor on serum TNF-α protein and hepatic TNF-α mRNA after LPS/RAN treatment

Rats were treated with LPS/RAN or LPS/Veh, and SB 239063 or its vehicle was administered as in Fig 3.4. Serum TNF-α protein and hepatic TNF-α mRNA were evaluated at 2hrs after RAN or Veh treatment. LPS/RAN treatment caused a significant increase in serum TNF-α concentration compared to LPS/Veh treatment (Fig 3.7A). SB 239063 reduced serum TNF-α concentration both after LPS/RAN and after LPS/Veh treatment. In contrast, LPS/RAN treatment did not affect hepatic TNF-α mRNA compared to LPS treatment, and SB 239063 did not change TNF-α mRNA after either treatment (Fig 3.7B).

3.4.6 Hepatic TACE activity after LPS/RAN treatment

Figure 3.5 Effects of a p38 inhibitor on PMN biomarkers. Rats were treated with LPS, and 2hrs later they were treated with RAN or its vehicle. SB 239063 or its vehicle was administered as in Fig 3.3. A: PMNs in the liver were enumerated 2hrs and 6hrs after RAN or its vehicle. B: Serum MIP-2 was measured 2hrs after RAN or its vehicle. C: HOCl adduct staining was measured 6hrs after RAN. * significantly different from the respective treatment without RAN. # significantly different from the respective treatment without SB 239063. n=4-6.

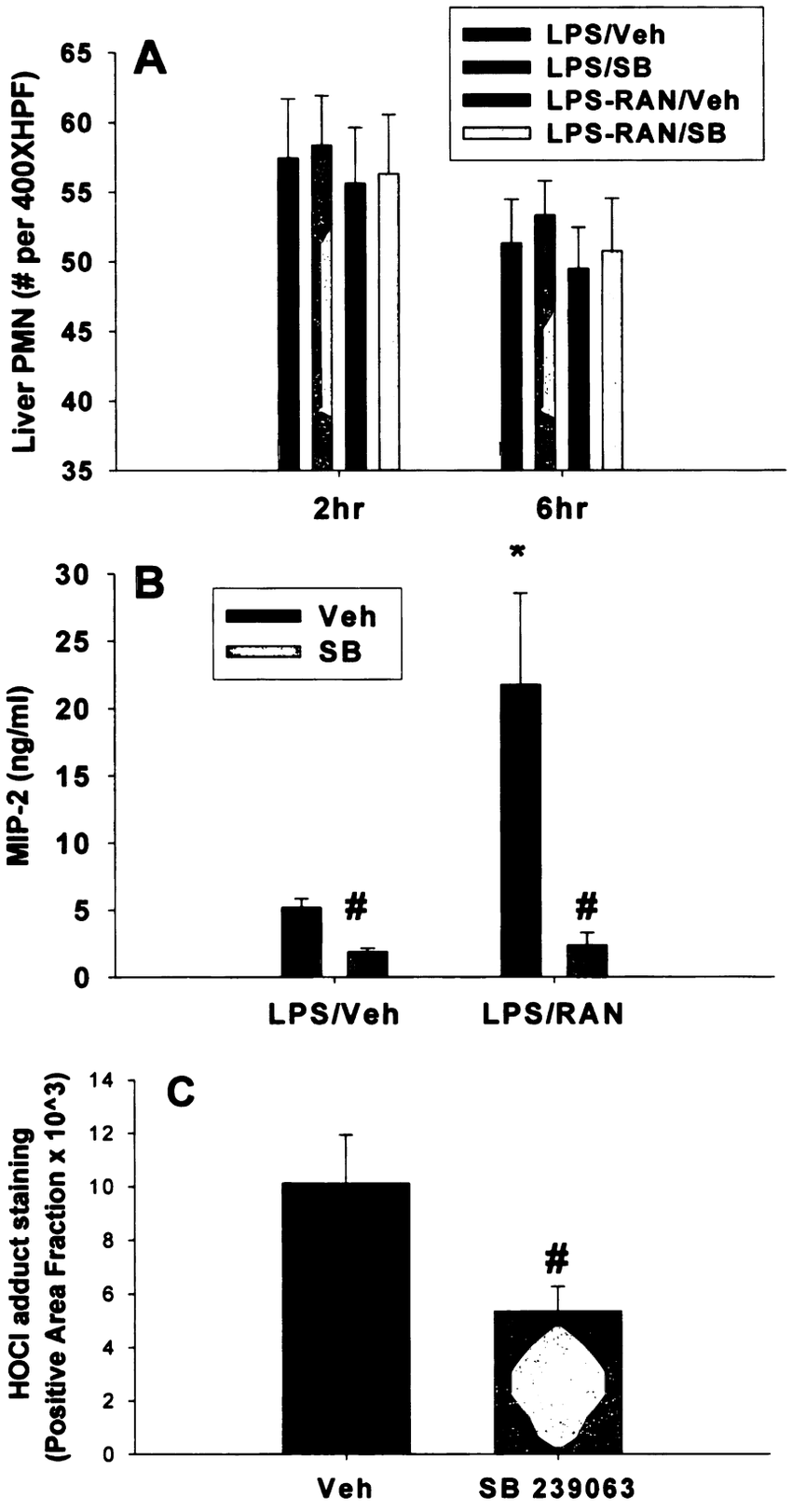

A
LPS/Veh
LPS/SB
LPS-RAN/Veh
LPS-RAN/SB
Liver PMN (# per 400XHPF)
65
60
55
50
45
40
35
2hr
6hr
B
Veh
SB
MIP-2 (ng/ml)
30
25
20
15
10
5
0
*
#
#
LPS/Veh
LPS/RAN
C
HOCl adduct staining
(Positive Area Fraction x 10^3)
14
12
10
8
6
4
2
0
#
Veh
SB 239063

Figure 3.6 Hepatic TNF-α mRNA after LPS/RAN treatment. Rats were treated with LPS or Veh and cotreated with RAN or Veh as in Fig 3.1. Hepatic TNF-α mRNA was evaluated 1hr, 2hr and 3hr after the drug treatment. * significantly different from the respective treatment without LPS. # significantly different from the respective treatment without RAN. n=4-8.

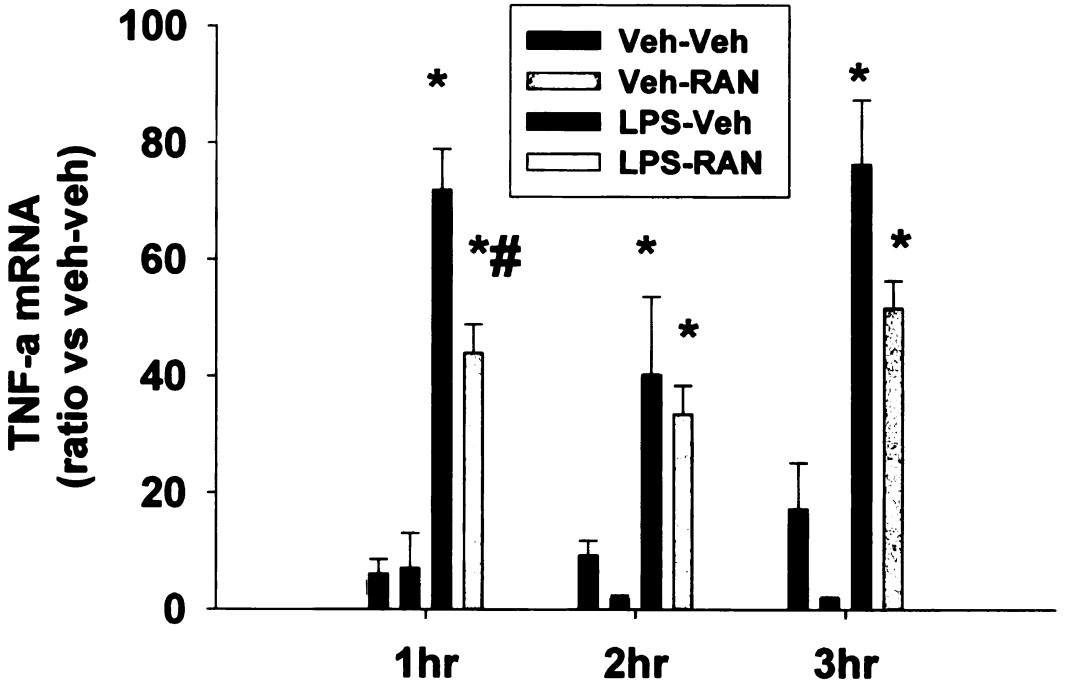

TNF-a mRNA
(ratio vs veh-veh)
100
80
60
40
20
0
Veh-Veh
Veh-RAN
LPS-Veh
LPS-RAN
*
*#
*
*
*
*
1hr
2hr
3hr

Figure 3.7 Effects of a p38 inhibitor on serum TNF-α protein and hepatic TNF-α mRNA. Rats were treated with LPS/RAN or LPS/Veh, and SB 239063 or its vehicle was also administered as in Fig 3.4. Serum TNF-α protein and hepatic TNF-α mRNA were evaluated at 2hrs after RAN or Veh treatment. * significantly different from the respective treatment without RAN . # significantly different from the respective treatment without SB 239063. n=4-7.

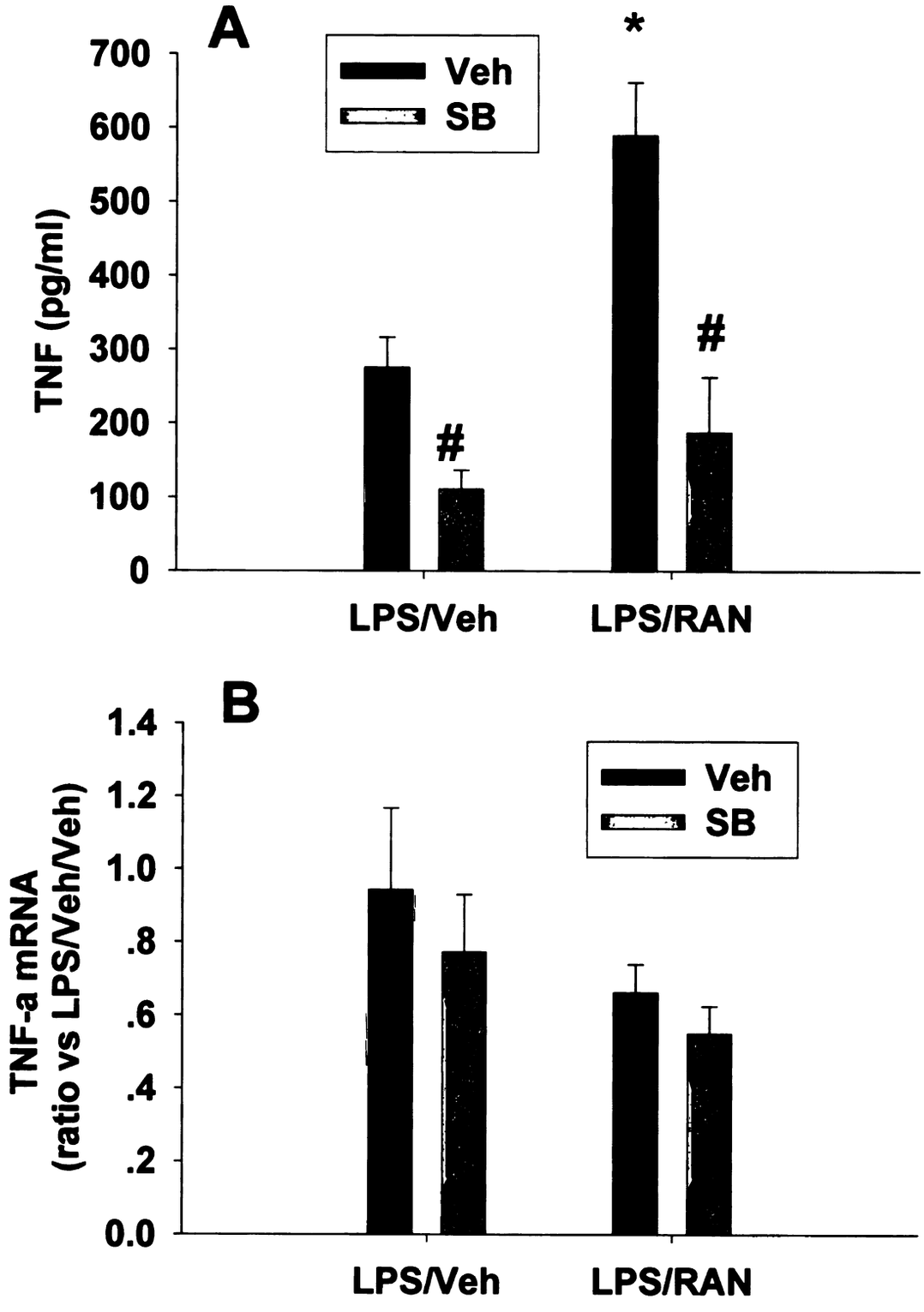
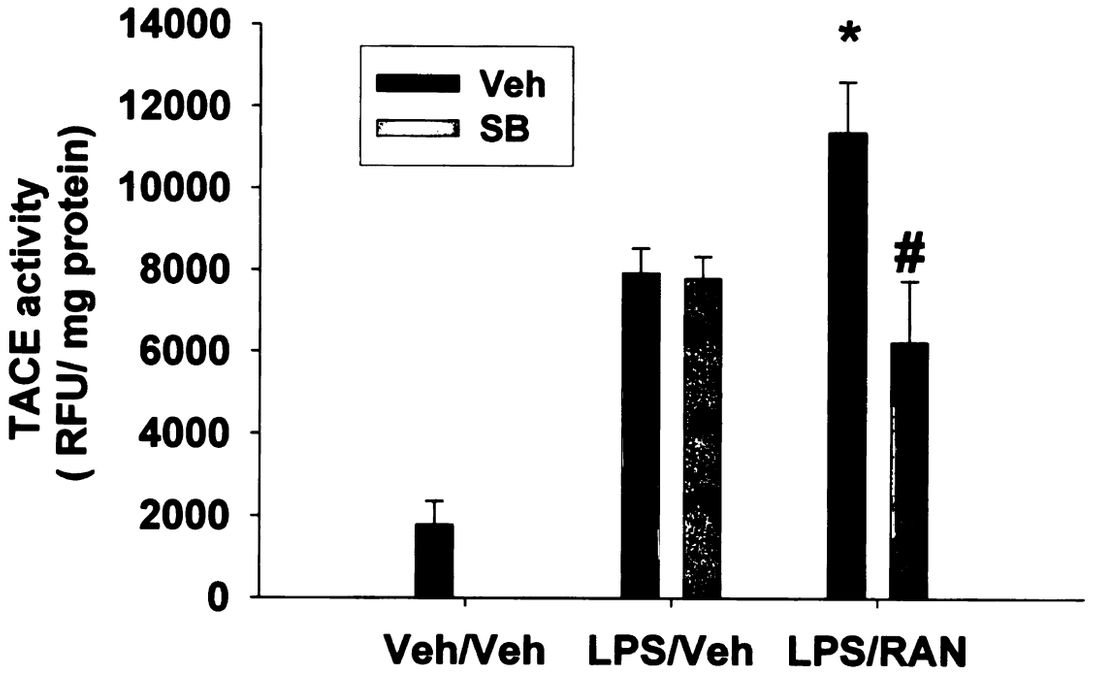

A
Veh
SB
TNF (pg/ml)
700
600
500
400
300
200
100
0
*
#
#
LPS/Veh
LPS/RAN
B
Veh
SB
TNF-a mRNA
(ratio vs LPS/Veh/Veh)
1.4
1.2
1.0
.8
.6
.4
.2
0.0
LPS/Veh
LPS/RAN

Figure 3.8 Hepatic TACE activity after LPS/RAN treatment. Rats were treated with LPS/RAN or LPS/Veh, and SB 239063 or its vehicle was also administered as in Fig 3.4. Hepatic TACE activity was measured 2hrs after RAN or Veh treatment. * significantly different from the respective treatment without RAN . # significantly different from the respective treatment without the SB compound. n=4-7.


