This is to certify that the
dissertation entitled

Inflammation and idiosyncratic reactions: Ranitidine as a model

presented by

James Parker Luyendyk

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Pharmacology and Toxicology

Major Professor's Signature

12-9-04

Date

INFLAMMATION AND IDIOSYNCRATIC DRUG REACTIONS: RANITIDINE AS A MODEL

By

James Parker Luyendyk

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pharmacology and Toxicology

2004

ABSTRACT

INFLAMMATION AND IDIOSYNCRATIC DRUG REACTIONS:

RANITIDINE AS A MODEL

By

James Parker Luyendyk

Idiosyncratic reactions 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, the mechanism of toxicity is unknown. Inasmuch as a modest inflammatory response can render rats susceptible to hepatotoxic effects of several xenobiotics, inflammation might also be a determinant of sensitivity to drug toxicity. This dissertation tested the hypothesis that an inflammatory response caused by a nonhepatotoxic dose of bacterial lipopolysaccharide (LPS) could precipitate idiosyncrasy-like RAN hepatotoxicity in rats. Administration of a small dose of LPS (44 X 10^6 EU/kg, iv) two hours before a nonhepatotoxic dose of RAN (30 mg/kg, iv) caused significant hepatotoxicity characterized by midzonal hepatocellular oncotic necrosis, the onset of which occurred between 2-3 h after drug treatment. These histopathologic findings and changes in clinical chemistry resembled idiosyncratic RAN hepatotoxicity in people. The H2-antagonist famotidine (FAM), which is not associated with idiosyncratic hepatotoxicity, was not rendered toxic by LPS cotreatment. Hepatic gene expression was evaluated at a time before injury in rats treated with LPS and/or RAN, and hierarchical clustering of active genes segregated rats to their respective treatment groups. Several genes related to hypoxia, inflammation and cell death were expressed to a greater degree in LPS/RAN-treated rats compared to either agent given

alone. This pattern of expression for plasminogen activator inhibitor-1 (PAI-1) was confirmed by real-time PCR and was mirrored by the PAI-1 concentration in the plasma of LPS/RAN-treated rats. Consistent with the antifibrinolytic function of PAI-1, hepatic fibrin deposition was observed only in livers of LPS/RAN-treated rats. To determine if hepatic fibrin deposition was a consequence of impaired fibrinolysis, coagulation system activation, or both, several biomarkers of coagulation were evaluated. Changes in serum hyaluronic acid, an indicator of altered sinusoidal endothelial cell (SEC) homeostasis, thrombin-antithrombin dimers, and fibrinogen suggested coagulation system activation in LPS/RAN-treated rats. Liver injury and hepatic fibrin deposition were attenuated by both the fibrinolytic agent streptokinase and the anticoagulant heparin, indicating that the hemostatic system is involved in LPS/RAN-induced liver injury. Liver hypoxia, one consequence of fibrin deposition, was observed only in livers of LPS/RAN-treated rats and was reduced by heparin coadministration. Neutrophils (PMNs) accumulated in livers of LPS/RAN-treated rats, and killing of primary rat hepatocytes by PMN elastase *in vitro* was augmented under hypoxic conditions. The results suggest that the hemostatic system is important for liver injury in LPS/RAN-treated rats and are consistent with resultant hypoxia interacting with inflammatory mediators to cause hepatocellular injury. Furthermore, these studies support the possibility of predicting and understanding the mechanisms of some idiosyncratic drug reactions by utilization of a drug/LPS-cotreatment model.

ACKNOWLEDGMENTS

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 Pharmacology and Toxicology, Department of Biochemistry and Molecular Biology, and the Center for Integrative Toxicology deserve tremendous thanks. In addition, my interactions with other collaborators and colleagues from Pharmacia Corporation, Bristol-Myers Squibb, University of Louisville, the Society of Toxicology, and the Michigan Regional Chapter of the Society of Toxicology have dramatically increased my excitement for Toxicology. In addition to laboratory funding I would like to thank the organizations which have supported my research through awards and fellowships. To paraphrase these thanks would be insufficient. Accordingly, grab your 64 oz. soda and get ready to read.

Let's start with the MSU folks, shall we. First and foremost I would like to thank my mentor and friend, 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. The advice Bob has given me will not be forgotten. Dr. Patricia Ganey also deserves my thanks. Patti played a very significant role in my graduate career by serving as a member of my graduate committee and as another mentor in the lab, and I am proud to have coauthored several manuscripts with her. I am perhaps more of a pest at times to Dr. Jane Maddox. However, this speaks strongly to Jane's involvement in the lab's activities. Jane's door is always open and she seems to know the answers to all my questions. I would like to

thank Jane for the significant amount of time she put in to training me in the lab as well as the teamwork on this dissertation work. I have enjoyed working with Jane and will miss greatly my daily interactions with her. I would also like to thank Dr. James Pestka for serving on my graduate committee and for his continued interest in my research and career goals.

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. Umesh Hanumegowda, Dr. Bryan Copple, and Dr. Francis Tukov. 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, Cathy Rondelli, John Phipps, John Buchweitz, Mary Kinser, Steve Yee, Xiaomin Deng, Chris Green, Steve Bezdecny, Shawn Kinser, Pat Shaw, Colin North, Kate Shores, Rohan Pradhan, Theresa Eagle, Peer Karmaus, Natasha Snyder, Brook Wooley, and Danielle Feldpausch. In addition to folks in the lab, I would like to thank Dr. Tim Zacharewski for his assistance with our microarray work. Along this line, I need to thank Lyle Burgoon for his patience and significant help with microarray analysis. I would also like to thank Dr. Larry Fischer and the administrative staff of the Center for Integrative Toxicology (Institute for Environmental Toxicology). I also thank Dr. Stephanie Watts for her continued leadership of the Graduate Program in Pharmacology and Toxicology and for her much needed advice on several occasions.

In addition to colleagues on campus I have offered the unique opportunity to collaborate with others in industry and academia on the work presented herein as well as other projects. Dr. Greg Cosma has served as another mentor to me and as a member of

my committee. Greg has been helpful in all aspects of my career from experimental design to encouragement. I met Greg through our collaboration with Pharmacia where I also had the chance to work with several other awesome folks including Dr. Gary Cockerell, Dave Blakeman, John Wijsman, and Dr. Glenn Cantor. Through the assistance of Glenn and Greg we were able to forge an incredible collaboration with Lois Lehman-McKeeman at Bristol-Myers Squibb. I would like to thank Lois, the members of her group including David Nelson, Vasanthi Bhaskaran, as well as Dr. Bruce Car, Dr. Glenn Cantor, and Dr. Bill Foster for the opportunity to visit BMS and analyze gene array data in the real world. I would also like to thank Glenn and Inge Cantor for a fantastic 3-day stay at the Cantor homestead in Princeton. In addition to industrial collaborators, I would like to thank Dr. Gavin Arteel for opening up both his lab and home to me for a fun 4-day visit to Louisville. Dr. Arteel's encouragement is also greatly appreciated.

I owe a big thank you to the Society of Toxicology for all that it has offered me during the past 4 years. The list of folks is too big, but I'll just say thanks. SOT has been, and will be a big part of my career. One person who deserves a big thank you is Betty Eidemiller. More or less, I should just thank Betty for everything. I would also like to thank the Committee and organizers of the Michigan Regional Chapter of the Society of Toxicology. Interactions at the Regional Chapter level have had an enormous impact on me personally and professionally.

I should take a moment to thank those who made my research possible. First off, Bob kept my studies going by seeking funding from multiple sources and deserves all the credit. In addition, I would like to thank the Center for Integrative Toxicology (NIEHS training grant), Michigan State University, the Society of Toxicology and Novartis

Corporation for their assistance with paying my stipend. Also, I would like to thank the MSU College of Veterinary Medicine, the Midwest Regional Chapter of the SOT, the Michigan Regional Chapter of the SOT, and the ASPET Toxicology Division for sponsoring awards enabling me to travel to several professional meetings.

I would also like to thank 7-11 for keeping the soda fountain running and Bryan and Eric at University Foreign Car for keeping the Volvo running. Finally, I would like to thank my wife Elizabeth for her unconditional support and love despite how crazy I get. Also, Liz deserves all the credit for my new and improved fashion sense. Finally, without my parents I would have missed out on the opportunities I have had and would not be looking forward to those in the future. Nor would I have such a cool car. So thanks for everything Mom and Dad...and yes, I checked the oil.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Images in this dissertation are presented in color

LIST OF ABBREVIATIONS

APAP	acetaminophen
ADR	adverse drug reaction
AFB_1	aflatoxin B_1
AA	allyl alcohol
ANOVA	analysis of variance
APC	antigen presenting cell
ALT	alanine aminotransferase
ALP	alkaline phosphatase
AST	aspartate aminotransferase
Btg-2	b-cell translocation gene 2
CL	centrilobular
CIM	cimetidine
Cxcl10	CXC chemokine ligand 10
COX-2	cyclooxygenase-2
CYP	cytochrome P450
CINC-1	cytokine-induced chemoattractant-1
DCF	diclofenac
Egr-1	early growth response-1
EU	endotoxin unit
ELISA	enzyme-linked immunosorbent assay
FDR	false discovery rate
FAM	famotidine
fMLP	f-Met-Leu-Phe
GC	gadolinium chloride
Gal	galactosamine
GGT	gamma-glutamyl transferase
GI	gastrointestinal
GLUT1	glucose transporter 1
GSH	glutathione
H2	histamine2-
HAL	halothane
HA	hyaluronic acid
HIF-1α	hypoxia inducible factor-1α
HPC	hepatic parenchymal cell
HPF	high power field
Igfbp-1	insulin-like growth factor binding protein-1
IL-1	interleukin 1
Iv	intravenous
Ip	intraperitoneal
JNK	c-Jun NH(2)-terminal kinase
KC	Kupffer cell
LAL	Limulus amoebocyte lysate
LP	lipid peroxidation

LPS	lipopolysaccharide
L	lipopolysaccharide/vehicle
R	vehicle/ranitidine
LR	lipopolysaccharide/ranitidine
MCT	monocrotaline
MPT	mitochondrial permeability transition
NF-κB	nuclear factor kappaB
NGFI-B	nerve growth factor-induced B
NSA	neoantigen-specific antibodies
LOX-1	lectinlike oxidized low-density lipoprotein receptor-1
PBS	phosphate-buffered saline
PDE4B	phosphodiesterase 4B
PIM	pimonidazole
PAI-1	plasminogen activator inhibitor-1
PAR	protease activated receptor
PMN	neutrophil
PMN-CM	neutrophil-conditioned medium
PMN-E	neutrophil elastase
PP	periportal
RAN	ranitidine
RECA-1	rat endothelial cell antigen-1
SEC	sinusoidal endothelial cell
SK	streptokinase
TAT	thrombin-antithrombin dimmer
TF	tissue factor
TFA	trifluoroacetyl
TGZ	troglitazone
TLR4	toll-like receptor 4
TNF	tumor necrosis factor-alpha

Chapter 1

General Introduction

1.1 Idiosyncratic hepatotoxicity

1.1.1 Background and proposed mechanisms

Despite continued advances in predicting and understanding adverse drug reactions (ADRs), they remain a large problem for patients and the pharmaceutical industry alike. Untoward drug reactions contribute significantly to hospitalization and mortality in the United States (Lazarou et al., 1998). In addition, the incidence of ADRs is likely under-reported, with one study suggesting an incidence about 16-fold higher than suggested by current reporting methods (Bagheri et al., 2000; Sgro et al., 2002). The impact of ADRs goes beyond the obvious relationship to human health. Removal of a drug from the market can cause a significant deficit in successful treatment of a disease or condition. One example of this situation is the anti-epileptic felbamate (Pellock, 1999). Felbamate is effective in treating severe cases of epilepsy, but its utilization in treatment of epilepsy was reduced as a consequence of rare hepatotoxicity, and it is left now only for those patients for which the therapeutic benefit outweighs the risk of toxicity (for review, see Dieckhaus et al., 2002). Furthermore, ADRs affect the pharmaceutical industry financially through loss of investment capital, future profits, and potential financial loss from lawsuits. Overall, the consequences of ADRs only emphasize the importance of studies aimed at understanding mechanisms and facilitating prediction.

ADRs affect numerous organ systems as well as circulating cells, with a frequent target organ being the liver. In fact, one report estimated more than half of acute liver failure cases occur as a result of ADRs (Gill and Sterling, 2001). Acute, drug-induced liver injury is associated with several therapeutic/pharmacologic classes of drugs and

may manifest as an array of histopathologic and clinical features including acute hepatocellular necrosis, biliary injury, or a combination of the two (Zimmerman, 1978). More than 25 years ago, Zimmerman described agents that caused liver toxicity as either "intrinsic hepatotoxins" or as "nonpredictable" hepatotoxins (Zimmerman, 1978), alternatively defined as type A or type B ADRs, respectively (Pirmohamed et al., 1998). Type B reactions are unpredicted reactions occurring in a small percentage of people taking a drug (typically < 5%) and are therefore frequently termed "idiosyncratic." Contrasting intrinsically hepatotoxic drugs (type A ADR), which cause dose-dependent hepatotoxicity over a predictable timecourse, the dose-response relationship for drugs that cause idiosyncratic hepatotoxicity is obscured or absent, and reactions occur at various times after the start of drug therapy (Zimmerman, 1993). Furthermore, unlike type A reactions, for which hepatotoxicity might be due to an exaggerated pharmacologic effect of the drug, type B reactions are seemingly unrelated to the drug's pharmacology. Overall, the features of idiosyncratic reactions make these reactions more dangerous, more difficult to understand, and unfortunately, more difficult to predict.

A recent example of a drug associated with type B drug-induced liver toxicity is that of troglitazone (Rezulin®), one of many thiazolidinediones marketed for treatment of type 2 diabetes. The fraction of patients that developed hepatotoxicity from troglitazone treatment was relatively small (about 1:20,000) (Faich and Moseley, 2001). Nevertheless, several cases of hepatotoxicity were documented in which the injury was severe enough to necessitate liver transplant or cause death of the patient (Kohlroser et al., 2000; Murphy et al., 2000). Interestingly, the mechanism of liver injury appears to be independent of the drug's pharmacologic action, as other PPAR-gamma agonists used to

date lack the same propensity to cause idiosyncratic hepatotoxicity (Tolman and Chandramouli, 2003). Another well-studied drug is acetaminophen (APAP), a component of numerous over-the-counter medications and a leading cause of drug-induced liver failure. APAP typically produces dose-dependent hepatotoxicity, and several mechanisms for the toxicity have been proposed (Kaplowitz, 2004b). However, the exact mechanism of APAP-induced liver injury is still incompletely understood, and in some cases even patients taking large doses of APAP do not develop liver injury (Tredger et al., 1995; Shayiq et al., 1999). This has led to the proposal that even APAP hepatotoxicity has characteristics of idiosyncratic hepatotoxicity (Kaplowitz, 2004b). Thus, it appears that idiosyncratic reactions still occur even for drugs for which the mechanisms of hepatotoxicity are relatively well understood.

Currently, there is no model for the accurate preclinical prediction of idiosyncratic drug reactions. Drugs continue to carry "black box" warnings or suffer curtailed use after being approved for market as a result of unpredicted human toxicity (Lasser et al., 2002). Inasmuch as these responses are not typically observed until a large population of patients has taken a drug, it is not surprising that they are not observed in small groups of animals in laboratory experiments. Lack of statistical power and/or lack of necessary susceptibility factors might both contribute to the inability to detect the potential for toxicity. Interestingly, a recent paper by Alden *et al* examined the preclinical toxicology studies of three drugs associated with idiosyncratic hepatotoxicity: bromfenac, zileuton, and troglitazone (Alden, 2003). The authors questioned the rationale for the assumption that idiosyncratic reactions cannot be predicted preclinically and offered evidence in the case of each drug that subtle, but important changes in liver biomarkers might have

predicted the toxicity. Ultimately, the ability to predict these reactions during preclinical development would allow the pharmaceutical industry to bring their safest candidates to market.

Better prediction of idiosyncratic liver injury will require a better understanding of the mechanisms underlying toxicity. Uetrecht and colleagues appropriately note that "No one model fits the characteristics of all idiosyncratic drug reactions" (Seguin and Uetrecht, 2003). Properties of the drug, the genetic background of the patient, and other factors including disease and cell stress probably influence the incidence of idiosyncratic reactions (Boelsterli, 2003a; Kaplowitz, 2001). Several hypotheses have been proposed to explain the underlying basis of idiosyncratic reactions. For example, genetic polymorphisms, resulting in altered expression of drug metabolizing enzymes might be important for causing drug idiosyncrasy (Pirmohamed et al., 1996). Ultimately, the "reactive metabolite hypothesis" suggests that changes in drug metabolism could result in formation of a reactive metabolite or reduced detoxification of reactive intermediates. An extension of the reactive metabolite hypothesis is the hapten hypothesis, in which a drug or reactive drug metabolite covalently binds to proteins such that an antibody-driven response is directed against "self" by the immune system, resulting in liver toxicity (Ju and Uetrecht, 2002). Furthermore, Uetrecht and others have applied Matzinger's Danger Hypothesis to idiosyncratic drug reactions (Pirmohamed et al., 2002; Seguin and Uetrecht, 2003). This hypothesis suggests that a second "danger signal," in addition to the presence of autoantibodies, is necessary to mount a specific immune 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. Although for

some drugs experimental evidence supporting these mechanisms is available, in some cases the exact etiology remains controversial. Furthermore, the basis of idiosyncratic reactions for the vast majority of drugs remains unclear. The following section will critically review the supporting evidence, shortcomings, and some alternative thinking surrounding underyling mechanisms of idiosyncratic hepatotoxicity for two different drugs, halothane (HAL) and diclofenac (DCF).

1.1.2 Examples: HAL and DCF

HAL. Hepatotoxicity of the inhalation anesthetic HAL was identified more than 40 years ago as two different manifestations of hepatotoxicity. A mild elevation in serum enzymes occurs in about 20% of exposed patients. However, a much more severe "HAL hepatitis" occurs in a much smaller fraction of exposed patients (for review, see Gut et al., 1993). Numerous animal models have been evaluated with the purpose of trying to understand the mechanism of idiosyncratic HAL hepatotoxicity. Interestingly, animal models have reproduced the variability in response observed in people, but not necessarily all the histopathologic features or severity of lesions in people. Complementing the body of work in laboratory animals, many epidemiological studies examining HAL hepatotoxicity in exposed populations have provided mechanistic clues about HAL idiosyncrasy. Despite this rather large body of literature, the exact mechanism of idiosyncratic HAL hepatotoxicity remains elusive.

HAL undergoes oxidative metabolism by cytochromes P450 (CYPs) to form the metabolite triflouroacetyl chloride (TFA; Gut et al., 1993). Hydrolysis of TFA-chloride yields triflouroacetic acid, the primary HAL metabolite identified in urine of HAL-

exposed people (Stier, 1964). In addition, TFA can form adducts with several types of cellular macromolecules, including proteins (Gut et al., 1993). CYP2E1 is probably the primary isoform responsible for HAL metabolism in experimental animals and in people (Kenna et al., 1990; Spracklin et al., 1997; Kharasch et al., 1996). Treatment of rat and human hepatocytes with HAL results in the formation of TFA-modified proteins (Ilyin et al., 1994; Van Pelt and Kenna, 1994), and increased metabolism of HAL by induction of CYP activity is associated with increased formation of TFA-modified proteins (Kenna et al., 1990). Furthermore, inhibition of CYP activity in people and experimental animals results in a reduction in the formation of TFA-modified proteins (Kharasch et al., 1996; Spracklin et al., 2003). Overall, the data suggest that the formation of TFA-modified proteins is probably a consequence of CYP-mediated generation of the reactive HAL metabolite TFA. Interestingly, some studies have shown that P450 induction in animals worsens HAL hepatotoxicity (Rice et al., 1987). Furthermore, experiments in outbred guinea pigs, which manifest a range of hepatotoxic responses to HAL, have suggested that TFA-modified proteins are a determinant of sensitivity to HAL-induced liver injury (Bourdi et al., 2001).

As mentioned above, one possible consequence of protein modification by reactive drug metabolites is recognition of a metabolite/protein hapten by the specific immune system. In the case of HAL, the haptens formed are frequently referred to as TFA-neoantigens. The hapten hypothesis has a strong foothold in theories about HAL idiosyncrasy, as several animal models have been utilized to study the specific immune response after exposure to HAL, and numerous epidemiological studies have suggested an antibody response occurs in people exposed to HAL.

Studies in animals have demonstrated that the liver is a target for the formation of TFA-modified proteins (Satoh et al., 1985). Inasmuch as the liver is the primary site of HAL metabolism, this is perhaps not surprising. How might the specific immune system recognize TFA-neoantigens in the liver? In addition to their participation in the innate immune response, several cell types in the liver can contribute to antigen presentation and control of a specific immune response (Knolle and Gerken, 2000). One possibility is that resident liver macrophages, otherwise known as Kupffer cells (KCs), process and present TFA-neoantigens to other immune cells. Indeed, studies in guinea pigs have detected TFA-neoantigens in KCs of HAL-treated guinea pigs but not in circulating monocytes or in the spleen or lymph nodes (Furst et al., 1997). In addition, KC adhesion to lymphocytes is increased in the presence of TFA-modified proteins or homogenate from livers of HAL-treated animals, and it is decreased in the presence of an anti-MHCII antibody (Furst and Gandolfi, 1997). These data are consistent with the generation of an immune response through processing and presentation of TFA-antigens by resident liver antigen-presenting cells (APCs). However, the exact role of KC's in HAL-induced hepatotoxicity is unclear, as in rats, a species only sensitive to HAL hepatitis after CYP induction and hypoxia exposure, TFA-neoantigens were observed in KCs in some, but not all studies (Amouzadeh and Pohl, 1995; Christen et al., 1991; McLain et al., 1979). Furthermore, studies examining the effect of KC inactivation on HAL hepatotoxicity in animal models are lacking.

Other clinical features of idiosyncratic HAL hepatotoxicity are somewhat suggestive of an immunologically based mechanism of hepatotoxicity. For example, there is usually a delay between exposure to HAL and development of liver injury.

Furthermore, patients frequently respond more rapidly and robustly upon rechallenge, suggesting immunologic memory. Indeed, TFA-neoantigen-specific antibodies (NSA) have been identified in patients who developed HAL hepatitis (Kenna et al., 1987; Martin et al., 1993b; Martin et al., 1993a; Pumford et al., 1993a; Vergani et al., 1980; Bird and Williams, 1989; Njoku et al., 2002), that are not always found in sera from unexposed donors (Kitteringham et al., 1995; Bird and Williams, 1989), suggesting a causal role between these antibodies and idiosyncratic HAL hepatitis. Overall, the observation that circulating TFA-NSA are frequently associated with HAL hepatitis in patients probably has led to the dogmatic association of a specific immune response with HAL hepatotoxicity. Although these data are supportive, downstream pathways of hepatotoxicity are not yet known, and controversy exists as to the role of a humoral immune response in HAL-induced liver injury.

Some experiments in laboratory animals have not placed the same emphasis on the humoral immune response as a mediator of HAL hepatitis. In one experiment, guinea pigs given TFA-modified proteins prior to exposure to HAL had an enhanced antibody response when exposed to HAL. However, this response was not accompanied by HAL hepatitis, leading the authors to suggest that enhancing the humoral immune response is not sufficient to cause HAL hepatotoxicity (Hastings et al., 1995). In addition, the connection between circulating TFA-NSA and HAL hepatotoxicity in people is controversial. In one study, Njoku *et al.* 2002 examined the serum of over 150 anesthesiologists, 20 HAL hepatitis patients, and 20 unexposed individuals for the presence of these antibodies. Although autoantibodies were identified in the sera of anesthesiologists, the vast majority did not develop HAL hepatitis (Njoku et al., 2002).

Another study by Kitteringham *et al.* also found autoantibodies in 3 of 6 patients exposed to HAL that did not develop HAL hepatitis (Kitteringham et al., 1995). In addition, TFA-NSA are not always identified in patients who develop of HAL hepatitis (Bird and Williams, 1989). The latter observation might be due to lack of assay sensitivity or selection of antigen for detection. Nevertheless, these studies call into question the relationship between autoantibodies resulting from HAL exposure and the manifestation of HAL-induced idiosyncrasy. Further study is warranted to determine the exact role of autoantibodies in HAL hepatotoxicity.