14000
12000
10000
8000
6000
4000
2000
0
TACE activity
(RFU/ mg protein)
Veh
SB
*
#
Veh/Veh
LPS/Veh
LPS/RAN

Hep

trea

trea

SB

TA

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Hepatic TACE activity was measured 2hrs after RAN or Veh treatment. LPS/Veh treatment caused a significant increase in hepatic TACE activity as compared to Veh/Veh treatment (Fig 3.8). RAN treatment enhanced TACE activity in livers of LPS-treated rats. SB 239063 had no effect on hepatic TACE activity after LPS/Veh treatment but reduced TACE activity after LPS/RAN treatment to the same level as LPS/Veh treatment.

3.4.7 Effects of TACE inhibitor on serum TNFα and liver injury

A selective TACE inhibitor (BMS-561392; DPC-333) or its vehicle was administered 15 min before RAN or its vehicle. Serum TNFα concentration was measured 1hr after RAN treatment, and serum ALT activity was measured at 6hr. LPS/RAN treatment caused a significant increase in serum TNF-α concentration as compared to LPS/Veh treatment (Fig 3.9A), confirming previous results (Tukov et al., 2007g). The inhibitor did not affect serum TNF-α concentration after LPS/Veh treatment. By contrast, the TACE inhibitor decreased serum TNF-α concentration after LPS/RAN treatment to the same level as LPS/Veh treatment. The TACE inhibitor also reduced serum ALT activity after LPS/RAN treatment, indicating attenuation of liver injury (Fig 3.9B).

3.4.8 Effects of TACE inhibitor on plasma PAI-1

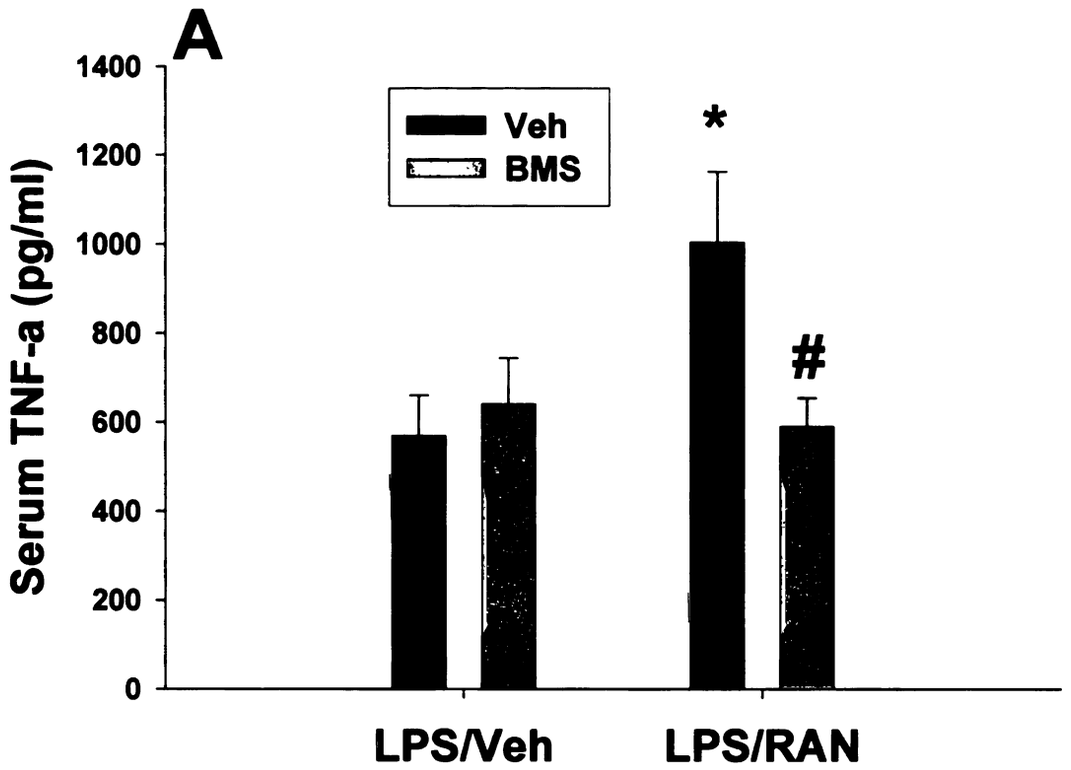
Rats were treated with LPS/RAN or LPS/Veh and with the TACE inhibitor as in Fig 3.9. Since previous results showed that TNF-α was important for PAI-1 production (Tukov et al., 2007f), plasma active PAI-1 concentration was measured 2hrs after RAN

treatment. Confirming previous results (Luyendyk et al., 2006d), LPS/RAN treatment caused a significant increase in plasma active PAI-1 concentration as compared to LPS/Veh treatment (Fig 3.10). The TACE inhibitor decreased plasma active PAI-1 concentration after LPS/RAN treatment to almost the same level as LPS/Veh treatment.

3.4.9 Effects of PAI-1 inhibitor on hepatotoxicity and markers of hemostasis and PMNs

A PAI-1 inhibitor, WAY-140312, was administered 1hr before RAN and again 1hr and 3hr later. Hepatotoxicity, plasma active PAI-1 concentration, hepatic fibrin deposition and liver HOCl adduct were measured 6hrs after RAN treatment. The PAI-1 inhibitor reduced plasma active PAI-1 concentration and serum ALT activity (Fig 3.11A and 11B). It also decreased hepatic fibrin deposition and HOCl adduct staining (Fig 3.11C and 11D). The PAI-1 inhibitor did not affect hepatic PMN accumulation or serum CINC-1 or MIP-2 concentrations after LPS/RAN treatment (data not shown).

Figure 3.9 Effects of a TACE inhibitor on serum TNF-α and liver injury. Rats were treated with LPS/RAN or LPS/Veh as in Fig 3.4. A selective TACE inhibitor, BMS-561392, or its vehicle was administered 15 min before RAN or its vehicle. (A) Serum TNF-α was measured 1hr after RAN treatment and (B) serum ALT activity was measured at 6hr. * significantly different from the respective treatment without RAN. # significantly different from the respective treatment without BMS-561392. n=6-10.


A
Serum TNF-a (pg/ml)
1400
1200
1000
800
600
400
200
0
Veh
BMS
*
#
LPS/Veh
LPS/RAN

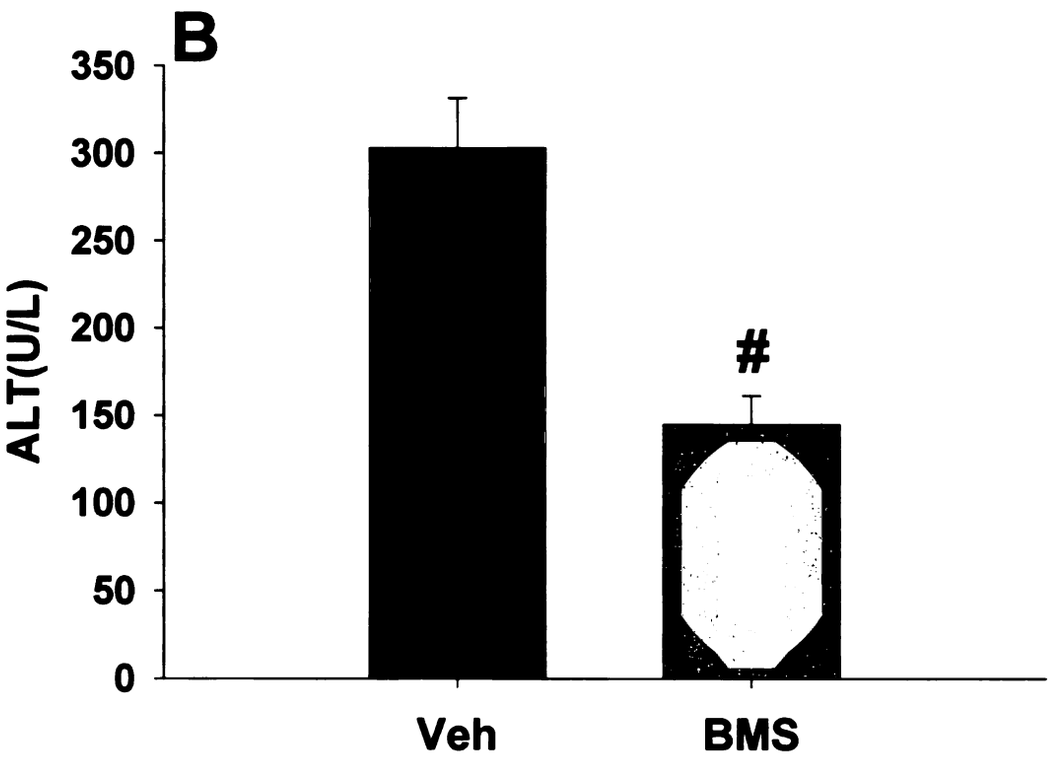

B
ALT(U/L)
350
300
250
200
150
100
50
0
#
Veh
BMS

Figure 3.10 Effects of TACE inhibitor on plasma active PAI-1. Rats were treated with LPS/RAN or LPS/Veh and with the TACE inhibitor as in Fig 3.9. Plasma active PAI-1 was evaluated 2hrs after RAN treatment. * significantly different from the respective treatment without RAN . # significantly different from the respective treatment without BMS-561392. n=6-10.

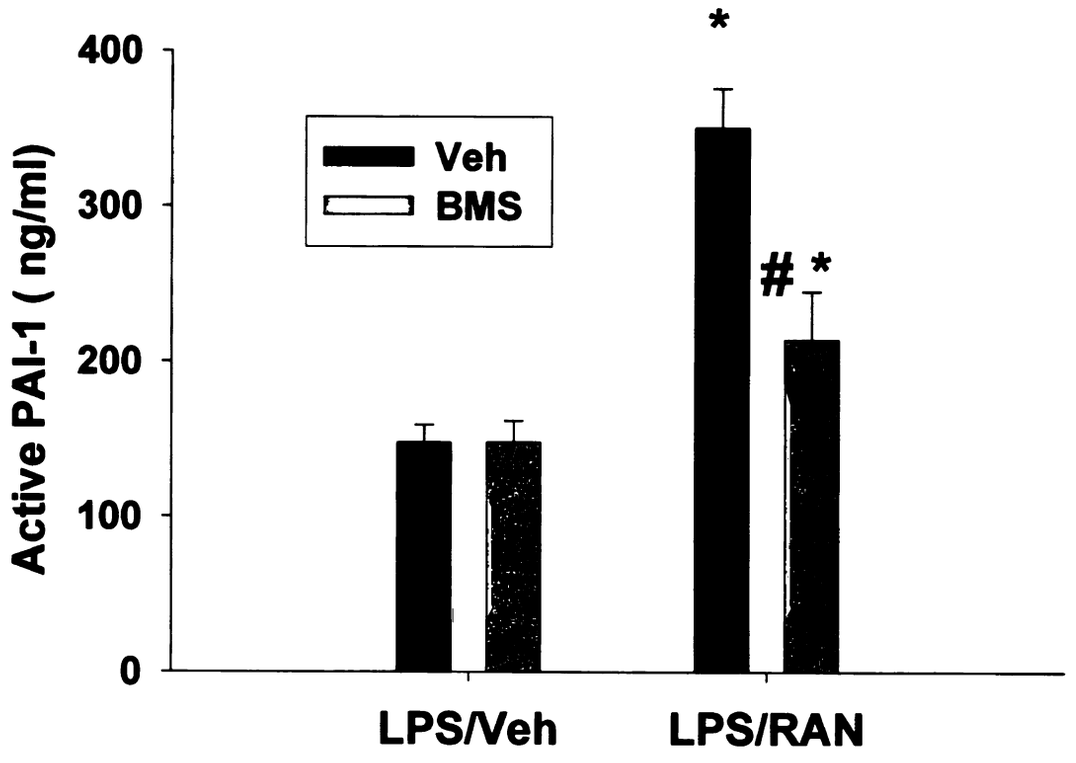
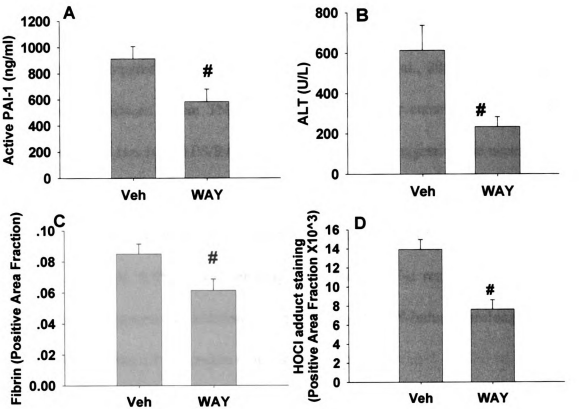

Active PAI-1 (ng/ml)
400
300
200
100
0
Veh
BMS
*
*
LPS/Veh
LPS/RAN

Figure 3.11 Effects of a PAI-1 inhibitor on hepatotoxicity and markers of hemostasis and PMN activation. Rats were treated with LPS and cotreated with RAN 2hrs later. A PAI-1 inhibitor, WAY-140312, was administered 1hr before RAN and again 1hr and 3hr later. Hepatotoxicity, plasma active PAI-1, hepatic fibrin deposition and liver HOCl adduct were measured 6hrs after RAN treatment. # significantly different from the respective treatment without WAY-140312. n=6-9.


A
Active PAI-1 (ng/ml)
1200
1000
800
600
400
200
0
#
Veh
WAY
B
ALT (U/L)
800
600
400
200
0
#
Veh
WAY
C
Fibrin (Positive Area Fraction)
.10
.08
.06
.04
.02
0.00
#
Veh
WAY
D
HOCl adduct staining
(Positive Area Fraction X10^3)
16
14
12
10
8
6
4
2
0
#
Veh
WAY

Discussion

At the dose used in the present study, LPS rapidly induced serum TNFα production but failed to cause liver injury (Tukov et al., 2007e). The serum TNFα concentration peaked at about 1.5 hours and rapidly decreased after that, returning almost to normal by 8 hours. This indicates that an increase in TNFα of this magnitude and duration is not sufficient to cause hepatocellular damage. RAN-cotreated rats had a longer lasting serum TNF-α increase compared to rats given LPS alone (Tukov et al., 2007d). Pretreatment with agents that reduced serum TNF-α (ie. pentoxyfylline or etanercept) before LPS challenge protected rats from LPS/RAN-induced liver injury, suggesting the necessity of TNF-α in producing the hepatocellular injury. However, since these agents were given before the LPS challenge, those results could not differentiate whether the LPS-induced TNF elevation or the RAN-induced prolongation of the TNF-α response to LPS was crucial for the pathogenesis. In addition, the mechanism of RAN-induced prolongation of TNF-α production after LPS exposure remains unknown.

p38 and its downstream MAPK Activated Protein Kinase 2 (MK-2) are involved in the production of several cytokines and chemokines including TNF-α (Hitti et al., 2006b;Neininger et al., 2002e;Numahata et al., 2003a). Interestingly, the p38-dependent cytokine/chemokines, such as TNF-α, MIP-2 and IL-6, are selectively upregulated in LPS/RAN-treated rats compared to rats treated with LPS/FAM or only with LPS (Luyendyk et al., 2006c;Tukov et al., 2007c). Furthermore, TNF-α has been shown to be critical for activation of the coagulation system, PMN chemokine production and

subsequent hepatotoxicity after LPS/RAN treatment (Tukov et al., 2007b). All of the above results suggested the possibility that p38 MAPK activation might be an upstream signal leading to the pathogenic cascade in LPS/RAN hepatotoxicity. Indeed, RAN, but not FAM, selectively augmented p38 activation early after LPS treatment. A p38 inhibitor given at the same time as the drug reduced the hepatotoxicity. This suggests that the p38 activation after RAN cotreatment in LPS-treated rats is critical for the liver injury.

The p38 inhibitor also reduced activation of the hemostatic system and PMN activation. The effect on the hemostatic sytem was reflected in the reduction of plasma TAT and PAI-1 concentration, whereas the effect on PMN activation was marked by the reduction in serum MIP-2 concentration and hepatic HOCl-adduct staining. Similar effects were observed after treatment with agents that reduced serum TNF-α, such as pentoxyfylline or etanercept (Tukov et al., 2007a). These results are consistent with the possibility that p38 activation was responsible for the prolonged TNF-α production caused by RAN cotreatment and that TNF-α precipitated the downstream effects on the hemostatic system and PMNs.

RAN did not increase the hepatic TNF-α mRNA level after LPS treatment nor it did not increase the TNF-α mRNA stability at least during 1 hr-3 hr after RAN treatment, suggesting a post-transcriptional mode of action. Moreover, the reduction in serum TNF-α protein concentration after inhibition of p38 MAPK was not accompanied by diminished hepatic TNF-α mRNA, suggesting that p38 regulated TNF-α production after

RAN treatment in a post-transcriptional manner. p38 and its downstream activation of MK-2 have been shown to regulate TNF-α production in macrophages mostly by increasing mRNA translation (Neininger et al., 2002f;Hitti et al., 2006a). This was mediated by the AU-rich 3'-untranslated region of TNF-α mRNA. This finding is consistent with our finding that a p38 inhibitor reduces TNF-α production in a post-transcriptional manner.

As mentioned in the introduction, increase in TNF-α protein can arise from the cleavage of pro-TNF-α by TACE (Aggarwal et al., 1985a;Mullberg et al., 2000b). Accordingly, another possibility is that p38 activates TACE, leading to increased TNF-α protein release into the circulation from Kupffer cells or other nonparenchymal cells in liver. Indeed, p38 is essential for ectodomain shedding of TNF-α in CHO cells (Fan and Derynck, 1999b). In the present study, LPS/RAN treatment increased hepatic TACE activity as compared to LPS/Veh treatment (Fig 8) the p38 inhibitor reduced hepatic TACE activity after LPS/RAN treatment to the same level as LPS/Veh treatment. Furthermore, a TACE inhibitor similarly reduced serum TNF-α concentration and liver injury. All of these results suggest that RAN prolonged TNF-α production after LPS treatment mainly through augmented p38-dependent TACE activity. However, further studies need to be conducted to check whether RAN could also prolong TNF-α production in some part by p38-induced TNF-α translation.

As mentioned above, previous results could not differentiate whether the LPS-induced TNF elevation or the RAN-induced prolongation of the TNF-α response to

LPS was crucial for the pathogenesis. In the present study, the TACE inhibitor was given immediately before RAN treatment so that the inhibitor did not affect serum TNF-α level until after RAN was administered. This treatment regimen was effective in reducing hepatocellular injury, suggesting that the RAN-induced prolongation of the TNF-α response was critical for LPS/RAN-induced hepatotoxicity. In contrast to RAN, FAM did not prolong TNF-α response after LPS treatment. Thus, the increase in LPS-stimulated TNF-α production could distinguish a drug that causes human IADRs from one that does not.

In human case reports of RAN-induced idiosyncratic hepatotoxicity, no direct evidence for enhanced serum TNF-α or other cytokines has been reported. However, most clinical samples are taken after the hepatotoxicity develops when the peak of TNF-α has likely passed. Furthermore, it is interesting that in 24 out of 34 human cases of RAN idiosyncratic hepatotoxicity, prodromal signs consistent with endotoxemia (i.e., increased LPS conc. in blood) or inflammation (e.g. diarrhea, fever, nausea/vomiting, and/or abdominal pain) were present (Luyendyk et al., 2003i). These clinical signs are consistent with increased production of TNF-α and other inflammatory cytokines.

The TACE inhibitor also reduced plasma active PAI-1 concentration after LPS/RAN treatment to almost the same level as after LPS/Veh treatment, suggesting enhanced PAI-1 production as a possible downstream effect of RAN-augmented TNF-α production. Since PAI-1 is an important negative regulator of the fibrinolytic system (Levi, 2005a;Levi et al., 2003a), this is consistent with the previous finding that fibrin

deposition resulting from the perturbed hemostatic system is crucial for the liver injury caused by LPS/RAN (Luyendyk et al., 2004b). Indeed, in the present study a PAI-1 inhibitor reduced the hepatocellular injury caused by LPS/RAN cotreatment. The inhibitor also reduced hepatic fibrin deposition and PMN activation. Fibrin deposition can lead to hypoxia, which occurs early in this model (Deng et al., 2007d;Luyendyk et al., 2005t). Hypoxia could potentiate the killing of hepatocytes by proteases (e.g. elastase) released from PMNs after their activation (Luyendyk et al., 2005u). Indeed, proteases released from activated PMNs have been shown to be important in the pathogenesis (Deng et al., 2007c).

Although the PAI-1 inhibitor decreased PMN activation, it did not affect hepatic PMN accumulation or serum PMN chemokine concentration, suggesting a direct effect of PAI-1 on PMN activation. A recent study showed that PAI-1 directly potentiates LPS-induced PMN activation through a Jun-N-terminal kinase (JNK)-dependent pathway (Kwak et al., 2006b). In the LPS/RAN model, LPS causes PMN accumulation in the liver, and RAN somehow activates the hepatic PMNs (Deng et al., 2007b). RAN itself did not enhance PMN activation. In fact, it has been shown to reduce PMN activation in vitro (Okajima et al., 2002c;Okajima et al., 2000c). All of these results suggest that RAN might induce activation of PMNs accumulated in the liver after LPS exposure indirectly by augmenting PAI-1 production. Polymorphisms in PAI-1 in the human population have been identified (Lane and Grant, 2000), and thus PAI-1 could represent a potential interaction between genetic and environmental factors (i.e. inflammatory stress) in

RAN-induced IADRs. For instance, patients with a more active PAI-1-producing allele might be more susceptible to RAN-induced IADRs caused by endotoxin exposure or some other inflammatory stress.

As summarized in Fig 3.12, RAN augmented TNF-α production after LPS treatment in a post-transcriptional manner by enhancing p38 activation. The increase in TNF-α protein appears to occur through the p38-dependent activation of TACE. The prolongation of LPS-induced TNF-α production by RAN appears to be crucial for the liver injury caused by LPS/RAN cotreatment. The injury appears to involve PAI-1, which is important for both the hepatic fibrin deposition and PMN activation. The hypoxia resulting from hepatic fibrin deposition could act synergistically with toxic proteases released from activated PMNs to kill the hepatocytes.

Figure 3.12 Diagram of pathogenic mechanism contributing to hepatocellular injury in LPS/RAN model. RAN augments TNF-α production after LPS treatment in a post-transcriptional manner by enhancing p38 activation. The increase in TNF-α protein appears to occur through the p38-dependent activation of TACE. The prolongation of LPS-induced TNF-α production by RAN causes more PAI-1 production, which is important for both the hepatic fibrin deposition and PMN activation. The hypoxia resulting from hepatic fibrin deposition could act synergistically with toxic proteases released from activated PMNs to kill the hepatocytes.

RAN
LPS
p38
Pro-TNF
TACE
TNF protein
PAI-1
Fibrin
Hypoxia
Liver injury
PMN accumulation
PMN activation

CHAPTER FOUR

Xiaomin Deng, Robert F. Stachlewitz, Michael J. Liguori, Eric A.G. Blomme, Jeffrey F. Waring, James P. Luyendyk, Jane F. Maddox, Patricia E. Ganey and Robert A. Roth. (2006) Modest inflammation enhances diclofenac hepatotoxicity in rats: role of neutrophils and bacterial translocation. J Pharmacol Exp Ther. 319(3):1191-9.

4.1

4.1 Abstract

Idiosyncratic adverse drug reactions (IADRs) represent an important human health problem, yet animal models for preclinical prediction of these reactions are lacking. Recent evidence in animals suggests that some IADRs arise from drug interaction with an inflammatory episode that renders the liver sensitive to injury. Diclofenac (DCLF) is one of those drugs for which the clinical use is limited by idiosyncratic liver injury. We tested the hypothesis that modest inflammation triggered in rats by a small dose of lipopolysaccharide (LPS) renders a nonhepatotoxic dose of DCLF injurious to liver. Cotreatment of rats with nonhepatotoxic doses of LPS and DCLF resulted in elevated serum alanine aminotransferase (ALT) activity and liver histopathologic change 6 hours after DCLF administration. Neither LPS nor DCLF alone had such an effect. Gene array analysis of livers revealed a unique gene expression pattern in the LPS/DCLF-cotreated group compared to groups given either agent alone. Antiserum-induced neutrophil (PMN) depletion in LPS/DCLF-cotreated rats protected against liver injury, demonstrating a crucial role for PMNs in the pathogenesis of this LPS/DCLF interaction. Gut sterilization of LPS/DCLF-treated rats did not protect against liver injury. In contrast, gut sterilization did attenuate liver injury caused by a large, hepatotoxic dose of DCLF, suggesting that hepatotoxicity induced by large dose of DCLF is caused in part by its ability to increase intestinal permeability to endotoxin or other bacterial products and to cause their translocation. These results demonstrate that inflammation-DCLF interaction

precipit

predict

precipitates hepatotoxicity in rats and raise the possibility of creating animal models that predict human IADRs.

4.2 Introduction

Idiosyncratic adverse drug reactions (IADRs) remain a challenging human health problem (Boelsterli, 2003e;Kaplowitz, 2005a). They are one of the leading causes for drug development failures and removal of medicines from the market. A well known example is troglitazone, which was removed from the market by the Food And Drug Administration in 2000, because of its potential to cause idiosyncratic liver injury (Chojkier, 2005a). IADRs appear to be independent of dose, and the onset of injury varies relative to the onset of drug treatment. Unfortunately, the mechanisms of idiosyncratic reactions are poorly understood despite the large number of drugs associated with these reactions. One of the common targets of idiosyncratic toxicity is liver. Animal models to predict these adverse reactions are lacking. It is therefore of interest to establish models that mimic human IADRs. In addition, mechanistic studies of such models could provide biomarkers for IADRs or strategies to prevent them.

There are two conventional hypotheses to explain IADRs. One is that the reactions occur as a consequence of drug metabolism polymorphisms, which result in different levels of toxic drug metabolites among patients (Williams and Park, 2003a). The other one argues that they arise from a specific immune response to a hapten formed by a drug or its metabolites (Pirmohamed et al., 2002a). However, convincing evidence for these hypotheses is lacking for the majority of drugs associated with idiosyncratic toxicity. It is equally plausible that other, unrecognized events render tissues susceptible to toxicity during drug therapy.

Results of several studies in experimental animals indicate that modest inflammation, triggered by a small dose of lipopolysaccharide (LPS), augments hepatotoxicity induced by several classes of xenobiotic agents. For example, coadministration of nonhepatotoxic doses of LPS and trovafloxacin (TVX), a quinolone antibiotic associated with hepatic IADRs, results in liver damage that resembles human TVX idiosyncrasy. By contrast, cotreatment with LPS and levofloxacin, a quinolone antibiotic without idiosyncratic liability, did not produce liver injury at a dose equipotent to that of TVX (Waring et al., 2006a). Episodes of modest, subclinical inflammation are commonplace in people. Since they occur irregularly and may go unnoticed during drug therapy, their interaction with drugs could explain the erratic temporal and dose relationships that characterize IADRs (Roth et al., 2003a).

Non steroidal anti-inflammatory drugs (NSAIDs) comprise another class of drugs, some of which cause hepatic IADRs. For example, diclofenac(DCLF) has caused rare but sometimes serious hepatotoxicity in humans (Boelsterli, 2003d). Although the apparent incidence of severe DCLF-induced hepatic adverse reactions is quite low (from 1 to 2 cases per million prescriptions or 6 to 18 cases/100,000 person-year.), the large number of patients treated with DCLF makes the absolute number of cases impressive (Walker, 1997a). In addition, cases of severe injury leading to liver transplantation comprise a large proportion of the reported cases of DCLF-induced hepatotoxicity (Lewis et al., 2003). The scarceness of liver biopsies from patients and the diverse histopathlogical presentations in available samples make it difficult to draw clues about mechanisms from

human liver pathology alone (Zimmerman et al., 1999). Thus, the pathogenesis of this low-incidence/ high-severity DCLF hepatotoxicity is largely unknown. Several mechanisms have been proposed, including formations of reactive drug metabolites, oxidative stress (Cantoni et al., 2003b), mitochondrial injury (Masubuchi et al., 2002b) and immune-modulated hypersensitivity (Greaves et al., 2001b). Experimental data supporting such mechanisms are incomplete, especially at the *in vivo* level (Cantoni et al., 2003c;Boelsterli, 2003c).

Based on the observation that a number of drugs that have the ability to cause IADRs in human patients cause liver injury in LPS-treated rats and are not hepatotoxic in naïve rats, we tested the hypothesis that a modest inflammatory episode in rats increases the sensitivity of the liver to hepatotoxic effects of DCLF. We found that pretreatment of rats with a small, nonhepatotoxic dose of LPS renders a nontoxic dose of DCLF injurious to liver. Furthermore, we determined whether gene expression profiles could distinguish a LPS/DCLF interaction that causes hepatotoxicity from the nontoxic treatments with LPS or DCLF alone and provide clues about the mode of action.