Although the focus on mechanisms of HAL hepatotoxicity has remained on autoantibodies, several other hypotheses have received relatively little attention. For example, the role of hypoxia in causing HAL hepatotoxicity has been explored and debated. Oxygen tension seems to be important for determining the relevant role of various CYP 450 isoforms in metabolism of HAL. As mentioned above, CYP 2E1 seems to be important in oxidative metabolism of HAL to TFA. However, under low oxygen tension, other CYP 450 isoforms might become important for reductive metabolism of HAL to a free radical form (Spracklin et al., 1996). Studies in animal models and human liver microsomes have demonstrated that lipid peroxidation (LP) occurs after HAL exposure (Minoda and Kharasch, 2001; Yamazoe et al., 1998; Sato et al., 1990; Akita et al., 1989; Younes et al., 1988; Wood et al., 1976). Interestingly, LP is enhanced under conditions of hypoxia (Yamazoe et al., 1998; Younes et al., 1988; El-Bassiouni et al., 1998; de and Noll, 1984) and is further enhanced by pretreatment with the P450-inducer phenobarbital (Awad et al., 1996). Furthermore, hypoxic rats exposed to HAL developed liver injury after phenobarbital treatment, but not after pretreatment with the CYP2E1

inducer izoniazid, calling into question the sole importance of CYP 2E1 in the toxicity (Awad et al., 1996). Interestingly, glutathione (GSH) depletion has also been associated with LP, both of which precede hepatic injury in guinea pigs exposed to HAL (Akita et al., 1988). Moreover, GSH depletion enhances LP (Younes et al., 1988) and the hepatic toxicity of HAL (Lind et al., 1994; Lind et al., 1992), suggesting that GSH status is an important determinant of HAL hepatotoxicity. Other compounds, with antioxidant effects, have provided protection seemingly unrelated to their effects on HAL metabolism (Lind and Gandolfi, 1997). Overall, evidence in these models suggests that LP and GSH depletion might be important for HAL-induced liver injury.

In summary, evidence supporting the involvement of the humoral immune response in HAL-induced liver injury includes correlation of autoantibody formation with the incidence of hepatotoxicity. However, this relationship has been called into question by some studies, and other mechanisms of HAL-induced injury have also been proposed. Observations including HAL-induced LP or GSH depletion might represent the missing link in connecting autoantibodies to the development of HAL-induced injury, especially if considered as danger signals in the context of the "danger hypothesis." For example, in the report by Kitteringham *et al.* 1995 in which autoantibodies were identified in patients with HAL-induced liver injury, a second but necessary signal such as decreased antioxidant capacity could be important. Thus, even for HAL, one of the "classic" examples of immune-mediated drug toxicity, current hypotheses are controversial, and the exact mechanism is not yet known.

DCF. Nonsteroidal anti-inflammatory drugs are used for the treatment of a variety of inflammatory conditions including arthritis. Several drugs in this class, including DCF,

are associated with infrequent hepatotoxicity in patients (Boelsterli et al., 1995). Small increases in liver enzymes in DCF patients were noted in early clinical studies, but these increases do not frequently progress to frank liver injury (Helfgott et al., 1990). The incidence of more severe DCF hepatotoxicity in people has been estimated to be as low as 0.001 % (Purcell et al., 1991; Walker, 1997), but this is probably an underestimate if one considers the potential for underreporting mentioned earlier. The severity of DCF-induced hepatotoxicity outcome is illustrated by a review of 250 published DCF case reports in which the mortality rate was found to be about 10% (Lewis, 2003). Severe DCF-induced liver injury is associated primarily with pronounced elevations in markers of hepatocellular injury (Lewis, 2003), and the time to onset of severe DCF hepatotoxicity ranges from 1 month to one year after the start of therapy (Banks et al., 1995). Overall, DCF is an important example of idiosyncratic toxicity.

DCF metabolism by CYP 450s including CYP2C9 and CYP3A4, and by glucuronidation results in formation of metabolites containing moieties capable of binding proteins (for review, see Boelsterli, 2003b; Tang, 2003). Indeed, rat and human liver microsomal systems are capable of metabolizing DCF to reactive metabolites, and DCF protein adducts have been identified in cultured hepatocytes and in livers of rodents treated with DCF (Kretz-Rommel and Boelsterli, 1994; Pumford et al., 1993b). Therefore, in the case of DCF, not unlike HAL, reactive metabolites are produced, and their adducts can be identified *in vitro* and in animals. However, the relationship of these adducts to the toxicity of DCF is debated. For example, large concentrations of DCF can induce cell death in cultured hepatocytes (Kretz-Rommel and Boelsterli, 1993) and inhibition of CYP-mediated metabolism reduces DCF cytotoxicity in hepatocytes (Bort et

al., 1999; Kretz-Rommel and Boelsterli, 1993), but in one study inhibition of metabolism was without effect on DCF-adduct formation (Kretz-Rommel and Boelsterli, 1993). Inhibition of UDP-glucuronosyltransferase activity decreased DCF-adducts but significantly increased DCF-toxicity. Furthermore, identified polymorphisms in the CYP2C9 gene do not appear to cause differences in DCF metabolism, and some studies have failed to find an association between CYP2C9 polymorphism and DCF hepatotoxicity (Yasar et al., 2001; Aithal et al., 2000). Overall, the relationship of DCF-metabolites and their protein adducts to DCF-induced liver injury is unclear.

As described previously for HAL, one possibility is that DCF-modified proteins are immunogenic and that an antibody-mediated response is responsible for DCF hepatitis. Indeed, some case reports of DCF-induced liver injury are consistent with an immune-mediated response, but not all (Boelsterli, 2003b). Antibodies directed against red blood cells and platelets have been identified in some patients taking DCF (Bougie et al., 1997; Sachs et al., 2004; Meyer et al., 2003), and antibodies against DCF-modified liver proteins were identified in 7 patients with DCF-induced liver injury (Aithal et al., 2004). Nevertheless, in this same study, antibodies were also identified in more than 50% of patients taking DCF that did not have hepatotoxicity, suggesting the presence of the antibodies was not sufficient to cause liver injury. In one interesting study, mice treated with DCF conjugated to the immunogenic carrier, keyhole limpet hemocyanin (KLH), developed antibodies against the DCF-modified protein. Splenocytes isolated from these animals were able to kill DCF-treated hepatocytes in culture. Furthermore, although supernatants from cultured splenocytes from KLH-DCF-treated mice were not sufficient to injure hepatocytes, addition of naïve splenocytes to hepatocytes cultures given

supernatant resulted in cytotoxicity (Kretz-Rommel and Boelsterli, 1995). Despite these intriguing results, antibody-mediated DCF hepatotoxicity has not been demonstrated in animal models, and a clear-cut, immune-mediated mechanism for DCF-induced liver injury in people has not been established.

Other effects of DCF on the liver and hepatocytes in culture might reveal novel pathways or danger signals for DCF-induced hepatotoxicity. For example, ATP content is decreased in hepatocytes treated with DCF and is associated with opening of the mitochondrial permeability transition (MPT) pore (Masubuchi et al., 2002). Indeed, DCF can induce MPT pore opening in isolated mitochondria at relatively small concentrations. Furthermore, antioxidants and inhibitors of MPT pore opening inhibit DCF-induced apoptosis in cultured hepatocytes (Gomez-Lechon et al., 2003a; Gomez-Lechon et al., 2003b). In and of themselves, these results provide insight into how DCF can kill cells *in vitro*. However, administration of large doses of DCF to mice does not cause acute liver injury, suggesting that if these mechanisms operate *in vivo*, they are not sufficient to cause liver injury. In this regard, other susceptibility factors must accompany mitochondrial effects of DCF on hepatocytes.

Another possible mechanism of DCF hepatotoxicity that might merit attention is related to accumulation of DCF metabolites in the bile canaliculi. Boelsterli and colleagues demonstrated that transport of DCF glucuronides into the bile of rats was dependent on activity of the canalicular pump mrp2 (Seitz et al., 1998). Moreover, DCF-modified proteins were not identified in canalicular membranes of rats lacking this transporter. The consequences of DCF-modified proteins in bile canaliculi is not clear, but a decrease in activity of several resident canalicular enzymes was found in DCF-

treated rats (Sallustio and Holbrook, 2001). Canalicular transporters are important regulators of drug and metabolite elimination from hepatocytes, and altered export of reactive DCF-metabolites might be one determinant of DCF hepatotoxicity. In this regard, it is interesting to note that genetic polymorphisms have been identified in the mrp2 gene (Suzuki and Sugiyama, 2002; Kerb et al., 2001). Given the importance of mrp2 in the elimination of DCF metabolites, characterization of these polymorphisms in patients with DCF-induced liver injury is of interest. Although little is known about the consequences of mrp2-mediated export of DCF metabolites on DCF-induced hepatotoxicity, it is interesting that the mechanism of idiosyncratic hepatotoxicity of other compounds might also relate to alterations in canalicular transport (Funk et al., 2001).

In summary, mechanisms of DCF-induced hepatotoxicity are not fully understood. Several studies have demonstrated that DCF treatment results in the formation of reactive metabolites capable of binding to proteins, and antibodies to these proteins have been identified in some patients with DCF hepatitis. However, these results still cannot account for individual susceptibility to DCF hepatotoxicity. Induction of oxidative stress and mitochondrial dysfunction by DCF are also of interest, but secondary susceptibility factors are likely required for manifestation of hepatotoxicity. Interestingly, for both DCF and HAL, about 20% of the people taking the drug experienced a mild elevation in liver enzymes, but full-blown hepatitis develops only in a small fraction of the patients. In light of the observations by Alden (2003) that subtle changes in serum enzymes might be predictive of idiosyncratic reactions, these minor elevations might in fact be predictive of more severe hepatotoxicity and perhaps are indicators of altered

hepatocellular homeostasis triggered by drug-induced oxidative stress, LP, or other factors.

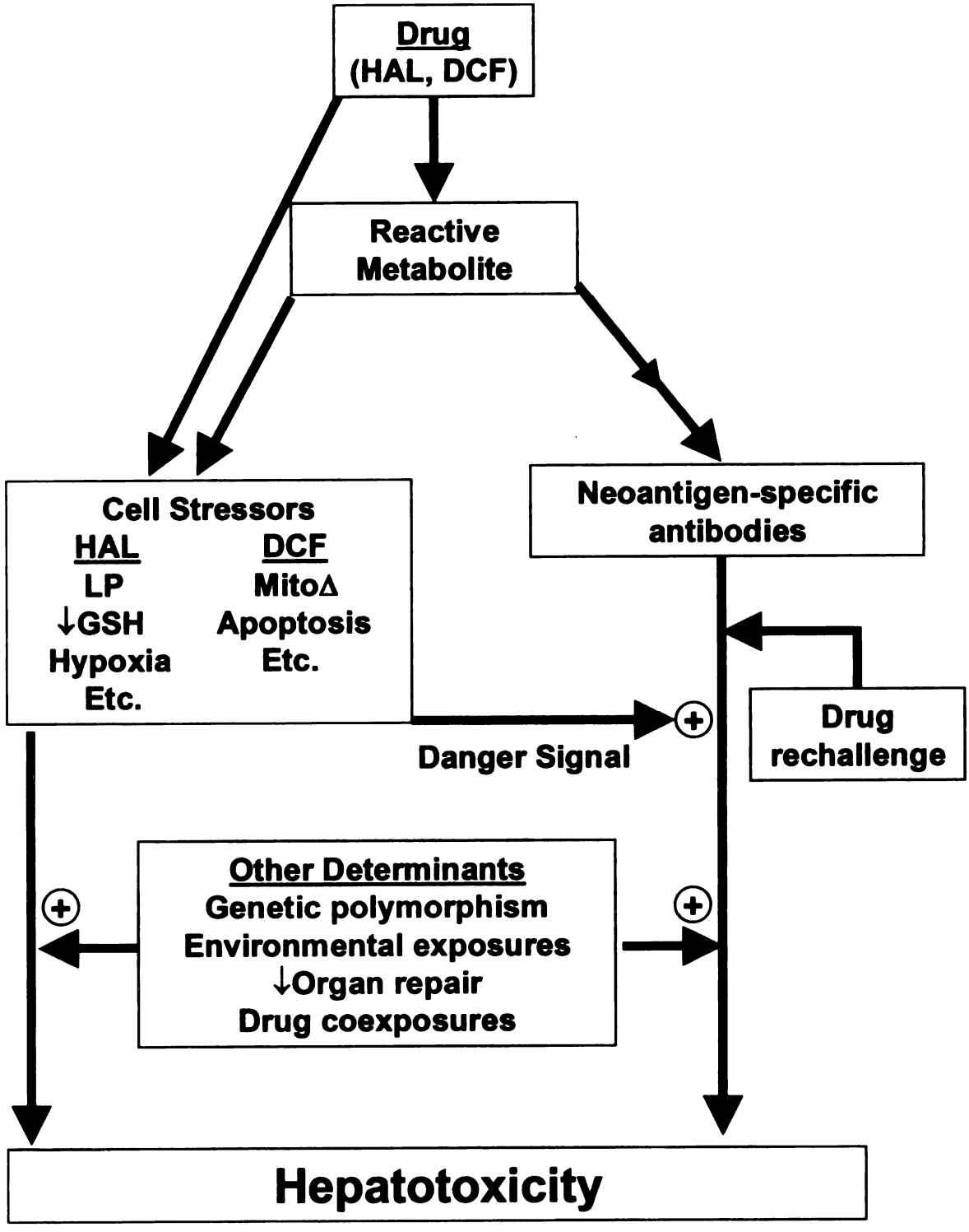
1.1.3 The "multiple determinant hypothesis."

The basis for all idiosyncratic reactions is probably not a single mechanism and therefore cannot be described by a single hypothesis or model. Figure 1.1 shows how this may be the case for both DCF and HAL. Each agent is associated with the formation of reactive metabolites, consistent with the reactive metabolite hypothesis of idiosyncrasy. Autoantibodies have been identified in people taking each drug, suggesting that the formation of drug-haptens is associated with the injury. Furthermore, consistent with the danger hypothesis of immune-mediated idiosyncrasy, cell stressors such as LP and mitochondrial dysfunction also occur after exposure to each of these agents. Gaps in each of these individual hypotheses have been identified in this brief review: taken together, this evidence paints a picture of multiple operative mechanisms.

Recently, a hypothesis has been proposed to explain the low occurrence of idiosyncratic reactions in people. The "multiple determinant hypothesis" proposed by Li states that the probability of an idiosyncratic reaction occurring for a drug is controlled by the product of the probabilities of a plethora of factors including, but not limited to drug exposure, environmental factors, genetic polymorphism, altered metabolism, formation of antigens, and inadequate liver repair (Li, 2002). Thus, the primary emphasis of this hypothesis is that one mechanism can't describe all idiosyncratic reactions, and instead it incorporates numerous possibilities (e.g., reactive metabolites and specific immune response) into a model describing the probability of idiosyncratic reactions. Li suggests

that both genetic and environmental factors also influence the overall probability of an idiosyncratic reaction occurring. Identification of individual susceptibility factors such as concurrent disease or environmental factors and development of models that can account for such factors might provide insight into mechanisms of idiosyncratic drug reactions.

Figure 1.1: Reactive metabolite, hapten, danger, and multiple determinant hypotheses of idiosyncratic hepatotoxicity from halothane (HAL) and diclofenac (DCF). DCF and HAL are associated with several cell stressors that might be caused by either the drug or a reactive drug metabolite. Reactive HAL and DCF metabolites are also capable of reacting with proteins, and circulating autoantibodies have also been identified in patients with hepatotoxicity from either agent. Cell stress induced by HAL or DCF might serve as danger signals which, in the presence of neoantigen specific antibodies (NSA), may cause hepatotoxicity. Cell stress might also cause hepatotoxicity independent of other factors. Furthermore, numerous other susceptibility factors might be necessary triggers for hepatotoxicity caused by cell stress or a specific immune response. Overall, this paradigm suggests that several mechanisms might be operative in idiosyncratic hepatotoxicity from DCF and HAL.


Drug
(HAL, DCF)
Reactive
Metabolite
Cell Stressors
HAL
LP
↓GSH
Hypoxia
Etc.
DCF
MitoΔ
Apoptosis
Etc.
Neoantigen-specific
antibodies
Drug
rechallenge
Danger Signal
Other Determinants
Genetic polymorphism
Environmental exposures
↓Organ repair
Drug coexposures
Hepatotoxicity

1.1.4 Ranitidine and idiosyncratic hepatotoxicity

Ranitidine (Zantac®, RAN) is one of several histamine 2 (H2) receptor-antagonists commonly used for the treatment of duodenal ulcers, gastric hypersecretory diseases and gastroesophageal reflux disease. Side-effects with RAN as well as other H2-antagonists including cimetidine (CIM, Tagamet®), famotidine (FAM, Pepcid®) and nizatidine (Axid®) are rare, allowing transition of these drugs to over-the-counter availability. Nonetheless, each carries an association with infrequent adverse effects in people. RAN is associated with several adverse effects, all of which occur in a very small fraction of treated patients (Vial et al., 1991). One of these is idiosyncratic hepatotoxicity. The incidence of idiosyncratic liver injury for RAN seems to be less than that of CIM, whereas reports of liver injury associated with FAM are few and suspect (Garcia et al., 1997; Luyendyk et al., 2003b). Mechanisms underlying RAN-induced liver injury in people remain unclear.

Although one H2-antagonist, oxmetidine, is hepatotoxic and produces concentration- and time-dependent cytotoxicity in isolated hepatocytes (Oldham et al., 1985; Rush et al., 1985), millimolar concentrations of RAN, CIM, and FAM did not produce cytotoxicity in isolated hepatocytes (Francavilla et al., 1989; Zimmerman et al., 1986). Additionally, RAN is not hepatotoxic when given to rats at large doses. Accordingly, RAN does not fit the classical paradigm (i.e., a type A hepatotoxicant) of producing liver injury in a dose and time-dependent manner.

RAN is, however, as mentioned above, associated with idiosyncratic hepatotoxicity in people. Elevations in liver enzymes are occasionally described during RAN treatment, and the incidence of RAN-associated liver injury in people has been

estimated to be less than 0.1% of those taking the drug (Vial et al., 1991; Mills et al., 1997), and has not always been apparent, even in large clinical trials. To characterize better the features of RAN-induced liver injury in people, a literature search was conducted using National Library of Medicine PubMed. This search identified 36 case reports of RAN-induced liver injury in patients or subjects in clinical trials. The results from this evaluation are displayed in Table 1.1. The age range identified for RAN-induced liver injury was from 19-82, with 19 males, 12 females, and 5 cases for which the patient's gender was not reported. The average time to onset (or report) of liver injury relative to start of RAN therapy was 4 weeks, with some responses occurring as early as 1 week and others 8 months after the patient started RAN therapy. Accordingly, no obvious pattern of age, gender, or time to onset was identified for RAN-induced idiosyncratic hepatotoxicity.

Injury to hepatic parenchymal cells and cholestasis were described in the majority of cases, as estimated by changes in clinical chemistry and in some cases, histopathological observations. Elevations in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were typically more robust as compared to markers of cholestasis (GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase; bile acids). Nonetheless, RAN has been associated with cholestatic injury without elevations in markers of parenchymal cell injury (Lee et al., 1986; Ramrakhiani et al., 1998; Coutellier et al., 1993). Liver biopsy samples were taken in some cases for evaluation of liver histopathology. Hepatocellular necrosis and inflammatory cell infiltration were frequently noted and accompanied occasionally by cholestasis, bile pigment in KCs and hepatocytes, and intralobular eosinophil infiltration. It is important to

recognize that evaluation of clinical chemistry and liver histopathology in these cases might not represent the time of onset or peak of toxicity since patients likely present with symptoms at various times. For example, in one case a liver biopsy was taken 2 weeks after elevations in clinical chemistry markers were observed (Ramrakhiani et al., 1998). The authors note that eosinophilic infiltration of the liver might have occurred secondarily to hepatotoxicity, bringing into question a causal role for these cells in the toxicity. Overall, hepatocellular oncotic necrosis and infiltration of mixed inflammatory cells seem to be primary features of RAN-induced liver lesions, with some evidence for a biliary component.

An interesting feature of idiosyncratic reactions is that rechallenge with the drug does not always lead to production of the idiosyncratic reaction. Rechallenge is performed only infrequently for obvious reasons, but in some cases it can be revealing. As shown in Table 1.1, 7 case reports describing the results of a RAN rechallenge were identified. Upon rechallenge with RAN, 4 patients experienced the idiosyncratic reaction a second time. However, this was not true of all the cases. For example, Graham *et al*, (1985) rechallenged a patient with RAN at a dose "sufficient to reproduce the changes" without any change in ALT or AST. In addition, RAN therapy was not discontinued despite elevation in serum enzymes (see Drug not stopped, Table 1.1) in two cases. In the report by Barr and Piper (1981), elevations in serum ALT resolved despite continued therapy, while GGT activity did not normalize until RAN treatment was discontinued. Another report by Colin-Jones (1984) described another case of suspected RAN idiosyncrasy where continued RAN therapy was not associated with worsening of symptoms.

One potential commonality among many cases of RAN idiosyncrasy was revealed by conducting this literature search. Each case report was examined for prodromal signs of inflammation, including fever, abdominal pain, nausea, vomiting, diarrhea, illness/infection, or others factors (e.g., ethanol consumption or recent surgery) known to increase the circulating concentration of inflammagens. One or more of these indicators was reported in greater than 60% of the cases. This result on its own does not prove a definitive connection between inflammation and RAN hepatotoxicity, and detailed epidemiological investigation would be required to establish such an association. However, it is consistent with inflammation as a potential susceptibility factor.

	Patient Information		Hepatotoxicity Features			Prodromal Inflammatory signs	Other		
Reference	Age	Gender	Onset	Parenchymal	Cholestatic	>1 reported	Rechallenge	Auto-antibodies	History of hepatitis
Bredfeldt, 1984	42	M	3 wk	YES	YES	none reported			NO
Halparin, 1984	62	F	8 month	YES	YES	YES			
Liberopoulos, 2002	73	F	3 wk	YES	YES	none reported		NO	NO
Offit, 1984	66	M	5 wk	YES	YES	YES			
Hiesse, 1985	38	M	1 wk	YES	YES	YES	Responded	NO	NO
Proctor, 1984	50	F	2 wk	YES		none reported			NO
Karachalios, 1985	65	M	3 wk	YES	YES	none reported		NO	
Graham, 1985	50	M	6 wk	YES	YES	none reported	No response		NO
Lauritsen, 1984	81	M	6 wk	YES	YES	none reported		NO	NO
Barr, 1981	63	F	2 wk	YES	YES	YES	Drug not stopped	anti-SM	Previous Hep A
Hirschowitz, 1986	70	M	4 wk	YES	YES	none reported		NO	NO
Souza Lima, 1984	77	F	2 wk	YES	YES	YES			
Black, 1984	63	M	3 wk	YES	YES	YES		NO	NO
Black, 1984	59	F	3 wk	NO	YES	YES		NO	NO
Black, 1984	19	F	4 wk	YES	YES	YES		NO	NO
Jones, 1982			2 wks	NO	YES	YES			
Jones, 1982			4 wks	YES		none reported			
Jones, 1982			4 wks	YES	YES	none reported			
Ribeiro, 2000	66	F	1-3 wks	YES	YES	YES		NO	NO
Cohen, 1983				YES		none reported			
Lee, 1986	60	M	4 wks		YES	none reported			
Lee, 1986	34	M	4 wk	YES	YES	none reported			
Lee, 1986	82	M	4 wk	YES	YES	none reported			
Ramrakhiani, 1998	29	M	2 wk		YES	none reported			
Colin-Jones, 1984	51	F	2 wk	YES	YES	not translated	Drug not stopped		
Luparini, 2000	46	F		YES	YES	YES	Responded		
Desaint, 1985	27	M	2 wk	YES	YES	YES		NO	NO
Devuyst, 1993	58	M	2 wk	YES	YES	YES		NO	NO
van Bommel, 1992	69	F	3 wk	YES	YES	YES			
van Bommel, 1992	43	M	3 month	YES	YES	YES	Responded		
van Bommel, 1992	49	F	1 wk	YES	YES	YES			
van Bommel, 1992	64	M	3 wk	YES	YES	YES			
van Bommel, 1992	61	M	3 wk			YES	Responded		
van Bommel, 1992	61	M	5 wk	YES	YES	YES			
Coutellier, 1993	65				YES	YES			
Valois, 2003	51	M	2-3 weeks	YES	YES	YES		NO	NO

Table 1.1 Clinical features associated with reported cases of RAN hepatotoxicity. A literature search was conducted using NLM Pubmed to identify cases of RAN idiosyncratic hepatotoxicity. Patient descriptors, markers of hepatotoxicity, and other relevant clinical details were noted. Complete references can be found in the bibliography. Blank spaces indicate that a given measure was not described, discussed, and/or measured.