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4.3 Methods

4.3.1 Experimental Design

Rats received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences, and procedures were approved by the Michigan State University Committee on Animal Use and Care. Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR; Charles River, Portage, MI) weighing 250 to 350 grams were used for these studies. Animals were fed standard chow (Rodent chow/Tek 8640, Harlan Teklad, Madison, WI) and allowed access to water *ad libitum*. They were allowed to acclimate for 1 week in a 12-hr light/dark cycle before use.

Rats were fasted for 16 hrs then given a nonhepatotoxic dose of LPS (29 X 10^6 endotoxin units (EU)/kg; Sigma-Aldrich, Inc., St. Louis, MO, Cat. No. L-2880; lot 072K4095) or sterile saline (i.v.). This activity was determined using a QCL Chromogenic LAL Endpoint Assay from Cambrex (East Rutherford, NJ). Two hours later, they were given 20 mg/kg DCLF (Sigma-Aldrich, Inc., St. Louis, MO) or sterile saline, ip. For the high dose DCLF study, 100mg/kg DCLF was given without LPS administration. Rats remained fasted and were sacrificed 6 hrs after DCLF treatment for evaluation of liver injury, histopathology and gene expression. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Blood was collected from the dorsal aorta and a portion of the blood was placed into a tube containing sodium citrate (final concentration, 0.38%) for collection of plasma. Another portion was allowed to clot at

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room temperature, and serum was collected and stored at -20°C until use. Representative (3-4-mm) slices of the ventral portion of the left lateral liver lobe were collected and fixed in 10% neutral-buffered formalin. A portion of the right medial lobe of the liver was flash-frozen in liquid nitrogen for subsequent gene expression analysis.

4.3.2 PMN Depletion

PMNs were depleted by intravenous administration of 0.25ml rabbit anti-rat PMN serum (anti-PMN serum, Inter-cell Technologies, Jupiter, FL), diluted 1:1 with sterile saline, sixteen hours before administration of LPS. Normal rabbit serum (normal serum) was administered to some animals as a control. Previous studies in which anti-PMN serum was administered to rats demonstrated a selective depletion of PMNs .

4.3.3. Gut Sterilization

Gut sterilization was achieved by treating rats with polymyxin B (150 mg/kg) and neomycin (450 mg/kg) orally for 4 days before LPS/DCLF treatment or high dose DCLF alone. This treatment has been shown to completely abolish the gram-negative bacterial growth in rat fecal culture and to reduce plasma endotoxin concentration after chronic ethanol treatment (Adachi et al., 1995).

4.3.4 Bacterial Culture in Fecal and Liver Homogenates

Fecal and liver bacterial cultures were performed 6 hrs after DCLF or saline treatment. Rat fecal pellets (400mg) were collected directly from the anus and homogenized in 4 mL trypticase soy broth containing 15% glycerol. A 10μl fecal slurry was plated on MacConkey plates (Becton, Dickinson Co., Sparks, MD). For the liver bacterial culture, 100mg of the liver from the left lateral lobe was collected in 0.5 ml trypticase soy broth containing 15% glycerol and homogenized. Liver homogenates were diluted serially to $1:10^6$, and 100 μl of each dilution was plated on MacConkey plates. The culture plates were incubated for 24 hrs at 37°C in an atmosphere of 95% air and 5% CO_2. The presence or absence of bacterial colonies was examined for the fecal homogenates. The colonies of bacteria were counted for each dilution of liver homogenate and averaged for each animal.

4.3.5 ALT Activity and Histopathology Assessment

Hepatic parenchymal cell injury was estimated by quantifying serum alanine aminotransferase (ALT) activity. ALT activity was determined spectrophotometrically using Infinity-ALT from Thermo Electron Corp. (Louisville, CO). Formalin-fixed liver samples were routinely processed and stained with hematoxylin and eosin (H&E). Slides were read by a pathologist (EB) blinded to the treatment.

4.3.6 RNA Preparation

Frozen liver samples (approximately 100 mg of tissue per sample) were immediately added to 2 mL of TRIzol reagent per sample (Invitrogen Life Technologies, Carlsbad, California) and homogenized using a Polytron 300D tissue grinder (Brinkman Instruments, Westbury, NY). One mL of the tissue homogenate was transferred to a microfuge tube, and total RNA was extracted with chloroform followed by nucleic acid precipitation with isopropanol. The pellet was washed with 80% ethanol and resuspended in molecular biology grade water. Nucleic acid concentration was determined spectrophotometrically at 260 nm (Smart-Spec, Bio-Rad Laboratories, Hercules, CA), and RNA integrity was evaluated using an Agilent bioanalyzer (Agilent Technologies, Model 2100, Foster City, CA).

4.3.7 Gene Array Analysis

Microarray analysis was performed using the standard protocol provided by Affymetrix, Inc. (Santa Clara, CA). Briefly, approximately 15 μg of total RNA was reversed transcribed into cDNA using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, California) according to the manufacturer's instructions with the exception that the primer used for the reverse transcription reaction was a modified T7 primer with 24 thymidines at the 5' end (Affymetrix). The sequence was 5'GGCCAGTGAATTGTAATA- CGACTCACTATAGGGAGGCGG-(dT)24-3'. cDNA was purified via phenol/chloroform/isoamylalcohol (Invitrogen Life Technologies, Carlsbad, California) extraction and ethanol precipitation. The purified cDNA was

resuspended in molecular biology grade water. Following this procedure, biotin-labeled cRNA was synthesized according to the manufacturer's instructions from the cDNA using the Enzo RNA Transcript Labeling Kit (Affymetrix). The labeled cRNA was then purified using RNeasy kits (Qiagen, Valencia, CA). Subsequently, cRNA concentration and integrity were evaluated. Approximately 20 μg of cRNA was then fragmented in a solution of 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate at 94°C for 35 minutes. Fragmented, labeled cRNA was hybridized to an Affymetrix rat genome RAE230A array, which contains sequences corresponding to roughly 15,900 transcripts, at 45°C overnight using an Affymetrix Hybridization Oven 640. The array was subsequently washed and stained twice with strepavidin-phycoerythrin (Molecular Probes, Eugen, OR) using a Gene-Chip Fluidics Workstation 400 (Affymetrix). The array was then scanned using the Affymetrix GeneChip® Scanner 3000.

The microarray scanned image and intensity files (.cel files) were imported into Rosetta Resolver gene expression analysis software version 4.0 (Rosetta Inpharmatics, Seattle, WA). Error models were applied and ratios were built for each treatment array versus its respective vehicle control (pooled in silico). Using Rosetta Resolver, a p-value is calculated for every fold change, using the Rosetta Resolver error model (Rajagopalan, 2003).

4.3.8 Statistical Filtering of Genes Changed After LPS/DCLF Treatment

Genes changed after treatment with LPS/DCLF, LPS/saline or saline/DCLF were identified with saline/saline as baseline using the following criteria: $P<0.01$ in at least 3 out of 4 animals (groups LPS/saline and LPS/DCLF); $P<0.01$ in at least 4 out of 5 animals (group saline/DCLF). To identify the genes that changed after LPS/DCLF treatment relative to LPS/saline group or saline/DCLF group, a second filter was applied with the LPS/DCLF group as baseline using the same criteria above.

The genes expressed to a greater degree after LPS/DCLF treatment than after LPS/saline and saline/DCLF treatment were classified based on the relationship of their gene products to one or more functions. The classification was based on the gene function described in Entrez Gene site (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene). Furthermore, this list of genes was imported into Genomatix Bibliosphere 5.13 software (München, Germany). This yielded a list of biological process terms ranked by their probability of over-representation or under-representation in a particular gene list. Functional annotations were based on the Genomatix knowledge base. "Expected" refers to the numbers of gene expression changes expected by chance in the gene list. The "Z Score" ranks the deviation of the observed number of genes in one biological process category of the Genomatix database found in the imported list of genes from the numbers expected to occur by chance. That is, it ranks the probability that the genes in this category are over-represented or under- represented in the gene list.

4.3.9 Serum MIP-2 Concentration and Blood/Liver Leukocyte Evaluation

Serum MIP-2 concentrations were determined using a commercially available ELISA kit (Biosource International, Camarillo, CA). Total blood leukocytes were quantified using a Unopette white blood cell (WBC) determination kit (Becton-Dickinson, Franklin Lakes, NJ) and a hemacytometer. Slides were prepared from whole blood and stained using the Hema 3® Staining System (Fisher Scientific, Middletown, VA), and differential counting was performed. Immunohistochemical staining for PMNs was performed on formalin-fixed liver sections as described previously (Kim et al., 2005d). Hepatic PMN accumulation was evaluated by counting PMNs in 20 randomly selected, high-power fields (HPF, 400X).

4.3.10 Statistical Analysis

All data are expressed as mean $\pm$ SEM. ALT activity, ELISA and circulating and liver leukocyte data were analyzed by two-way ANOVA with Tukey's posthoc test. For Figures 1 and 5 and Table 5, one-way ANOVA was applied. Data were transformed if they did not pass the normality and equal variance tests for ANOVA. For microarray analysis, error models were applied and ratios were built for each treatment array versus its averaged respective vehicle control using the Rosetta Resolver system. Gene expression was considered significantly changed if the p-value was less than or equal to 0.01. Agglomerative cluster analysis was performed using the average link heuristic criteria and the Euclidean distance metric for similarity measure.

4.4 Results

4.4.1 Hepatotoxicity from DCLF/LPS Interaction

In a preliminary study, a large dose of DCLF caused liver injury within 6 hrs. Accordingly, in an initial dose response study, rats were given DCLF (i.p) at doses from 0-100mg/kg, and serum ALT activity was evaluated 6 hrs after DCLF treatment. Doses up to 40mg/kg did not cause elevation in serum ALT activity, indicating no liver injury. Administration of DCLF at doses of 50mg/kg and 100mg/kg caused a significant increase in ALT activity (Fig 4.1), indicating hepatocellular injury.

To determine the influence of a nonhepatotoxic dose of LPS on DCLF hepatotoxicity, rats were pretreated with LPS ($29X10^6$ EU/kg, iv) or sterile saline, Two hrs later they were treated with DCLF (20mg/kg, ip) or saline. Serum ALT activity was evaluated 6 hrs later. Neither LPS nor DCLF when given alone altered ALT activity. However, cotreatment with LPS and DCLF caused a significant increase in serum ALT activity (Fig 4.2).

Livers from both saline/saline-treated and saline/ DCLF-treated rats had no or minimal histopathological changes (Table 4.1). LPS/saline treatment caused moderate leukocyte infiltration, occasional hepatocellular apoptosis, and modest parenchymal edema and hemorrhage. LPS/DCLF cotreatment enhanced hepatocellular apoptosis, parenchymal edema and hemorrhage relative to LPS/saline treatment.

4.4.2 Microarray Analysis of Livers from Rats Treated with LPS/DCLF

Figure 4.1 Dose-responsive DCLF hepatotoxcity. DCLF was given at doses ranging from 0-100mg/kg, i.p, and ALT was measured 6 hrs later. n=3-4; * significantly different from saline-treated group. $p<0.05$.

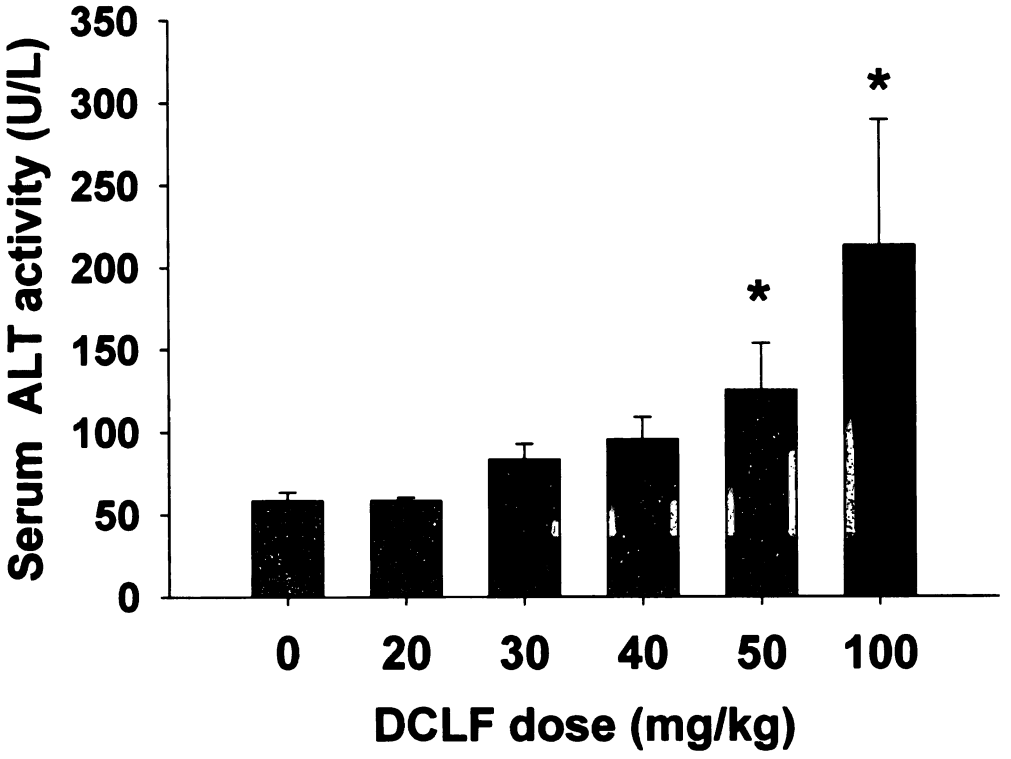
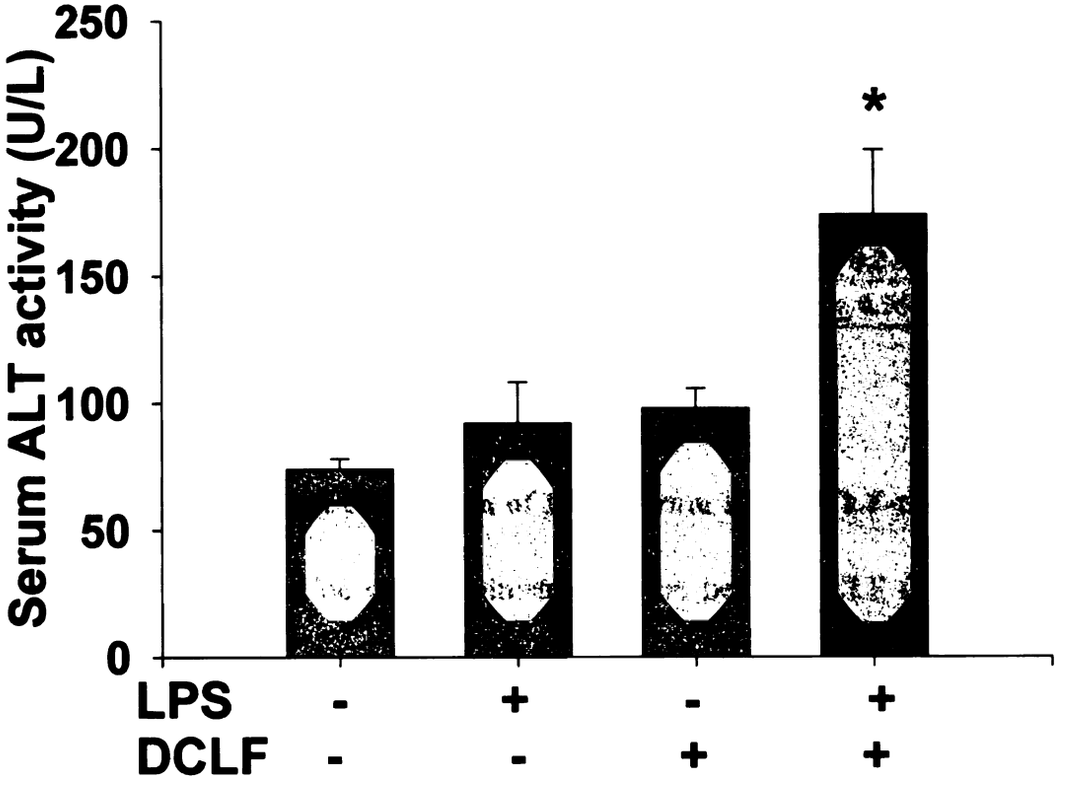

Serum ALT activity (U/L)
350
300
250
200
150
100
50
0
*
*
0
20
30
40
50
100
DCLF dose (mg/kg)

Figure 4.2 LPS potentiates DCLF hepatotoxicity. LPS ($29X10^6$ EU/kg, i.v) or vehicle was administered 2 hrs before DCLF (20mg/kg, i.p) or vehicle. ALT was measured 6 hrs after DCLF or its vehicle. n=4-5; * significantly different from all other groups. $p<0.05$.