1.2 Inflammation

1.2.1 Incidence and triggers

Individual susceptibility factors to drug-induced toxicity are probably not represented in animal models of drug toxicity (e.g., underlying disease). This section will provide evidence for the possibility that inflammation is one such factor. Our understanding of inflammation and the effects of inflammatory mediators on tissues has stretched beyond "a localized protective reaction of tissue to irritation, injury, or infection, characterized by pain, redness, swelling..." (The American Heritage® Dictionary of the English Language, Fourth Edition) to include inflammatory cells, the mediators they produce, as well as altered cellular signaling and gene expression. Although inflammation is important for warding off infection, it probably has other protective and sometimes deleterious effects as well. Indeed, as we learn more about inflammatory processes, it has become clear that inflammation, both acute and chronic, plays a role in the pathogenesis of many diseases, can cause tissue injury, and can increase sensitivity of tissues to the toxic effects of xenobiotics.

Inflammation is a feature of numerous diseases, and may be identified as either an innocent bystander or as a contributor to disease outcome. Several obvious examples for which inflammation is involved in disease are conditions for which treatment focuses on inhibition of inflammatory mediators (e.g, arthritis, asthma, inflammatory bowel disease) However, inflammation is now recognized as a component of several other conditions, including those affecting the cardiovascular system to cancer. Some of these are listed in Table 1.2.

Table 1.2: Some conditions for which inflammation is a consequence or contributor

Condition	For review, see:
viral hepatitis	(Nakamoto and Kaneko, 2003)
Parkinson's disease	(Barcia et al., 2003)
diabetes	(Tracy, 2003)
acute stroke	(Price et al., 2003)
obesity	(Cottam et al., 2004)
multiple sclerosis	(Martino et al., 2002)
pulmonary hypertension	(Tuder and Voelkel, 1998)
cancer	(Platz and De Marzo, 2004;Whitcomb, 2004)
cardiovascular disease	(Willerson and Ridker, 2004)
HIV	(Dalgleish and O'Byrne, 2002)
atherosclerosis	(Lind, 2003;Shishehbor and Hazen, 2004)
periodontitis	(Van Dyke and Serhan, 2003)
osteoporosis	(Pfeilschifter et al., 2002)

As an example, inflammation is a recognized component of obesity. Adipocytes actively produce several factors, including pro-inflammatory mediators such as cytokines and lipid-derived products (for review, see Cottam et al., 2004). Correlations of the acute phase protein, C-reactive protein, and obesity have been observed in numerous epidemiological studies (Visser et al., 2001; Visser et al., 1999). In addition, weight loss is associated with a decrease in circulating concentrations of pro-inflammatory mediators (Cottam et al., 2004). Interestingly, obese animals are also more sensitive to inflammatory liver injury (Yang et al., 1997). Accordingly, obesity is associated with inflammation, and might be a condition sufficient to render tissues sensitive to injury from inflammatory mediators. Indeed, the inflammatory component of obesity might be a determinant of obesity-related disease (e.g, diabetes, heart disease) (Cottam et al., 2004). Overall, inflammation accompanies several different diseases, either as a consequence or a key player.

Bacterial lipopolysaccharide (LPS, endotoxin) is an outer cell wall component of gram-negative bacteria and a potent inflammagen in people. Exposure to large amounts of LPS, such as during Gram-negative bacterial sepsis, can result in damage to several organs, including the liver, as well as mortality (Hewett and Roth, 1993). LPS activates toll-like receptors, resulting in a cascade of inflammatory events. This makes it an effective and commonly used model inflammagen in animal studies. Human exposure to small amounts of LPS is commonplace and can occur by multiple modes, including environmental exposure and translocation of LPS released by dividing or dying resident gut flora across the gastrointestinal (GI) mucosa into the circulation. Exposure to small

amounts of LPS might be beneficial to the host, but it might also increase susceptibility to other toxicities (see section 1.2.3).

Inhalation of environmental LPS occurs by occupational exposure to grains or dusts and from contaminated household air (Lane et al., 2004; Gereda et al., 2001). Contamination of household air with LPS can vary with season and is dependent on several factors including household pets and central air conditioning (Gereda et al., 2001; Park et al., 2000; Heinrich et al., 2001). Under several conditions, enhanced exposure to LPS in humans also occurs by translocation of endogenous GI tract LPS into the portal circulation (for review, see Roth et al., 1997). For example, liver and GI diseases are associated with increased circulating LPS (Gardiner et al., 1995), as are surgeries in which the GI tract is disrupted. Changes in diet, alcohol consumption, and antibiotic treatment are also associated with increased LPS translocation (Roth et al., 1997; Lepper et al., 2002). Interestingly, even strenuous exercise has been shown to cause mild endotoxemia in humans and other species (Ashton et al., 2003; Jeukendrup et al., 2000; Camus et al., 1998; Camus et al., 1997). Overall, exposure of people to LPS is episodic and commonplace, and can occur under numerous conditions in the absence of any acute tissue injury.

1.2.2 LPS-induced liver injury.

Inasmuch as episodes of inflammation occur commonly in people, either as a component of a disease or in response to inflammagens like LPS, it is likely that simultaneous exposure to xenobiotics, including drugs, occurs in some people. The following section briefly discusses the ability of LPS to incite an inflammatory response

and the current understanding of mediators important for liver injury from large doses of LPS. Furthermore, studies evaluating the effect of xenobiotic/inflammagen coexposure on liver injury will be reviewed, and the role of inflammatory mediators in this interaction discussed.

LPS activation of toll-like receptors (TLRs) on several cell types in the liver leads to changes in gene expression, altered sinusoidal endothelial cell homeostasis, activation of the coagulation system, accumulation and/or activation of inflammatory cells such as macrophages (KCs resident in liver), neutrophils and platelets, as well as production of cytokines such as IL-1 and TNF and other soluble factors including lipid mediators. This inflammatory response is in turn governed by both positive and negative regulation. Among this laundry list of inflammatory factors, numerous cell types and pro-inflammatory mediators have been identified as critical mediators of LPS-induced liver injury. The summary below pertains primarily to hepatic events that occur after a large, hepatotoxic dose of LPS:

KCs: KCs might be classified as "first responders" to LPS in the liver. Activation of Toll-like receptor-4 on KCs is thought to be one of the earliest events triggered by LPS in the liver. This results in production of inflammatory mediators, including reactive oxygen species, cytokines, etc. Inactivation of KCs with gadolinium chloride (GC) attenuates liver injury in rats from a hepatotoxic dose of LPS (Pearson et al., 1996a; Vollmar et al., 1996) but is without effect on circulating fibrinogen (Pearson et al., 1996a), suggesting that thrombin generation occurs by KC-independent mechanisms. However, KC inactivation does result in a decrease in hepatic accumulation of platelets. Stimulation of KCs with LPS results in production of TNF (Su et al., 2000), and

inhibition of KCs with GC reduced plasma TNF concentration in some (Vollmar et al., 1996), but not all studies (Pearson et al., 1996a).

TNF: TNF production is triggered in several cell types by exposure to LPS. Hepatic mRNA and plasma TNF concentration are increased early after exposure to LPS (Hewett and Roth, 1993). TNF-neutralizing antibodies and inhibition of TNF synthesis significantly attenuate LPS-induced injury in rodents (Hewett et al., 1993). After an hepatotoxic dose of LPS, inhibition of TNF biosynthesis or neutralization of TNF does not influence hepatic PMN accumulation, suggesting either that TNF injures the liver after exposure to LPS by a neutrophil-independent mechanism or that TNF affects a critical function of PMNs (e.g., activation and release of cytotoxic proteases) (Hewett et al., 1993).

Platelets: Platelet depletion prior to LPS exposure significantly reduces LPS-induced activation of the coagulation system and liver injury (Pearson et al., 1995). A reduction in the number of circulating platelets was not associated with a change in hepatic PMN accumulation (Pearson et al., 1995).

PMNs: PMN accumulation occurs in liver rapidly after treatment with LPS. Hepatic gene expression of the chemokines, cytokine-inducible neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-2 (MIP-2), is increased after LPS exposure, and this is mediated by effects of cytokines such as IL-1 (Calkins et al., 2002). Activated PMNs release a variety of cytotoxic factors that can kill hepatocytes *in vitro* including ROS and the lysosomal proteases, cathepsin G and elastase (Ho et al., 1996; Ganey et al., 1994; Mavier et al., 1988). Neutrophil depletion or inhibition of hepatic PMN accumulation by neutralization of these chemokines

significantly attenuates liver injury (Li et al., 2004; Hewett et al., 1992). Furthermore, inhibitors of PMN elastase reduce hepatocellular damage in models of LPS-induced liver injury (Ishii et al., 2002).

Despite these results, the role of PMN-derived proteases has been debated. Liver injury develops in LPS-treated rodents within 6 h, whereas PMN protease killing of hepatic parenchymal cells (HPCs) takes longer to manifest *in vitro* (Ho et al., 1996). Accordingly, other PMN-derived factors might act in concert with proteases to kill parenchymal cells. For example, an inhibitor of PMN NADPH oxidase activity significantly reduced liver injury in galactosamine-sensitized rats given LPS. This inhibition was associated with a decrease in chlorotyrosine staining (indicator of myeloperoxidase activity), suggesting that PMN-derived reactive oxygen species (ROS) are important for injury in galactosamine (gal)/LPS-cotreated rats (Gujral et al., 2004). However, PMNs stimulated with fMLP plus PMA to release proteases and ROS, respectively, do not kill HPCs with a timecourse different from that of proteases alone (Ganey et al., 1994). This result suggests that an interaction of proteases with other inflammatory mediators, with other liver cell types, or with some change in hepatocellular homeostasis not represented *in vitro* is necessary for full manifestation of LPS-induced liver injury.

Hemostatic system: Treatment of rodents with LPS causes activation of the coagulation system (Hewett and Roth, 1995; Akahane et al., 2001), but the exact mechanism of LPS-mediated coagulation activation is not yet known. Several studies have suggested a role of tissue factor (TF) in activation of the coagulation system after LPS exposure. LPS exposure induced TF (for review, see Mackman, 1996) and it is

stored in circulating platelets (Engelmann et al., 2003). When expressed on the cell surface, it can activate the coagulation system through interactions with coagulation factor VIIa. Mice deficient in tissue factor expression were less susceptible to LPS-induced coagulation activation (Pawlinski et al., 2004). Furthermore, expression of human TF pathway inhibitor in endothelial cells, platelets and monocytes dramatically reduced coagulation system activation and fibrin deposition in a mouse model of endotoxemia (Chen et al., 2004). Overall, the expression of TF after LPS exposure appears to be important for LPS-induced thrombin generation.

Inhibition of thrombin activation significantly attenuates LPS-induced liver injury (Pearson et al., 1996b; Moulin et al., 2001), but this protection is independent of circulating fibrinogen (Hewett and Roth, 1995). Although fibrin clots are observed in liver after treatment of rodents with LPS, this result suggests that thrombin activation is important for liver injury from LPS, but that the formation of fibrin clots is not required for the injury. Thrombin has activities outside the traditional scope of the hemostatic system. For example, thrombin-mediated activation of its receptor, protease activated receptor-1 (PAR-1), influences inflammatory cell accumulation and/or activation in the liver. Although inhibition of thrombin activation did not attenuate accumulation of PMNs in liver after LPS-treatment, it reduced PMN activation as estimated by increased plasma elastase concentration (Pearson et al., 1996b; Copple et al., 2003). Furthermore, perfusion of livers from LPS-treated rats with thrombin or the PAR-1 agonist TFLLR caused hepatocellular injury (Moulin et al., 2001;Copple et al., 2003), an effect eliminated by prior depletion of PMNs (Moulin et al., 2001). Overall, these results suggest that

thrombin-mediated activation of PAR-1 is important for PMN activation and that these two events are sufficient for injuring livers of LPS-treated rats.

Overall, several inflammatory mediators are associated with LPS-induced liver injury including KCs, TNF, PMNs and their chemokines, platelets, and thrombin. These are summarized in Table 1.3. Much remains to be learned about how these mediators network to cause liver injury from LPS. Moreover, though not discussed in detail here, changes in the activation state of other cell types such as SECs and stellate cells might be important. Interestingly, even though thrombin activation is paramount for LPS hepatotoxicity, its action is seemingly unrelated to coagulation and fibrin deposition; rather, thrombin is important for receptor-mediated activation of PMNs and perhaps other inflammatory cells.

Table 1.3: Examples of inflammatory mediators important for LPS-induced liver injury. Several inflammatory cells, the mediators they produce, and components of the hemostatic system cause events critical to LPS-induced liver injury. For example, KCs, neutrophils, chemokines, platelets, TNF, and the coagulation system are all involved in LPS-induced injury.

Mediator	**Reference demonstrating contribution to liver injury**
TNF	(Hewett et al., 1993)
PMN chemokines	(Li et al., 2004)
PMNs	(Hewett et al., 1992;Gujral et al., 2004;Ishii et al., 2002)
Platelets	(Pearson et al., 1995)
Thrombin	(Hewett and Roth, 1995;Moulin et al., 1996)
KCs	(Pearson et al., 1996a;Vollmar et al., 1996)

1.2.3 LPS-induced sensitivity to hepatotoxicity

A robust inflammatory response, such as that observed after exposure to a large, hepatotoxic dose of LPS, can result in frank injury to organs including the liver. By contrast, smaller doses of LPS produce a modest, but noninjurious inflammatory response. Inasmuch as inflammatory mediators can alter tissue homeostasis, our group and others tested the hypothesis that a modest underlying inflammatory response can render the liver sensitive to hepatotoxic effects of xenobiotics. One way to evaluate this hypothesis is to examine the sensitivity of animals to xenobiotic-induced liver injury in the face of normally noninjurious inflammation. Accordingly, several animal models have been developed in which rats are treated with a small dose of inflammagen (typically LPS) to elicit a nonhepatotoxic inflammatory response and then are cotreated with a nonhepatotoxic dose of a xenobiotic agent. LPS coexposure augments the hepatotoxicity of numerous organic chemicals, heavy metals, and some drugs (Table 1.4). Although this discussion focuses on models of LPS-augmented hepatotoxicity, LPS has also been shown to enhance extrahepatic toxicity of some chemicals (Wagner et al., 2001; Rumbeiha et al., 2000; Zhou et al., 2000). Overall, these studies demonstrate that an underyling inflammatory response has the potential to lower the threshold for xenobiotic toxicity. Furthermore, the results suggest that inflammation might be a determinant of sensitivity to chemically induced liver injury in people.

The effect of inflammation on hepatotoxicity has been investigated in several experiments by examining the dose-response relationship in the presence or absence of inflammation. In one model, rats are cotreated with a small, nonhepatotoxic dose of the fungal toxin aflatoxin B_1 (AFB_1) and a small dose of LPS. Whereas liver injury does not

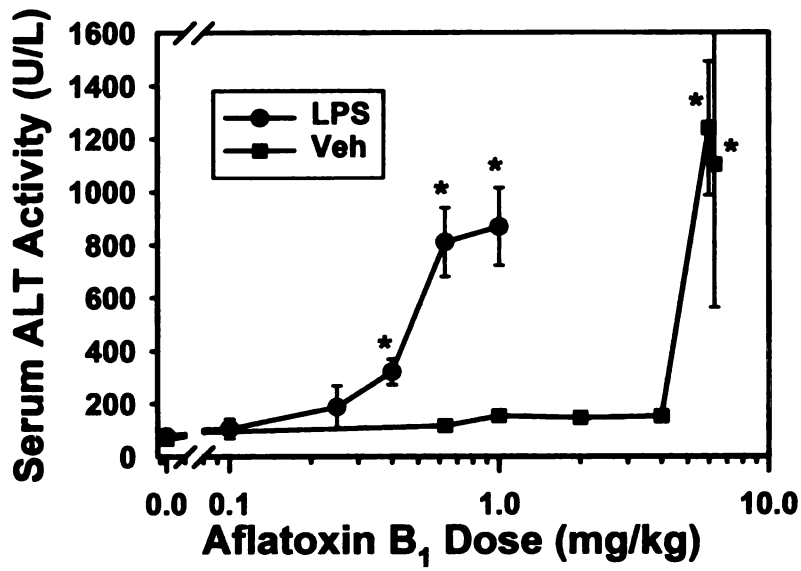
develop in rats treated with either agent alone, AFB_1/LPS-cotreated rats develop marked hepatotoxicity characterized by periportal hepatocellular oncotic necrosis and injury to intrahepatic bile ducts (Barton et al., 2000b). At the timepoint of maximal injury in AFB_1/LPS-treated rats, sensitivity to AFB_1-induced liver injury increases 10-20 fold in rats coexposed to a small dose of LPS (Luyendyk et al., 2002; Fig 1.3). A leftward shift in the xenobiotic dose-response curve has also been observed in other models of LPS-augmented hepatotoxicity (Sneed et al., 1997; Yee et al., 2000). Overall, these experiments suggest that an underlying inflammatory response has the potential to decrease the threshold for xenobiotic-induced hepatotoxicity in rats.

Animal models of LPS-potentiated liver injury have not only suggested that inflammation is a determinant of sensitivity for liver injury but have provided models in which the specific mediators of toxicity can be evaluated. Regarding mechanisms of toxicity in these models, one possibility is that the xenobiotic agent has the ability to directly render hepatocytes more sensitive to a normally nontoxic inflammatory response. For example, the cytotoxicity of allyl alcohol (AA) in cultured rat hepatocytes is augmented by cotreatment with a prostaglandin released by activated KCs (Maddox et al., 2004). Another possibility is that the xenobiotic can boost the LPS-induced inflammatory response such that it exceeds the threshold for inflammation-induced hepatotoxicity. One example of this is the ability of the plant toxin monocrotaline (MCT) to activate PMNs (Yee et al., 2003c). In all likelihood, both of these mechanisms are operative in models of LPS-potentiated hepatotoxicity.

Table 1.4: Xenobiotics for which hepatotoxicity is augmented by LPS.

Xenobiotic	Reference
Aflatoxin B1	(Barton et al., 2000b)
Monocrotaline	(Yee et al., 2000)
Allyl alcohol	(Sneed et al., 1997)
Carbon tetrachloride	(Chamulitrat et al., 1995)
Galactosamine	(Bahrami et al., 1994)
Ethanol	(Hansen et al., 1994)
T2-toxin	(Tai and Pestka, 1988)
Cadmium	(Cook et al., 1974)
HAL	(Lind et al., 1984)
Cocaine	(Labib et al., 2002)
Chlorpromazine	(Buchweitz et al., 2002a)
Lead	(Honchel et al., 1991)
PCBs	(Brown et al., 1996)
TCDD	(Patterson et al., 2003)

Figure 1.2: Noninjurious endotoxemia increases sensitivity of rats to AFB_1-induced hepatotoxicity. Rats given a small, nonhepatotoxic dose of LPS are rendered 10-20-fold more sensitive to AFB_1-induced hepatotoxicity (Luyendyk et al., 2002). Hepatotoxicity was estimated by increased serum alanine aminotransferase (ALT) activity. *Significantly different from respective group not given AFB_1.

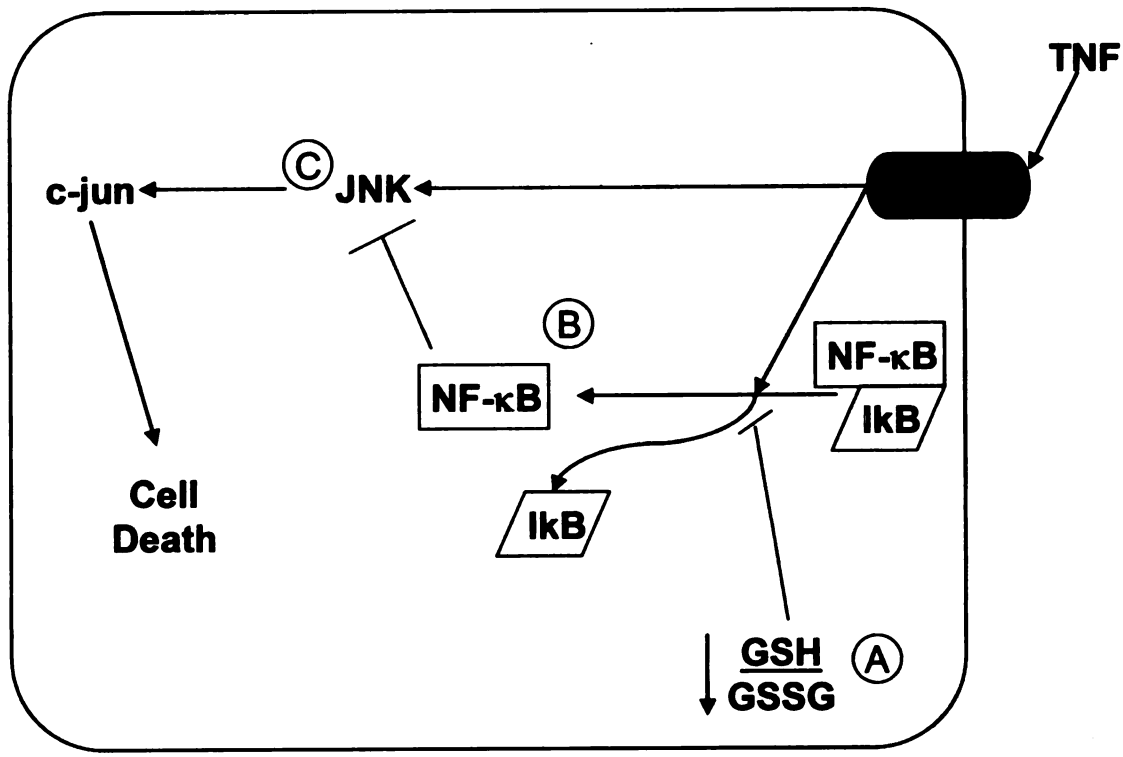

Serum ALT Activity (U/L)
1600
1400
1200
1000
800
600
400
200
0
LPS
Veh
*
*
*
*
*
0.0
0.1
1.0
10.0
Aflatoxin B1 Dose (mg/kg)

Identification of inflammatory mediators involved in models of LPS-potentiated hepatotoxicity has revealed a great deal of commonality with mechanisms involved in liver injury from large, hepatotoxic doses of LPS. For example, KCs are important for liver injury in rats cotreated with LPS and monocrotaline (Yee et al., 2003a). TNF has also been identified as a mediator of hepatotoxicity in some (Barton et al., 2001;Yee et al., 2003a), but not all models (Sneed et al., 2000) of LPS-potentiated hepatotoxicity. Similarly, cyclooxygenase 2 (COX-2) is important for the toxicity from AA/LPS-cotreatment (Ganey et al., 2001), but despite its augmented expression in AFB_1/LPS-treated rats, is not important for hepatotoxicity (Barton et al., 2001). Thus, augmented expression of an inflammatory mediator does not prove its involvement in liver injury. Interestingly, some common ground can be found across models of LPS-augmented hepatotoxicity despite the differences described above. For example, PMNs are important for liver injury in AFB_1/LPS, MCT/LPS, and AA/LPS cotreated rats (Barton et al., 2000a; Yee et al., 2003c; Kinser et al., 2004). Furthermore, activation of the coagulation system occurs in each of these models, and anticoagulation provides marked if not complete protection against the liver injury (Luyendyk et al., 2003a; Yee et al., 2003d; Kinser et al., 2002). Overall, mediators important for LPS/xenobiotic-induced liver injury likely depend on the xenobiotic agent.