Serum ALT activity (U/L)
250
200
150
100
50
0
*
LPS - + - +
DCLF - - + +

Treatment	Parenchymal Edema Multifocal	Parenchymal Hemorrhage Multifocal	Apoptosis Random	Leukocyte Infiltration
Saline/Saline	**0.0 ± 0**	**0.0 ± 0**	**0.0 ± 0**	**0.0 ± 0**
LPS/Saline	**1.6 ± 0.25**	**1.2 ± 0.2**	**1.25 ± 0.22**	**1.8± 0.38**
Saline/DCLF	**0.8 ± 0.2**	**0.0 ± 0**	**0.6 ± 0.24**	**0.0 ± 0**
LPS/DCLF	**3.0 ± 0**	**2.25 ± 0.25**	**2.25 ± 0.25**	**2± 0**

Table 4.1 Histopathological Evaluation of Livers from LPS/DCLF-treated Rats.LPS ($29X10^6$ EU/kg, i.v) or saline was administered 2 hrs before DCLF (20mg/kg, i.p) or saline. Livers were taken 6 hr after DCLF and stained with H&E. Liver histopathology was evaluated on a scale of 0-4 based on severity (0: normal; 1: minimal; 2: mild; 3: moderate; 4: severe). n=4-5.

Rat livers were subjected to RNA extraction and subsequent microarray analysis for global gene expression. Hierachical clustering analysis revealed mostly treatment-related clusters (Fig 4.3). Saline/DCLF treatment caused few gene expression changes. All rats treated with LPS irrespective of cotreatment clustered together. Within this major cluster, LPS/DCLF-treated rats formed a distinct subcluster. One LPS/saline-treated rat clustered with the LPS/DCLF-treated group. Interestingly, this animal had the largest serum ALT value in the LPS/saline group.

The number of gene expression changes after LPS/saline, saline/DCLF and LPS/DCLF treatments were identified with saline/saline-treated rats as the baseline. The numbers of genes changed in expression are depicted as a Venn diagram (Fig 4.4). A large number of gene expression changes occurred after either LPS/DCLF or LPS/saline treatment, but many more expression alterations occurred in the former group. A large number of gene expression changes occurred after both of these treatments (L and LD intersection in Fig 4.4). Numerous genes were differentially expressed specifically in the LPS/DCLF group (LD in Fig 4.4). DCLF alone caused few changes in gene expression based on the criteria used.

The genes altered in expression after LPS/DCLF treatment were further filtered by their expression pattern. Normalization to LPS/DCLF as the baseline was used to identify the genes changed to a greater degree after LPS/DCLF treatment compared with LPS alone and DCLF alone. This group of genes was interesting because liver injury occurred

Figure 4.3 Hierachical cluster analysis of hepatic gene expression after LPS/DCLF treatment. LPS ($29X10^6$ EU/kg, i.v) or vehicle was administered 2 hrs before DCLF (20mg/kg, i.p) or vehicle. Livers were taken at 6hr after DCLF, and mRNA was extracted and subjected to Affymetrix RAE230A gene array analysis. Gene expression changes were determined using saline/saline group as baseline and hierachical cluster analysis was performed by Rosetta Resolver using Euclidean average link distance.

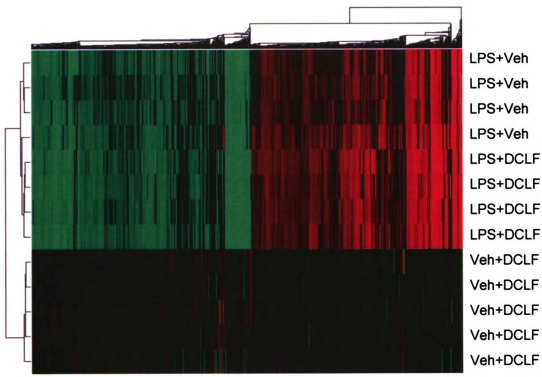
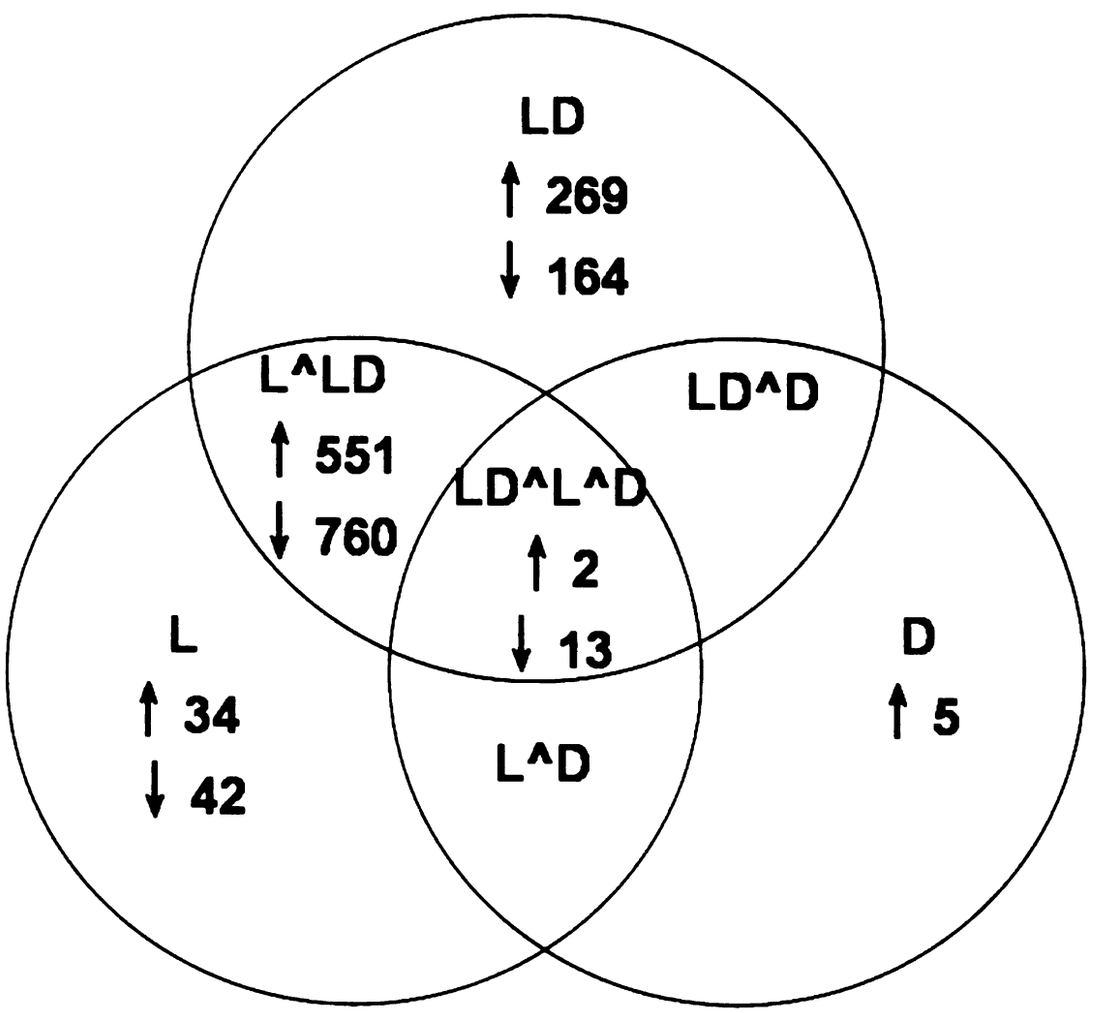

LPS+Veh
LPS+Veh
LPS+Veh
LPS+Veh
LPS+DCLF
LPS+DCLF
LPS+DCLF
LPS+DCLF
Veh+DCLF
Veh+DCLF
Veh+DCLF
Veh+DCLF
Veh+DCLF

Figure 4.4 Venn Diagram of gene expression changes caused by LPS/DCLF. Gene array analysis was performed as described in Fig 3. Active genes were identified as described in Methods, using saline/saline as the baseline. L indicates LPS/saline, LD indicates LPS/DCLF and D indicates saline/DCLF. The intersection of treatment groups indicates the gene expression changed after both or all three of the indicated treatments. Up arrow indicates numbers of genes with enhanced expression and down arrow indicates reduced expression.


LD
269
164
L^LD
551
760
LD^D
LD^L^D
2
13
L
34
42
L^D
D
5

Expression Pattern	Inflammation	Cell death and survival	Stress	Hemostasis	Hypoxia inducible	Total
LD&L, LD>L and LD>D	15	8	6	2	4	104
LD only,LD>L and LD>D	10	5	3	3	1	111

Table 4.2 Functional Classification of Genes Expressed to Greater Degree after LPS/DCLF Treatment than after LPS or DCLF Given Alone. Genes with two expression patterns were identified from the sets of genes in the Venn Diagram, either in the LD only group and LD intersect L group (refer to Fig 4.). Those two groups comprised genes expressed to a greater degree after LPS/DCLF treatment than after treatment with either agent alone. Genes were classified based on the relationship of their gene products to one or more functions described in the Entrez Gene web site. A gene can be assigned to one or more than one function.

Table 4.3 Gene Ontology (GO) Biological Process Categories for Which Gene Expression Was Affected to a Greater Degree after LPS/DCLF Treatment than after Treatment with LPS or DCLF Alone Genes with the expression pattern [LD>L and LD>D] were identified as in Table 2. This gene list was imported into Genomatix Bibliosphere 5.13. Functional annotations were based on the Genomatix knowledge base. "Expected" refers to the numbers of gene expression changes expected by chance in the gene list. The "Z Score" ranks the deviation of the observed number of genes in one biological process category of the Genomatix database found in the imported list of genes from the numbers expected to occur by chance. It indicates the ranking of whether the genes in this biological process category are over-represented or under-represented in the imported gene list.

Term	GO ID	Observed	Expected	ZScore
neutrophil chemotaxis	GO:0030593	2	0	35.25
immune cell chemotaxis	GO:0030595	2	0	32.63
taxis	GO:0042330	3	0.01	28.94
chemotaxis	GO:0006935	3	0.01	28.94
immune cell migration	GO:0050900	2	0	28.76
inflammatory response	GO:0006954	3	0.02	19.25
innate immune response	GO:0045087	3	0.02	19.04
response to chemical substance	GO:0042221	3	0.03	17.72
response to wounding	GO:0009611	3	0.04	14.67
response to pest/pathogen/parasite	GO:0009613	3	0.04	14.13
response to abiotic stimulus	GO:0009628	3	0.05	13.79
cell migration	GO:0016477	2	0.02	13.4
immune response	GO:0006955	3	0.08	10.74
defense response	GO:0006952	3	0.08	10.23
cell motility	GO:0006928	2	0.04	10.05
response to biotic stimulus	GO:0009607	3	0.1	9.19
response to stress	GO:0006950	3	0.12	8.61
response to external stimulus	GO:0009605	3	0.17	7.03
response to stimulus	GO:0050896	3	0.2	6.43
organismal physiological process	GO:0050874	3	0.22	6.15
signal transduction	GO:0007165	2	0.33	3.08
cellular process	GO:0009987	3	0.92	2.61
cell communication	GO:0007154	2	0.45	2.5
cellular physiological process	GO:0050875	2	0.58	2.09
physiological process	GO:0007582	3	1.23	2.07
biological_process	GO:0008150	3	1.49	1.74

in the LPS/DCLF group but not in the LPS alone or the DCLF alone group. Accordingly, genes changed to a greater degree in the LPS/DCLF group than in the LPS/saline and saline/DCLF groups are associated with liver injury. These genes were classified based on the relationship of their gene products to one or more biological functions (Table 4.2). Many genes related to inflammation, cell death/survival, stress response, hemostasis or hypoxia were selectively upregulated by LPS/DCLF treatment.

The genes changed to a greater degree after LPS/DCLF treatment compared to LPS alone or DCLF alone were imported into Genomatix Bibliosphere 5.13. This yielded a list of gene ontology biological process terms ranked by their probability of over-representation or under-representation on the gene list (Table 4.3).

4.4.3 Neutrophil-related Gene Expression Changes after LPS/DCLF Treatment and Effects of Neutrophil Depletion on LPS/DCLF-induced Hepatotoxicity

Transcripts for the genes encoding the neutrophil chemokines such as MIP-2 and MIP-1α and the adhesion molecule ICAM-1 were changed to a greater degree in the LPS/DCLF cotreated group compared to LPS or DCLF given alone (Fig 4.5). Serum MIP-2 protein levels in both saline/saline and saline/DCLF groups were below the detection limit of the assay (Fig 4.6). LPS/saline treatment increased serum MIP-2 protein concentration, whereas LPS/DCLF treatment caused a much greater increase (Fig 4.6).

Both LPS/saline and LPS/DCLF treatment caused hepatic PMN accumulation (Table

Figure 4.5 Neutrophil-related gene expression changes after LPS/DCLF treatment. Gene array analysis was performed as described in Fig 3. The fold changes in MIP-2, MIP-1α and ICAM-1 transcripts were calculated with the saline/saline group as baseline. n=4-5; * significantly different from saline/DCLF group; # significantly different from LPS/saline group. $p<0.05$.