One question at the core of these experiments is what critical change in hepatocellular homeostasis (e.g., signal transduction, gene expression) is initiated by coexposure of the cell to a particular inflammatory factor and xenobiotic? That is, what effects does a particular xenobiotic have on cells (e.g., hepatocytes) that render them sensitive to deleterious effects of inflammatory mediators? One example of how altered

HPC homeostasis can influence the outcome of exposure to inflammatory mediators is TNF-induced cytotoxicity in hepatocytes. Hepatocytes are not sensitive to TNF-induced cell death *in vitro* unless sensitized by one or more changes in signaling. For example, inhibition of NF-κB function is associated with an increase in the activity of c- Jun NH-terminal kinase (JNK) that renders hepatocytes sensitive to TNF-induced cytotoxicity (Jones et al., 2000; Liu et al., 2002). This increase in JNK activity may be due to lack of upregulation of negative JNK regulators by NF-κB. Consistent with this observation, JNK and c-Jun activity are required for TNF-induced cytotoxicity under conditions of NF-κB inhibition (Schwabe et al., 2004; Liu et al., 2002). Depletion of GSH also renders hepatocytes sensitive to TNF-induced cytotoxicity and is associated with decreased NF-κB activation and increased JNK activity (Nagai et al., 2002; Matsumaru et al., 2003). These studies illustrate how alterations in signaling pathways can influence the sensitivity of hepatocytes to TNF-induced cytotoxicity (Fig. 1.3). Similarly, xenobiotics capable of 1) depleting GSH, 2) inhibiting NF-κB activation, and/or 3) activating JNK pathways might be expected to sensitize hepatocytes to TNF-induced cytotoxicity. The example of TNF is only one of several possible ways that cellular alteration could interact with LPS-generated inflammatory mediators to result in toxicity. Another mode of xenobiotic/LPS interaction is the focus of the thesis research.

Figure 1.3: Factors influencing sensitivity of hepatocytes to TNF-induced cytotoxicity. Activation of cell death pathways involving JNK is important for TNF-induced killing of hepatocytes. Activation of TNFR1 receptors by TNF results in activation of NF-kB-mediated transcription and prevention of JNK activation. Inhibition of NF-kB translocation (B) leads to an increase in JNK activity and sensitizes the hepatocytes to TNF-induced cell death. GSH depletion (A) is associated with decreased NF-kB activation, an increase in JNK activity, and sensitization of hepatocytes to TNF-induced killing. Agents modulating JNK activity (C) can also modulate TNF-mediated cell death signaling. Xenobiotics that alter cell signaling at points A, B, or C might increase sensitivity to TNF-induced cytotoxicity.


TNF
C
c-jun
JNK
B
NF-κB
NF-κB
IkB
Cell
Death
IkB
GSH
GSSG
A

1.3 Inflammation-induced idiosyncrasy: a paradigm for hepatotoxicity

The previous section presented data supporting the hypothesis that inflammation increases the sensitivity of the liver to hepatotoxic effects of xenobiotics. Furthermore, potential molecular effects of inflammatory mediators were discussed in the context of increasing sensitivity of target cells to xenobiotic toxicity. We have hypothesized that underlying inflammatory responses might be a determinant of sensitivity to ADRs and could precipitate some idiosyncratic responses to drugs (Roth et al., 2003). Modest, episodic exposures to inflammagens (e.g. LPS), although noninjurious by themselves, might bring about a leftward shift in the dose-response curve for drug-induced hepatotoxicity. If this leftward shift is as dramatic as in the case of AFB_1 (Fig. 3), it is conceivable that a normally therapeutic (and noninjurious) dose of a drug could be rendered hepatotoxic in the face of an inflammatory response.

An interesting point about the inflammation hypothesis is that it can adequately describe the features classically associated with idiosyncratic reactions. This is summarized in Table 1.5. For example, in idiosyncratic reactions the affected organ is typically not the pharmacologic or therapeutic target. Why might the liver be a common target for idiosyncratic reactions under the inflammation hypothesis? Not only is the liver the site of first pass for orally administered drugs, it also receives the greatest exposure to gastrointestinal LPS via the portal vein (Jacob et al., 1977; Nolan, 1989). Furthermore, the response of resident KCs to LPS via TLR4 signaling (Su et al., 2000) and elaboration of inflammatory mediators by stellate cells, endothelial cells, and hepatocytes makes the liver both a first responder to and target for LPS (Hewett and Roth, 1993).

Table 1.5: The inflammation hypothesis of idiosyncratic drug reactions and its relationship to typical features of idiosyncratic drug reactions.

Idiosyncratic drug reactions	**"Inflammation hypothesis"**
Liver is a frequent target organ	Exposure to GI-derived LPS Resident liver cells (KCs, SECs) can respond to LPS
Seemingly unrelated to dose	Episodic leftward shift in dose-response curve
Inconsistent time to onset, Rechallenge does not always cause the response	Exposure to inflammagens is episodic
Relatively rare	Intersection of drug exposure and inflammagen exposure is infrequent

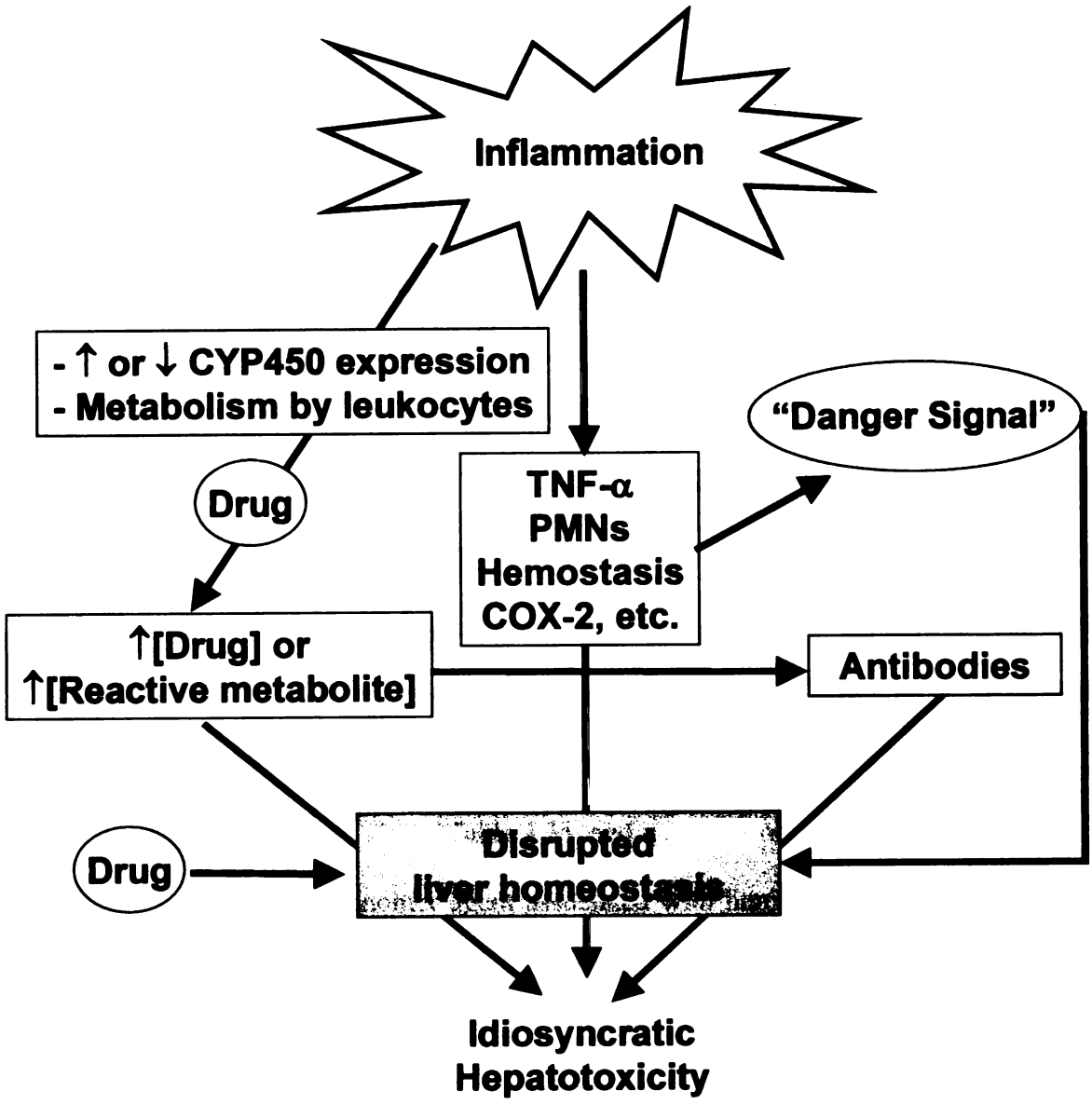
Accordingly, localized LPS-induced inflammatory responses might render the liver sensitive to hepatotoxicity. Idiosyncratic reactions are also frequently described as dose-independent or as not having a simple dose-response relationship for toxicity. Idiosyncratic reactions may indeed be dependent on dose, but this relationship could be concealed by a varying threshold for toxicity caused by episodic inflammation (Roth et al., 2003). For this reason, drugs associated with idiosyncratic reactions might behave as Paracelsus postulated (ie., the dose makes the poison), with an additional factor (ie., inflammation) required to reveal hepatotoxicity at a therapeutic dose in people. In this regard, it is interesting that Uetrecht suggested that drugs for which the dose is below 10 mg/kg have less propensity to cause idiosyncratic reactions (Uetrecht, 2001). Accordingly, the therapeutic plasma concentration of more potent drugs might lie below the new threshold for hepatotoxicity established by inflammation, even after a leftward shift in the dose-response curve. This observation is consistent with the much smaller propensity of FAM to cause idiosyncratic hepatotoxicity compared with RAN, as the therapeutic dose is considerably smaller than that of RAN. Idiosyncratic hepatotoxicity occurs at variable times after the start of drug therapy (i.e., for RAN, 1 week to 9 months). This variability can be explained by the episodic nature of exposure to inflammagens (Roth et al., 2003). Finally, the relatively small occurrence of idiosyncratic reactions might be due to numerous factors related to the genesis of the inflammatory response (e.g., timing of drug exposure, magnitude of inflammation, tolerance to inflammatory mediators, etc) or to polymorphisms in genes encoding important regulators or members of the inflammatory response. Overall, the hypothesis

that inflammation might precipitate some idiosyncratic drug reactions is consistent with features associated with this type of drug toxicity.

The inflammation hypothesis of idiosyncratic reactions need not be distinct from other hypotheses of mechanisms underlying idiosyncratic drug reactions (Figure 1.4), including formation of reactive metabolites. For example, exposure to LPS can decrease hepatic cytochrome P450 content, and could therefore result in drug accumulation. Interestingly, activated leukocytes, such as PMNs, and the products they release can metabolize drugs to reactive metabolites (Uetrecht, 1991). Accordingly, exposure to LPS can potentially increase drug concentration or reactive drug metabolite generation by altered hepatic metabolism and by activation of inflammatory cells. Inflammation might also be an important factor in antibody-mediated hepatotoxicity. For example, in one model of allergic hepatitis, cotreatment with LPS markedly increased the hepatotoxicity (Mizoguchi et al., 1990). Inflammation might also be a sufficient danger signal, such that in the presence of autoantibodies hepatotoxicity occurs. Proinflammatory cytokines such as TNF have been hypothesized to provide a necessary danger signal, and there is certainly evidence supporting a role for both inflammation and autoantibodies in HAL idiosyncrasy. Finally, some change in liver homeostasis caused by either the drug or inflammatory mediators might be a necessary factor for idiosyncratic hepatotoxicity.

Experimental evidence of reactive metabolites or an immune-mediated response as mediators of idiosyncratic hepatotoxicity from DCF and HAL is available. Is there evidence for inflammation as a component of the toxicity from either of these two drugs?