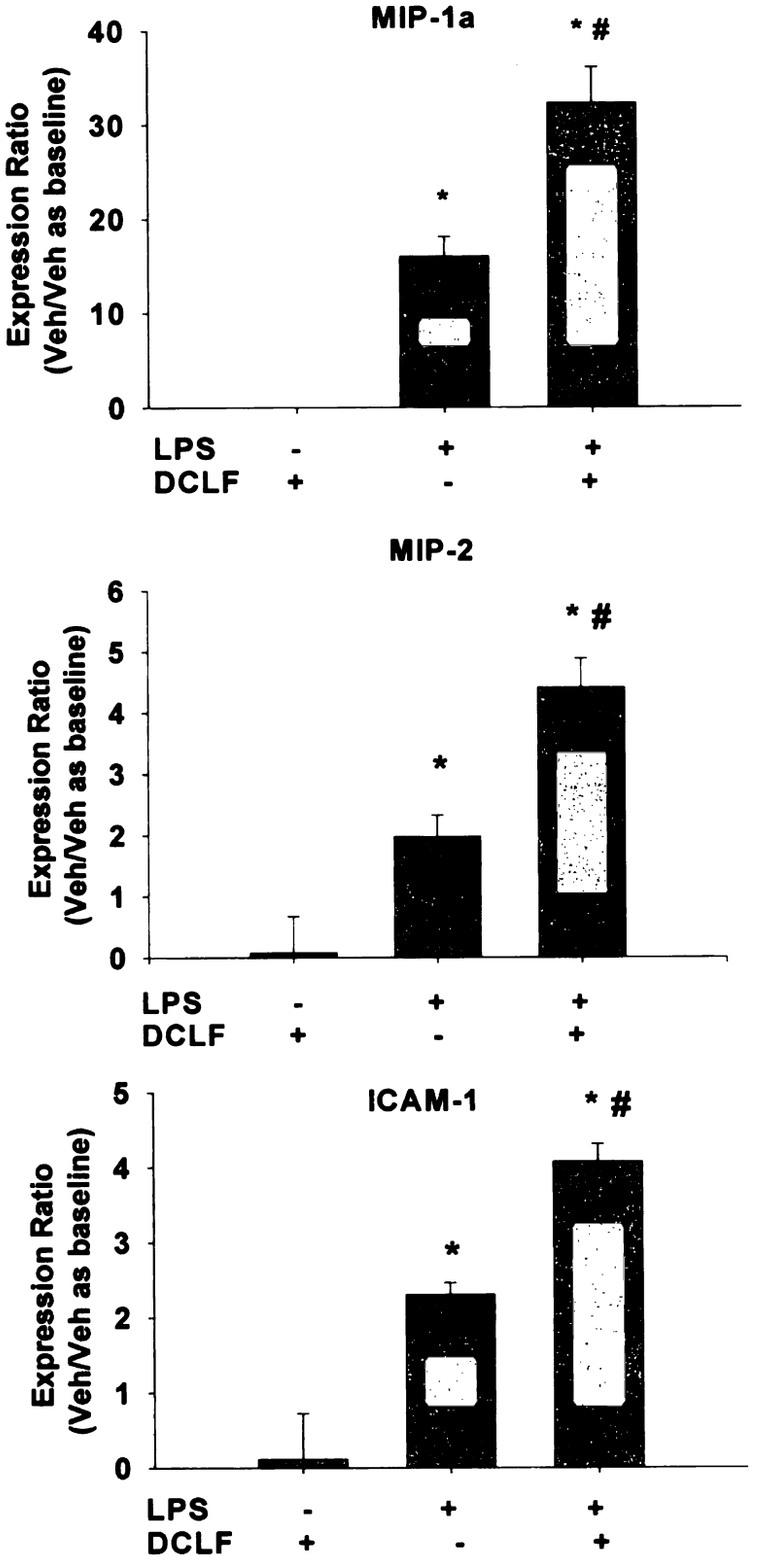

MIP-1a
* #
Expression Ratio
(Veh/Veh as baseline)
40
30
20
10
0
*
LPS - + +
DCLF + - +
MIP-2
* #
Expression Ratio
(Veh/Veh as baseline)
6
5
4
3
2
1
0
*
LPS - + +
DCLF + - +
ICAM-1
* #
Expression Ratio
(Veh/Veh as baseline)
5
4
3
2
1
0
*
LPS - + +
DCLF + - +

Figure 4.6 Serum MIP-2 protein concentration after LPS/DCLF treatment. LPS ($29X10^6$ EU/kg, i.v) or saline was administered to rats 2 hr before DCLF (20mg/kg, i.p) or saline. Serum MIP-2 was measured 6 hr after DCLF treatment. n= 4-5; * significantly different from group without LPS; # significantly different from group without DCLF. $p<0.05$.

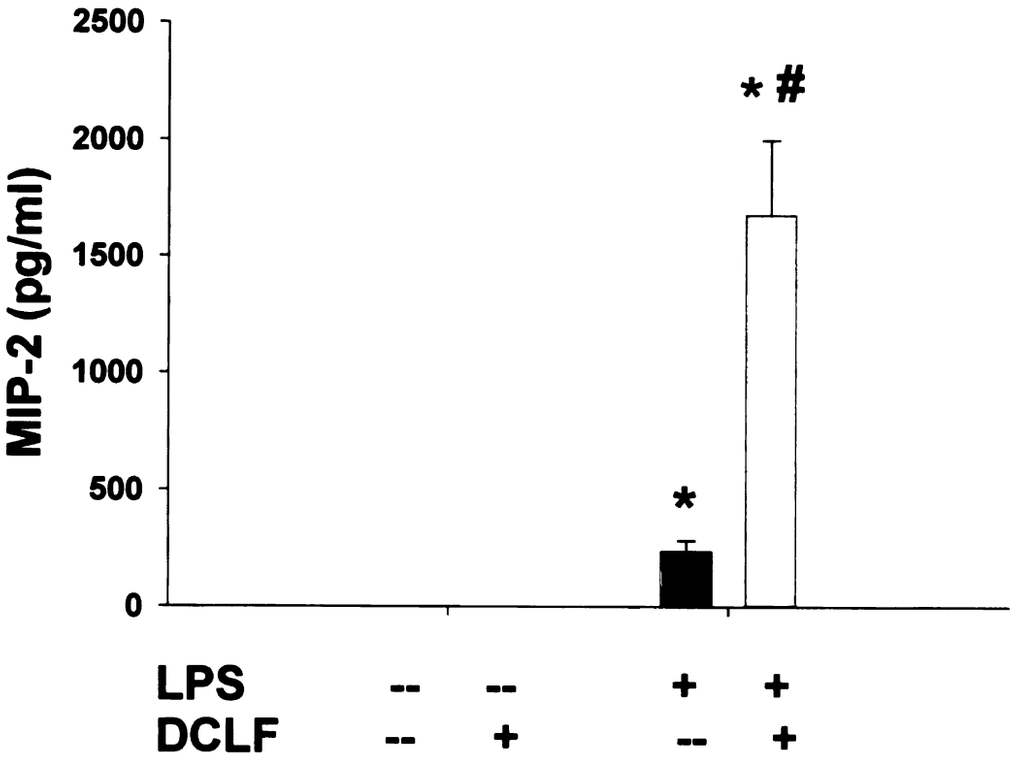
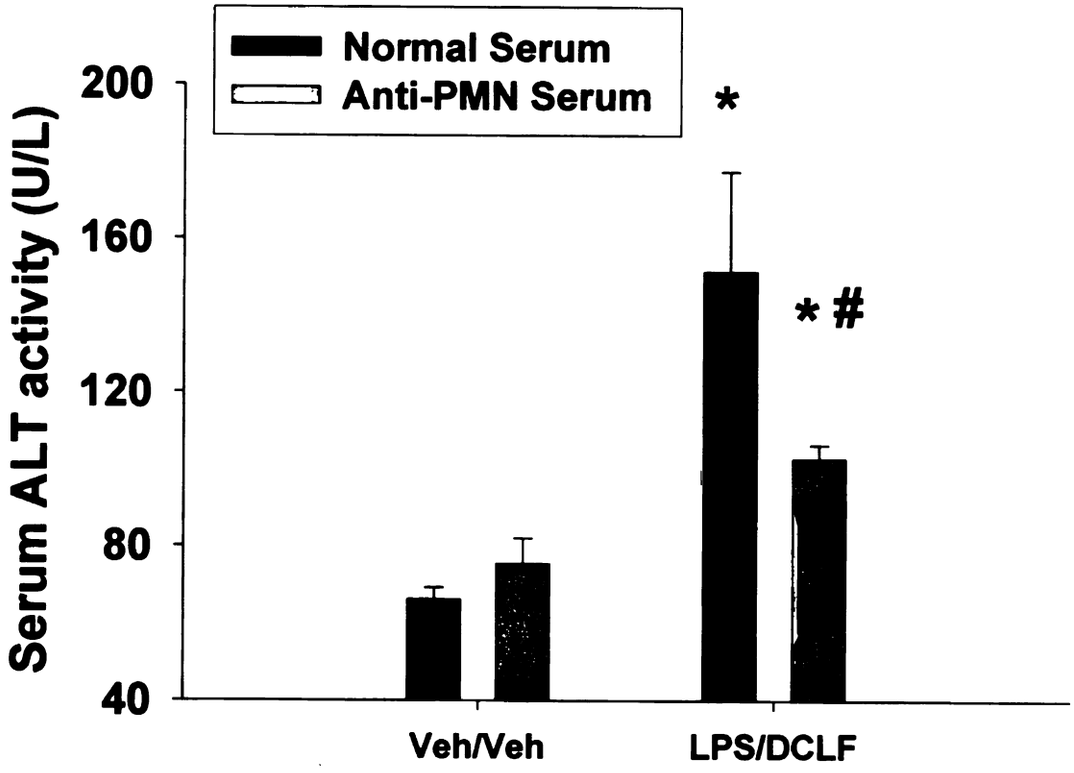

2500
2000
1500
1000
500
0
MIP-2 (pg/ml)
* #
*
LPS -- -- + +
DCLF -- + -- +

Figure 4.7 Effects of PMN depletion on LPS/DCLF-induced liver injury. Rats were pretreated with normal rabbit serum or rabbit anti-PMN serum 16 hrs before LPS ($29X10^6$ EU/kg, i.v) or its vehicle. DCLF (20mg/kg, i.p) or its vehicle was administered 2 hr after LPS. Serum ALT activity was evaluated 6hr after DCLF. n= 3-7; * significantly different from respective group without LPS/DCLF; # significantly different from respective group without antiserum. $p<0.05$.


Normal Serum
Anti-PMN Serum
Serum ALT activity (U/L)
200
160
120
80
40
*
* #
Veh/Veh
LPS/DCLF

4.1 and 4.4). To investigate the requirement for neutrophils in LPS/DCLF hepatotoxicity, neutrophil numbers in liver were reduced by treating rats with rabbit anti-rat PMN serum 16 hrs before LPS treatment. This treatment selectively reduced circulating and hepatic PMNs (Table 4.4). The anti-PMN serum alone did not alter ALT activity but did attenuate LPS/DCLF hepatotoxicity, as reflected by reduction in serum ALT activity (Fig 4.7). LPS/DCLF cotreatment reduced circulating lymphocytes (Table 4.4), similar to effects of LPS alone (data not shown).

4.4.4 Effects of Gut Sterilization on LPS/DCLF Hepatotoxicity

DCLF is known to cause gastrointestinal injury and bacterial translocation from intestine to liver (Kim et al., 2005c). The observation in the gene array study that the rat with the largest serum ALT activity after LPS/saline treatment clustered with the LPS/DCLF-treated rats suggested that the LPS/DCLF interaction might be due to bacteria/endotoxin exposure that resulted from a DCLF-induced intestinal permeability increase, which added to the effects of exogenous LPS given before DCLF exposure. To test this hypothesis, rats were treated with the nonabsorbable antibiotics, polymyxin B and neomycin, orally for 4 days before LPS/DCLF treatment. This antibiotic treatment completely abolished the colony growth from homogenized rat fecal samples on MacConkey agar plates, which are selective for gram-negative bacteria (data not shown).Gut sterilization did not significantly affect LPS/DCLF hepatotoxicity (Fig 4.8).

This result suggested that the LPS/DCLF interaction is not due to DCLF enhancing bacteria/endotoxin translocation from intestine to liver.

4.4.5 Effects of Gut Sterilization on Liver Injury in Rats Treated with a Hepatotoxic Dose of DCLF.

Large doses of DCLF have been shown to cause bacterial translocation from the intestine to liver (Kim et al., 2005b;Seitz and Boelsterli, 1998b). Consistent with this, DCLF (100mg/kg) did increase the level of gram-negative bacteria in the liver (Table 4.5). Although gut sterilization had no significant effect on LPS/DCLF hepatotoxicity, the possibility remained that LPS or bacterial translocation from intestine to liver plays an important role in the hepatotoxicity from a large, toxic dose of DCLF (100mg/kg). To test this hypothesis, rats were treated with nonabsorbable antibiotics as above before they were given a toxic dose of DCLF (100mg/kg). This antibiotic treatment attenuated the gram-negative bacterial colonies detected in liver (Table 4.5), and it reduced the serum ALT activity after administration of a hepatotoxic dose of DCLF, indicating attenuation of liver injury (Fig 4.9).

Treatment	Total Leukocytes (#/ul)	Blood PMNs (#/ul)	Blood Lymphocytes (#/ul)	Liver PMN (# per field)
NS/Veh/Veh	4350 ± 153	1105 ± 50	2684 ± 527	1.5 ± 0.3
AS/Veh/Veh	2817 ± 394 #	45 ± 23 #	2356 ± 503	1.7 ± 0.5
NS/LPS/DCLF	2479 ± 283 *	1228 ± 234	1063 ± 95 *	45.8 ± 6.0 *
AS/LPS/DCLF	1183 ± 223 #	122 ± 64 #	1001 ± 263 *	26.2 ± 1.8 *#

Table 4.4 Effects of Anti-PMN Serum on Circulating Leukocytes and Liver PMNs. Rats were pretreated with normal rabbit serum (NS) or rabbit anti-PMN serum (AS) 16 hrs before treatment with LPS (29X10^{6}EU/kg, i.v) or its vehicle. DCLF (20mg/kg, i.p) or its vehicle was administered 2 hr after LPS. Total leukocytes, PMNs and lymphocytes in blood and liver PMNs were counted 6hr after DCLF. n= 3-7; * significantly different from respective group without LPS/DCLF; # significantly different from respective group without antiserum. $p<0.05$.

Figure 4.8 Effects of gut sterilization on LPS/DCLF-induced liver injury. Rats were pretreated with polymyxin B (150mg/kg/day) and neomycin (450mg/kg/day) orally for 4 days before LPS ($29X10^6$ EU/kg) or its vehicle. DCLF (20mg/kg, i.p) or its vehicle was administered 2 hr after LPS or vehicle. Serum ALT activity was evaluated 6hr after DCLF or its vehicle. n=3-8; * significantly different from all other groups with the same antibiotic treatment. $p<0.05$.

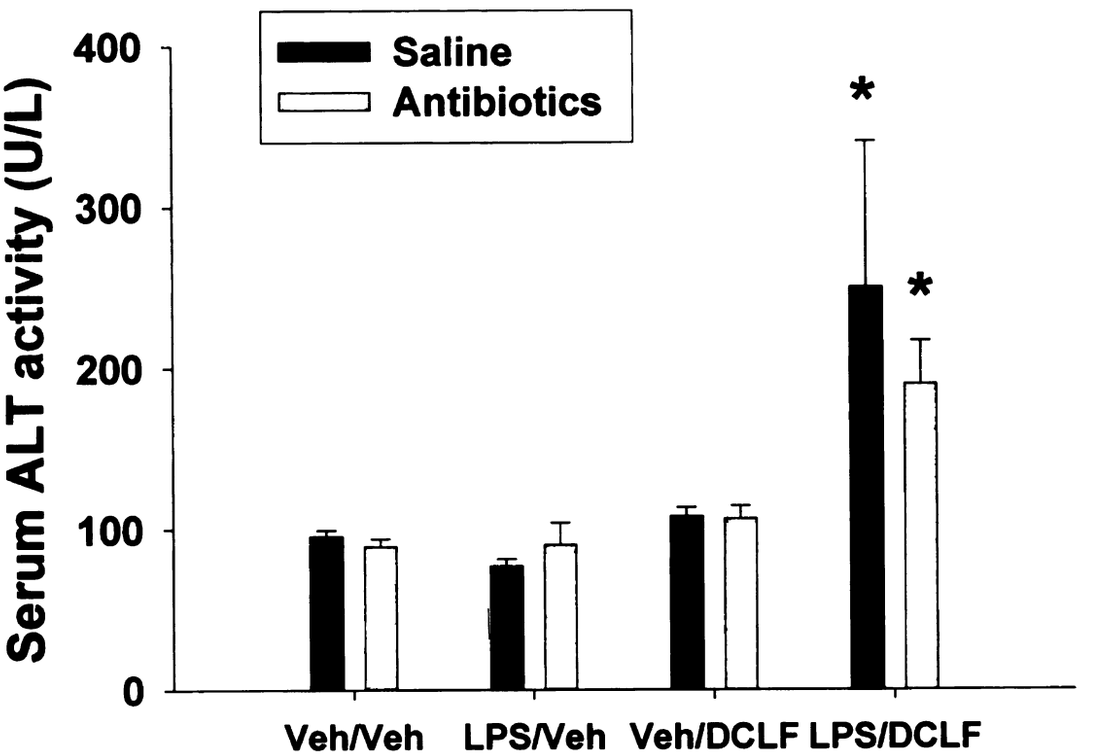
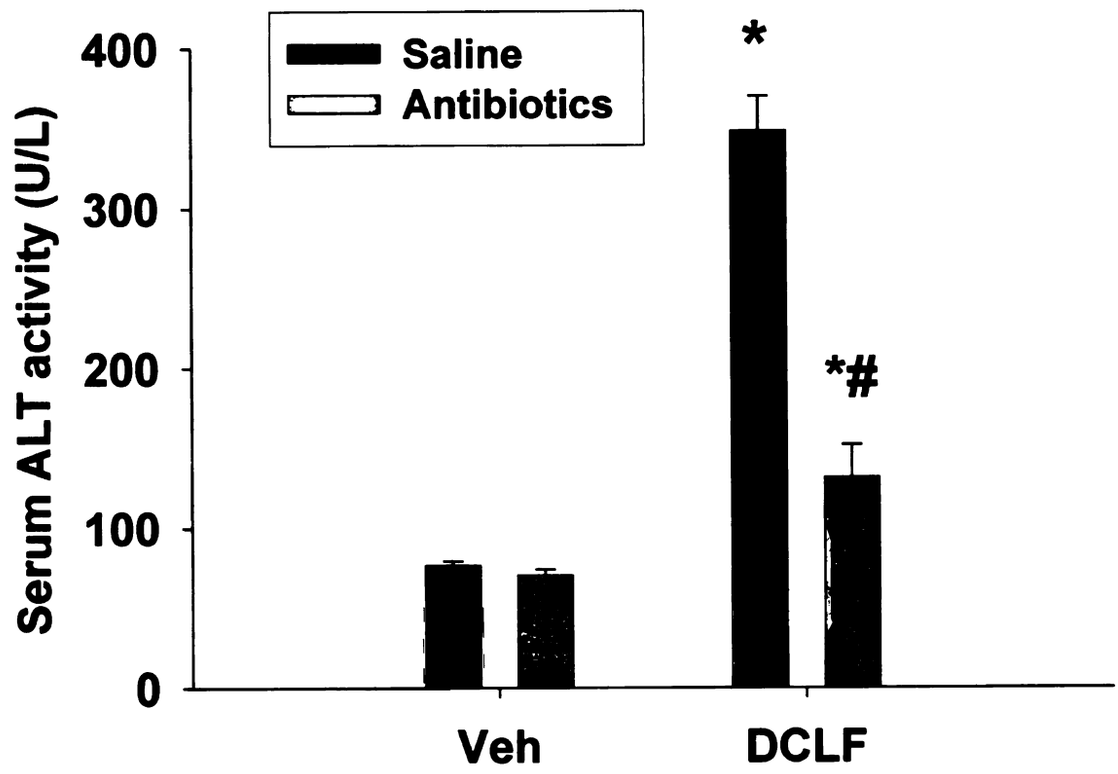

Saline
Antibiotics
Serum ALT activity (U/L)
400
300
200
100
0
*
*
Veh/Veh
LPS/Veh
Veh/DCLF
LPS/DCLF

Figure 4.9. Effects of gut sterilization on hepatotoxicity induced by a large dose of DCLF. Rats were pretreated with polymyxin B (150mg/kg/day) and neomycin (450mg/kg/day) orally for 4 days before administration of DCLF (100mg/kg, i.p) or saline. Serum ALT activity was evaluated 6hr after DCLF or vehicle. n=3-6; * significantly different from respective group without DCLF; # significantly different from respective group without antibiotics. $p<0.05$.


Saline
Antibiotics
Serum ALT activity (U/L)
400
300
200
100
0
*
*#
Veh
DCLF

Treatment	CFU/g (liver)
Saline/Vehicle	**209 ± 68**
Saline/DCLF	**5,845,596 ± 1,580,295 ***
Antibiotics/DCLF	**264,943 ± 167,660 * #**

Table 4.5. Effects of Gut Sterilization on liver Bacterial Colony Growth after High Dose DCLF Treatment. Rats were treated with polymyxin B and neomycin for 4 days before DCLF (100mg/kg) or vehicle treatment. Liver bacterial growth was quantified as described in Methods 6 hrs after DCLF or vehicle treatment. n= 6-8; * significantly different from respective group without DCLF; # significantly different from respective group without antibiotics. $p<0.05$.

4.5 Discussion

DCLF is well known to cause idiosyncratic hepatotoxicity (Boelsterli, 2003b). In this study, we showed that pretreating rats with a small dose of LPS converts a dose of DCLF that is normally noninjurious to one that is hepatotoxic. This result suggests the possibility that human patients might become susceptible to DCLF hepatotoxicity during a modest inflammatory episode. This appears even more intriguing, since most patients take DCLF for inflammation-related diseases such as rheumatoid arthritis or osteoarthritis. In fact, osteoarthritis has been associated with increased risk for DCLF-induced liver injury (Banks et al., 1995b).

Mechanisms of DCLF-induced liver injury remain incompletely understood. Mitochondrial injury leading to cell death has been proposed as a mode of hepatocellular injury, but by itself this does not explain the occurrence of injury only in a minority of patients (Masubuchi et al., 2002c;Gomez-Lechon et al., 2003). In addition, to our knowledge no direct evidence of mitochondrial injury *in vivo* has been reported. Given our results, it would be interesting to see DCLF induces mitochondrial injury *in vivo* or *in vitro* in the presence of inflammatory mediators.

Whether inflammation in human patients acts as a risk factor for DCLF-induced idiosyncratic liver injury requires additional study. Interestingly, polymorphisms in genes encoding cytokines IL-4 and IL-10 have been identified in patients developing DCLF-induced liver injury (Aithal et al., 2004c). These polymorphisms cause less IL-10 and more IL-4 production. Since IL-10 is an anti-inflammatory cytokine and IL-4

participates in B cell activation, these changes could enhance hepatotoxic interactions between DCLF and LPS or other inflammagens to which patients are exposed during drug therapy.

The potentiation of DCLF induced hepatotoxicity by LPS might appear paradoxical, since DCLF acts as a NSAID to dampen inflammatory responses. The primary mechanism of action of DCLF in this respect is inhibition of the enzyme cyclooxygenase (COX). COX catalyzes production of prostaglandins (PGs) and thromboxanes (Tx) from arachidonic acid. PGs and Tx have diverse biological actions, either proinflammatory or anti-inflammatory. However, as a nonselective COX inhibitor (i.e., inhibits both isoforms of the enzymes COX-1 and COX-2), it inhibits the synthesis of cytoprotective and anti-inflammatory PGs produced by constitutive COX activity as well as products of inducible COX-2, which are mostly proinflammatory. Moreover, the toxic interaction of DCLF with LPS might arise from inflammatory mediators (eg., cytokines) that are not products of the COX pathway and are not inhibited by NSAIDs.

Analysis of hepatic gene expression revealed a unique pattern of gene expression for animals treated with LPS/DCLF. The genes expressed to a greater degree in the LPS/DCLF group than in LPS/saline and saline/DCLF groups were selected for further functional analysis because liver injury followed this pattern. Interestingly, many genes involved in inflammation, cell death/survival, and response to stress were expressed to a greater degree in the LPS/DCLF group, consistent with the hypothesis that DCLF exaggerates the inflammatory stress caused by LPS.

Neutrophil/immune cell chemotaxis was at the top of the biological process rankings of the GO functional analysis for genes expressed to a greater degree in the LPS/DCLF group, and transcripts representing several neutrophil chemokines and adhesion molecules were upregulated to a great degree in this group, suggesting that PMNs might be involved in the pathogenesis. Serum MIP-2 concentration reflected the increase in the transcripts for this chemokine. The mechanism by which DCLF enhances MIP-2 protein expression remains unknown. There are reports that COX-2 inhibition enhances MCP-1/MIP-2 production in models of inflammation caused by radiation (Kyrkanides et al., 2002b). In addition, it has been proposed that in the face of inhibition of COX, arachidonic acid is metabolized more extensively by lipoxygenase. In this regard, it is interesting that the lipoxygenase products derived from arachidonic acid, such as 15-hydroxyperoxyeicosatetraenoic acid (15-HPETE), can increase adhesion molecules and transmigration of neutrophils across endothelial cell barriers (Sultana et al., 1996a). Thus, shifting of the generation of eicosanoids from the COX pathway to the lipoxygenase pathway by DCLF during inflammation (eg. LPS exposure) could contribute to enhancing the expression of MIP-2 and subsequent PMN activation. Depletion of PMNs in LPS/DCLF-treated rats attenuated liver injury, indicating the involvement of PMNs in the pathogenesis. PMNs cause cytotoxicity through the release of reactive oxygen species and other toxic factors. It is interesting in this regard that the addition of noncytotoxic concentrations of peroxidase/H_2O_2 to hepatocyte cultures markedly increased DCLF cytotoxicity (Tafazoli et al., 2005). In fact, several novel

reactive metabolites of DCLF formed by incubation of the drug with PMN-derived myeloperoxidase (Miyamoto et al., 1997c;Zuurbier et al., 1990a).

DCLF, as other NSAIDS, is known to cause gastrointestinal injury and bacteria translocation from the intestine to liver (Kim et al., 2005a;Banks et al., 1995a;Seitz and Boelsterli, 1998a). The resultant excessive bacteria/endotoxin exposure of liver could combine with the small dose of administered LPS to cause liver injury. This is not likely, because gut sterilization with nonabsorbable antibiotics did not dampen the hepatic damage caused by coadministration of LPS and DCLF. In contrast, gut sterilization did reduce liver injury caused by a larger, hepatotoxic dose of DCLF. It is possible that DCLF becomes hepatotoxic only in the presence of bacteria/endotoxin or other inflammagens and that the larger dose of DCLF is hepatotoxic because it causes translocation of LPS or bacteria from the intestine to liver.

LPS can increase intestinal permeability by inducing epithelial barrier dysfunction. This is reflected, for example, as an increase in dextran absorption (Moriez et al., 2005;Han et al., 2004). In addition, LPS administration to rats can change drug bioavailability through alterations in expression and activity of transporters and drug metabolizing enzymes in the intestinal epithelium (Roth et al., 2003b). Accordingly, it seems possible that LPS exposure could alter the toxicity of orally administered diclofenac by increasing its absorption from the gastrointestinal tract. This potential mode of action likely did not pertain to our results, since we administered the drug

intraperitoneally. However, in human patients who typically take diclofenac orally, this could provide an additional mode by which LPS exposure could influence drug toxicity.

Diclofenac causes IADRs in people in a time frame varying from a few weeks to a year after onset of therapy with the drug (Boelsterli, 2003). If drug-inflammation interaction underlies some human IADRs, then a reaction would be expected to occur only in patients with an inflammatory episode occurring during drug therapy and having sufficient magnitude to precipitate a hepatotoxic interaction with the drug. Modest inflammatory episodes likely vary in both timing of onset and magnitude among and within people (Roth et al., 2003c). Accordingly, this hypothesis of drug-inflammation interaction could explain the variable onset of diclofenac IADRs in human patients.

In summary, we established a potential model of DCLF-induced idiosyncratic liver injury by pretreating rats with a small dose of LPS before an otherwise nonhepatotoxic dose of DCLF. The molecular mechanisms of this inflammation-DCLF interaction remain unknown, but gene array analysis and PMN depletion studies indicated a role for PMNs. Gut sterilization of LPS/DCLF-treated rats failed to protect against liver injury, suggesting that this interaction is not due to excessive bacteria/endotoxin translocation from intestine to liver caused by DCLF. However, gut sterilization attenuated the liver injury caused by a large, toxic dose DCLF, suggesting that DCLF hepatotoxicity is partially due to bacteria/endotoxin translocation caused by injuring the intestinal barrier.

CHAPTER FIVE

Summary and Conclusions

5.1 Summary and Conclusions

The overall goal of the thesis project was to investigate how PMNs contribute to hepatocellular injury in LPS/RAN-cotreated rats and the mechanism by which PMNs become activated in the livers of LPS/RAN-cotreated rats. These studies will contribute to the understanding of mechanisms by which inflammation acts as a susceptibility factor for toxicity due to drugs and other xenobiotic agents. In addition, the completed study could lead to improved strategies for preventing or treating conditions involving PMN-dependent tissue injury.

In the thesis research conducted, a mechanism was proposed that PMNs contribute to liver injury by enhancing hemostasis. Both PMN antiserum and CD18 antiserum reduces the hepatic fibrin deposition at 6hrs after RAN treatment, confirming the role of PMNs in the hemostatic system. Previous in vitro studies showed that PMNs could cause thrombin activation and fibrin deposition by platelet-dependent and -independent mechanisms and PMNs even express functional tissue factor upon stimulation (MAUGERI et al., 2006;Goel and Diamond, 2003b;Goel and Diamond, 2003a); however, there have been no reports on the contribution of PMNs to fibrin formation in vivo to our knowledge. Data in this thesis showed that PMNs are involved in hepatic fibrin deposition in an animal model of liver injury in vivo. The PMN antiserum did not reduce fibrin deposition that occurs early after LPS/RAN treatment, suggesting that PMNs only contribute to the late phase of sinusoidal fibrin deposition. Furthermore, the PMN antiserum did not affect plasma TAT concentration, indicating this effect of PMNs on

fibrin formation is not due to thrombin activation. These findings contrast with the in vitro findings mentioned above. Furthermore, neutrophil proteases inhibitor (eglin C) studies showed that eglin C did reduced the fibrin deposition whereas had not effect on thrombin activation. Thus in the LPS/RAN model, PMNs contribute to sinusoidal fibrin deposition in a thrombin-independent manner.

An alternative mechanism by which PMNs could contribute to deposition of fibrin is through inhibiting fibrinolysis by increasing active PAI-1. PMN lysosomal proteases cathepsin B, D and G could increase PAI-1 activity in the medium of human umbilical vein endothelial cells by cleaving PAI-1 from extracellular matrix (Kimura and Yokoi-Hayashi, 1996d;Pintucci et al., 1993b;Pintucci et al., 1992d). Consistent with this, PMN depletion reduced active PAI-1 at 6hr after RAN (Fig. 2.5). A PMN proteases inhibitor also reduced active PAI-1 (Fig. 2.12). This suggests that PMNs contribute to fibrin deposition after 2hr by releasing proteases to activate PAI-1 and thereby inhibit fibrinolysis. The lack of earlier effects of PMNs on active PAI-1 (i.e., 2hr) might be due to PMNs are not being activated to release proteases during that time but begin to degranulate thereafter. In this regard, the shedding of PAI-1 from endothelial matrix by PMNs proteases might play a more dominate role at later times (i.e., 6hr). This is consistent with the results showing that formation of HOCl-modified epitopes/proteins, a marker of PMN degranulation/activation, did not increase until 3hr after LPS/RAN treatment.

In LPS/RAN-treated rats, PMN depletion reduced PIM staining at 2hr after LPS/RAN treatment, suggesting that PMNs contribute to early development of liver hypoxia. This early contribution of PMNs to hypoxia did not depend on sinusoidal fibrin deposition, since PMN deletion in LPS/RAN-treated rats did not affect liver fibrin at 2hr after RAN (Fig. 2.2). Furthermore, PMNs accumulated in livers were not activated at 2hr (Fig. 2.8), suggesting that enhancement of hypoxia by PMNs at this time does not require their activation and might be mediated directly by plugging sinusoids. LPS-RAN cotreatment caused a greater degree of tissue hypoxia than LPS given alone, although these two treatments had a similar effect on PMN accumulation (Luyendyk et al., 2004a;Luyendyk et al., 2005v). It is possible that RAN cotreatment causes PMNs to adhere more firmly to sinusoidal endothelium and/or to undergo a shape change that results in reduced sinusoidal blood flow and consequent hypoxia. In this regard, it would be interesting to examine the rolling and adhesion of PMNs on vessel walls in rats treated with LPS/RAN with intravascular microscopy. Also, flow cytometry could be applied to examine the ciruculating PMN size change after LPS/RAN treatment.

In the LPS/RAN model, PMN accumulation in liver was caused largely by LPS (Luyendyk et al., 2005w). However, LPS exposure alone did not lead to activation of the accumulated PMNs. RAN did cause activation of the PMNs that accumulated in liver after LPS treatment, as reflected by an increase in HOCl-protein adduct formation. RAN can reduce PMN activation in vitro (Okajima et al., 2000b;Okajima et al., 2002b); thus, the effects of RAN on hepatic PMN activation must be an indirect effect of RAN. This

might occur through MIP-2 or PAI-1, both of which are increased in LPS/RAN-treated rats and are known to enhance PMN activation. Anti-CD18 serum did not alter PMN accumulation but reduced PMN activation in liver (Fig. 2.9), suggesting that the early, LPS-induced sinusoidal accumulation of PMNs does not require CD18 whereas their activation is CD18-dependent. In addition, anti-CD18 serum markedly reduced hepatocellular injury (Fig. 2.9), suggesting that PMN transmigration/activation is required for LPS/RAN-induced liver injury.

There exists controversy regarding the mechanisms of activated PMN-induced cell injury in the liver. Reactive oxygen species and PMN protease are both proposed to be important for PMN-induced hepatocyte death (Jaeschke, 2006). PMN proteases can cause hepatocelluar death directly in vitro (Luyendyk et al., 2005x), but the time frame for the development of cell death is longer than that by which the liver injury develops in vivo after LPS/RAN treatment. This suggests that PMN proteases either did not participate in the hepatocyte killing in vivo or they need a second stress to manifest the process. The observation that PMN proteases inhibitors protected from LPS/RAN-induced liver injury ruled out the first possibility. Interestingly, hypoxia renders hepatocytes more sensitive to killing by PMN protease (i.e. elastases), and the time frame reflects the one by which liver injury develops in LPS/RAN-treated rats (Luyendyk et al., 2005y). Since hypoxia occurs early in livers of LPS/RAN treated rats (Luyendyk et al., 2005z;Deng et al., 2007a), these results suggest that PMNs contribute to the pathogenesis by releasing proteases that kill hepatocytes in an environment made hypoxic by fibrin deposition.

In the next section of the thesis, the role of RAN in TNFα production, PMN activation was explored. The results suggested that RAN augmented TNFα production after LPS treatment, and the prolongation of LPS-induced TNFα production by RAN appears to be crucial for the liver injury caused by LPS/RAN cotreatment. This was supported by the observation that a TACE inhibitor, given immediately before RAN, reduced both serum TNFα protein and the hepatocellular injury. The augmentation of TNF-α by RAN occured in a post-transcriptional manner through enhanced p38-dependent TACE activation. In addition, since RAN augmented hepatic p38 activation induced by LPS, p38 and downstream MK-2 may increase TNFα protein production by accelerating translation as reported by Neinigers and the colleagues (Neininger et al., 2002g).

The TACE inhibitor also reduced plasma active PAI-1 concentration after LPS/RAN treatment to almost the same level as after LPS treatment. Moreover, a PAI-1 inhibitor reduced the hepatocellular injury caused by LPS/RAN cotreatment. This suggested that enhanced PAI-1 production was a downstream effect of RAN-augmented TNF-α production. Further studies showed that PAI-1 was important for both hepatic fibrin deposition and PMN activation. As mentioned above, hypoxia resulting from fibrin deposition could potentiate the killing of hepatocytes by proteases (e.g. elastase) released from PMNs after their activation (Luyendyk et al., 2005).

Although the PAI-1 inhibitor decreased PMN activation, it did not affect hepatic PMN accumulation or serum PMN chemokine concentration, suggesting a direct effect of

PAI-1 on PMN activation. A recent study showed that PAI-1 directly potentiates LPS-induced PMN activation through a Jun-N-terminal kinase (JNK)-dependent pathway (Kwak et al., 2006a). As mentioned above, RAN by itself did not enhance PMN activation. In fact, it has been shown to reduce PMN activation in vitro (Okajima et al., 2002a;Okajima et al., 2000a). All of these results suggest that RAN might induce activation of PMNs indirectly by augmenting PAI-1 production. It would be interesting to see whether incubating primary rat PMNs with PAI-1 can increase fMLP- or PMA-induced degranulation of PMNs.

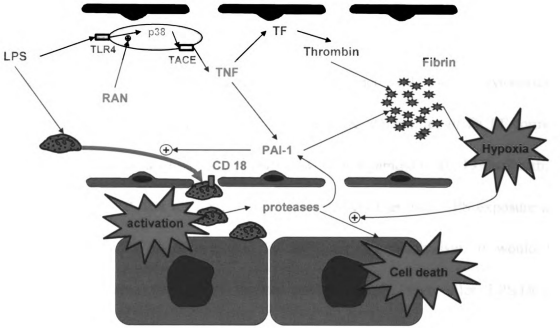
Although RAN augmented TNFα production and downstream cascades though activating TACE, the mechanism behind the TACE activation by RAN is largely unknown. The enhanced TACE activation after RAN treatment was p38 dependent; this was consistent with the finding that p38 is essential for ectodomain shedding of TNFα in CHO cells (Fan and Derynck, 1999a). Although ERK, another MAPK kinase, is able to phosphorylate TACE (az-Rodriguez et al., 2002), there are no reports to our knowledge that p38 directly phosphorylates and activates TACE. Thus, it is likely that p38 downstream kinases such as MK-2 participate in the activation of TACE. Further studies need to be conducted to pursue this possibility.

In the LPS/RAN model, it seems plausible that RAN augmented the LPS-induced inflammatory response to precipitate the hepatocellular injury. However, the initial molecular target of RAN remains largely unknown in this model. The lack of liver injury after cotreatment of LPS with FAM at a dose pharmacologically comparable to RAN

rules out the involvement of H2 receptor antagonism. Since RAN, not FAM, augmented hepatic p38 activation induced by LPS and since p38 is crucial for the downstream cascade of TNFα production, coagulation activation and PMN activation, it is possible that the p38 activation pathway might be where RAN exerts its initial effect. RAN itself did not activate p38, whereas it enhanced the p38 activation after LPS exposure. Thus, RAN might perturb one or more of the signaling molecules in the pathway of LPS-induced p38 activation. This pathway is well studied (Guha and Mackman, 2001). Toll-like receptor 4 (TLR4) on macrophages serves as a pattern recognition receptor for LPS. After recognizing LPS, TLR4 causes the recruitment of a set of intracellular Toll/IL-1 receptor (TIR) domain-containing adaptors, including myeloid differentiation factor 88 (MyD88). The association of TLR4 and MyD88 activates p38 through a signaling cascade involving IRAK4, TRAF6 and TAK1. It would be interesting to examine the phosphorylation states of these signaling molecules after LPS/RAN cotreatment to identify the possible molecular target of RAN.

The proposed pathogenic mechanism of LPS/RAN-induced liver injury is summarized in Figure 5.1. RAN augments TNF-α production after LPS treatment in a post-transcriptional manner by enhancing p38 activation. The increase in TNF-α protein appears to occur through the p38-dependent activation of TACE. The prolongation of LPS-induced TNF-α production by RAN appears to be crucial for the liver injury caused by LPS/RAN cotreatment. TNF-α leads to coagulation system activation and PAI-1 production, both of which increase hepatic fibrin deposition. PAI-1 might also be directly

Figure 5.1 Hypothetical pathogenesis of LPS/RAN-induced liver injury. RAN augments TNF-α production after LPS treatment in a post-transcriptional manner by enhancing p38 activation. The increase in TNF-α protein appears to occur through the p38-dependent activation of TACE, and the resultant prolongation of LPS-induced TNF-α production by RAN appears to be crucial for the liver injury caused by LPS/RAN cotreatment. TNF-α leads to coagulation system activation and PAI-1 production, both of which cause hepatic fibrin deposition. PAI-1 might also be directly responsible for the activation of hepatic PMNs accumulated after LPS exposure. The hypoxia resulting from hepatic fibrin deposition could act synergistically with toxic proteases released from activated PMNs to kill hepatocytes. PMN proteases are also involved in enhancing PAI-1 production and fibrin deposition.


LPS
TLR4
p38
TACE
TF
Thrombin
Fibrin
TNF
RAN
PAI-1
Hypoxia
CD 18
proteases
activation
Cell death

responsible for the activation of hepatic PMNs accumulated after LPS exposure. The hypoxia resulting from hepatic fibrin deposition could act synergistically with toxic proteases released from activated PMNs to kill hepatocytes. PMN proteases are also involved in enhancing PAI-1 production and fibrin deposition.

In addition to the LPS/RAN model, PMNs are involved in the liver injury caused by LPS/DCLF cotreatment. This raises the possibility that PMNs are one of the universal participants in liver injury in LPS-drug interaction models. Since PMNs cause cytotoxicity through the release of reactive oxygen species and other toxic factors, it is interesting in this regard that the addition of noncytotoxic concentrations of peroxidase/H_2O_2 to hepatocyte cultures markedly increased DCLF cytotoxicity (Miyamoto et al., 1997b). In fact, several novel reactive metabolites of DCLF formed when the drug was incubated with PMN-derived MPO (Miyamoto et al., 1997a;Zuurbier et al., 1990b). Thus, PMNs accumulated and activated in liver after LPS exposure are likely to render DCLF more cytotoxic by MPO-derived metabolism. It would be interesting to examine these MPO-derived metabolites in vivo after LPS/DCLF cotreatment.

The mechanism of PMN activation in the LPS/DCLF model is largely known. In a gene array study, transcripts representing several neutrophil chemokines (MIP-2 and MIP-1α) and adhesion molecules (ICAM-1) were upregulated to a greater degree in livers of LPS/DCLF-treated rats than in rats treated with LPS alone. Serum MIP-2 concentration reflected the increase in the transcripts for this chemokine. There are

reports that COX-2 inhibition enhances MCP-1/MIP-2 production in models of inflammation caused by radiation (Kyrkanides et al., 2002a). In addition, it has been proposed that in the face of inhibition of COX, arachidonic acid is metabolized more extensively by lipoxygenase. In this regard, it is interesting that lipoxygenase products derived from arachidonic acid, such as 15- hydroxyperoxyeicosatetraenoic acid (15-HPETE), can increase adhesion molecules and transmigration of neutrophils across endothelial cell barriers (Sultana et al., 1996b). Thus, shifting of the generation of eicosanoids from the COX pathway to the lipoxygenase pathway by DCLF during inflammation (eg. LPS exposure) might contribute to enhancing the expression of MIP-2 and subsequent PMN activation.

In summary, the research conducted in this thesis revealed the crucial roles of PMNs in models of idiosyncratic hepatotoxicity resulting from LPS-drug interactions. In the LPS/RAN model, LPS induced the accumulation of PMNs in the liver, and RAN caused the activation of these PMNs. This might occur through TNFα-dependent production of PAI-1. Activated PMNs can release toxic proteases to kill hepatocytes or to enhance fibrin deposition, both of which could lead to hepatocellular injury. In addition, PMNs are also important in the liver injury caused by LPS/DCLF cotreatment. These results contribute to the understanding of mechanisms by which inflammation could act as a susceptibility factor for IADRs and to the possible identification of biomarkers for IADRs resulting from inflammation-drug interaction. In addition, the conducted study

could lead to improved strategies (i.e., proteases inhibitors) for preventing or treating conditions involving PMN-dependent tissue injury.

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