Figure 1.4: Integration of the "inflammation hypothesis" with previously proposed hypotheses for mechanisms of idiosyncratic hepatotoxicity. Inflammation can influence a drug's propensity to cause idiosyncratic toxicity by several modes. For example, inflammation might increase the concentration of a drug by inhibiting P450 metabolism. Activated leukocytes have also been implicated in metabolism of drugs to reactive species. Modification of proteins by these metabolites could result in formation of autoantibodies, which during concurrent inflammation (i.e., a danger signal) can cause hepatotoxicity. Furthermore, the ability of the drug or inflammatory mediators to alter hepatocellular homeostasis might render the liver sensitive to injury from normally noninjurious activation of inflammatory mediators.


Inflammation
- ↑ or ↓ CYP450 expression
- Metabolism by leukocytes
Drug
↑[Drug] or
↑[Reactive metabolite]
TNF-α
PMNs
Hemostasis
COX-2, etc.
"Danger Signal"
Antibodies
Drug
Disrupted
liver homeostasis
Idiosyncratic
Hepatotoxicity

For example, some evidence supports the possibility that inflammation plays a role in idiosyncratic HAL hepatotoxicity. Cotreatment with LPS causes hepatotoxicity in hypoxic rats exposed to HAL (Lind et al., 1984). The case of DCF is perhaps a bit confusing, as DCF is an anti- inflammatory agent. Nonetheless, gastrointestinal damage caused by DCF might cause an increase in circulating LPS (Boelsterli, 2003b), and COX-2 is not a critical mediator of injury in some models of LPS-augmented hepatotoxicity (Barton et al., 2001). Accordingly, the anti-inflammatory action of DCF might be masked by its propensity to cause release of GI LPS, which could prompt expression of non-prostanoid inflammatory mediators. In addition, recent studies have suggested a relationship between cytokine polymorphisms and DCF hepatotoxicity (Aithal et al., 2004). Accordingly, some evidence supports inflammation as a mediator of hepatotoxicity for two drugs for which the preponderance of studies have focused on other hypotheses.

Unfortunately, epidemiological studies demonstrating a connection between inflammation and idiosyncratic responses have not been done. However, as described earlier, signs of inflammation frequently accompany idiosyncratic reactions to RAN (e.g., see prodromal inflammatory signs, Table 1.1), and a similar observation has been made for chlorpromazine (CPZ), another agent associated with human idiosyncratic reactions that can be mimicked in rats cotreated with LPS (Buchweitz et al., 2002). In addition, underlying diseases with inflammatory components might be susceptibility factors for idiosyncratic drug reactions (Boelsterli, 2003b; Ganey et al, 2004). Overall, retrospective evaluation of case reports and some experiments in animals have suggested a relationship between inflammation and idiosyncratic hepatotoxicity.

1.4 Overview of dissertation

Inasmuch as modest concurrent inflammation can render rats sensitive to hepatotoxicity from several xenobiotics, we have hypothesized that some idiosyncratic responses to drugs might be precipitated by inflammatory responses that occur during drug therapy (Roth et al., 2003; Buchweitz et al., 2002). Accordingly, the primary goal of this dissertation is to characterize the dose-response relationship and timecourse of hepatotoxicity in LPS/RAN-cotreated rats. Furthermore, experiments aimed at identification of mechanisms of toxicity in this RAN idiosyncrasy model are described. In the chapters that follow, the hypothesis to be tested is that RAN is rendered hepatotoxic by concurrent, noninjurious inflammation in rats. The features of this model are discussed in the context of RAN idiosyncrasy in people. Hepatic gene expression is evaluated in this model, and the possible relationship of gene expression changes to LPS/RAN-induced liver injury is discussed. The effects of LPS/RAN on activation of the hemostatic system and generation of liver hypoxia as critical toxicologic consequences are emphasized. Finally, preliminary evidence supporting a role for PMNs and a PMN/hypoxia interaction in the LPS/RAN-model is presented.

CHAPTER TWO

Luyendyk, J.P, Maddox, J.F., Cosma, G.N., Ganey, P.E., Cockerell, G.L., and Roth, R.A. (2003). Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. J Pharmacol Exp Ther. **307(1)**:9-16.

2.1 Abstract:

Drug idiosyncrasy is an adverse event of unknown etiology that occurs in a small fraction of people taking a drug. Some idiosyncratic drug reactions may occur from episodic decreases in the threshold for drug hepatotoxicity. Previous studies in rats have shown that modest underlying inflammation triggered by bacterial LPS can decrease the threshold for xenobiotic hepatotoxicity. The histamine2 (H2)-receptor antagonist RAN causes idiosyncratic reactions in people, with liver as a usual target. We tested the hypothesis that RAN could be rendered hepatotoxic in animals undergoing a modest inflammatory response. Male rats were treated with a nonhepatotoxic dose of LPS (44 x 10^6 EU/kg, iv) or its vehicle, then 2 hours later with a nonhepatotoxic dose of RAN (30 mg/kg, iv) or its vehicle. Liver injury was evident only in animals treated with both RAN and LPS as estimated by increases in serum alanine aminotransferase, aspartate aminotransferase and γ-glutamyl transferase activities within 6 h after RAN administration. LPS/RAN cotreatment resulted in midzonal liver lesions characterized by acute necrosuppurative hepatitis. FAM is an H2-antagonist for which the propensity for idiosyncratic reactions is far less than RAN. Rats given LPS and FAM at a dose pharmacologically equipotent to that of RAN did not develop liver injury. *In vitro,* RAN sensitized hepatocytes to killing by cytotoxic products from activated PMNs, whereas FAM lacked this ability. The results indicate that a response resembling human RAN idiosyncrasy can be reproduced in animals by RAN exposure during modest inflammation.

2.2 Introduction

As described in section 1.3, the ability of modest inflammation to potentiate the toxicity of numerous xenobiotic agents led us to hypothesize that some drug idiosyncrasies result from episodes of mild inflammation occurring during drug therapy (Roth et al., 2003). The hepatotoxicity of two drugs associated with idiosyncratic reactions, CPZ and HAL, is augmented by coexposure to a small, noninjurious dose of LPS. The purpose of this study was to test the hypothesis that underlying inflammation triggered by a nonhepatotoxic dose of LPS renders RAN hepatotoxic in rats, revealing a response resembling human RAN idiosyncrasy. Additionally, the hypothesis was tested that the H2 antagonist famotidine (FAM), for which the propensity to cause idiosyncratic reactions is markedly smaller, would not be rendered hepatotoxic by LPS. Finally, the ability of RAN to influence the killing of rat hepatocytes by cytotoxic products released by activated inflammatory cells was explored *in vitro*.

2.3 Materials and Methods

2.3.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). LPS derived from *E. coli* serotype O55:B5 with an activity of 6.6 X 10^6 EU/mg was used for these studies. This activity was determined using a colorometric, kinetic Limulus Amebocyte Lysate (LAL) assay (Kit #50-650U) purchased from Biowhittaker (Walkersville, MD).

2.3.2 Animals

Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR; Charles River, Portage, MI) weighing 250-350 grams (*in vivo* studies) or 90-150 grams (*in vitro* studies) 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-h light/dark cycle prior to use.

2.3.3 Experimental Protocol

Rats fasted for 24 hours were given 44.4 X 10^6 EU/kg LPS or its saline vehicle, iv Two hours later 30 mg/kg RAN, 6mg/kg FAM or sterile phosphate-buffered saline (PBS) vehicle was administered iv RAN solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. Three, 6, 12 and 24 hours later, separate groups of rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and killed by exsanguination. Blood was allowed to clot at room temperature, and serum was collected and stored at -20° C until use. Three 100 mg sections of liver were taken from the interior portion of the right media lobe, flash frozen individually in liquid nitrogen, and stored at –80 degrees C for RNA isolation. Representative (3-4 mm) slices of the ventral half of the left lateral liver lobe were collected and fixed in 10% neutral buffered formalin.

2.3.4 Assessment of hepatotoxicity

Hepatic parenchymal cell injury was estimated as an increase in serum alanine aminotransferase (ALT) activity was determined spectrophotometrically using Infinity-ALT reagent from Sigma Chemical Co. (St. Louis, MO). Hepatic parenchymal cell injury

was estimated as increases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Biliary injury was estimated from increases in gamma-glutamyl transferase (GGT) activity.

2.3.5 Histopathology

Formalin-fixed liver samples (3-4 samples/rat) were embedded in paraffin, sectioned at 5 μm, stained for hematoxylin and eosin (H&E), and examined by light microscopy. All tissue sections were examined by the pathologist without knowledge of treatment (i.e., performed in blinded fashion). All lesions were assigned a score of 0 to 5, with 0 representing no significant lesion and increasingly higher numbers representing progressively more severe lesions.

2.3.6 Hepatocyte isolation

Hepatocytes were isolated using the Gibco Hepatocyte Product Line (Invitrogen, Carlsbad, CA) including liver perfusion medium, liver digest medium, and hepatocyte wash buffer (Cat. Nos 17701, 17703, 17704). All reagents were warmed to 37 °C prior to perfusion. Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and the portal vein was cannulated. The liver was initially perfused with 150 ml perfusion medium at a rate of 12 ml/min with excess medium draining from the severed vena cava. This was followed immediately by perfusion with 100 ml of liver digestion medium at a rate of 12 ml/min. The liver was transferred carefully to a culture dish containing hepatocyte wash medium and gently scraped to separate cells. The resulting liver digest was passed through sterile gauze and spun briefly at 50xg to pellet hepatocytes. The

resulting pellet was washed two additional times with 50 ml volumes of hepatocyte wash medium. Hepatocytes were then resuspended at a density of 2.5 X 10^5 cells/ml in Williams' Medium E (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and plated in 12-well cell culture plates (Corning-Costar) at 0.80 ml/well. Cells were allowed to attach for 4-5 h before treatment.

2.3.7 Polymorphonuclear leukocyte (PMN) isolation and conditioned medium (CM) preparation

Rat PMNs were isolated by glycogen elicitation, as described previously (Ganey et al., 1994). Washed PMNs were suspended at a concentration of 2.5 X 10^6 cells/ml in Williams' Medium E. PMN-conditioned medium (PMN-CM) was prepared by treating PMNs with cytochalasin B (final concentration, 5 μg/ml), then 1 minute later with the PMN activator f-Met-Leu-Phe (fMLP) at a concentration of 100nM. PMNs were then incubated at 37°C for 30 min. They were removed by centrifugation and the supernatant (PMN-CM) collected. Aliquots were stored at –80° C until use.

2.3.8 Effect of RAN and FAM on PMN-CM induced cytotoxicity

Serum free Williams' Medium E containing various concentrations of PMN-CM (0, 25 or 50%) and either RAN (0, 175, 526, or 877 μg/ml) or FAM (0, 35, 105.2, or 175.4 μg/ml) was added after hepatocytes were attached (4-5h). After 16 h, the medium was collected, and the remaining attached cells were lysed with 1% triton X-100 followed by brief sonication. Media and lysates were centrifuged at 600xg for 5 min. Cytotoxicity was assessed by measuring ALT release into the medium using Sigma Diagnostics

Infinity ALT reagent (Sigma, St. Louis, MO). ALT activity in the medium was expressed as a percent of total ALT activity (i.e., medium activity plus lysate activity).

2.3.9 Statistical Analyses

Results are presented as mean ± SEM. For studies *in vivo*, one-way or two-way analysis of variance (ANOVA) was utilized as appropriate. Histopathology scores were compared using a rank sum test. For hepatocyte studies *in vitro*, one- or two-way repeated measures ANOVA was applied after appropriate data transformation. All individual group comparisons were made using Tukey's test. The criterion for significance was $p<0.05$ for all studies.

2.4 Results

2.4.1 Dose-ranging studies.

Rats were given LPS (44.4 X 10^6 EU/kg) 2 h before various doses of RAN (0, 10, 20, 25 or 30 mg/kg). Serum ALT activity was evaluated 24 h after RAN administration. A statistically significant increase in ALT activity was not observed in rats cotreated with LPS and RAN at doses of less than or equal to 25 mg/kg, whereas ALT activity was significantly increased in animals cotreated with LPS and 30 mg/kg RAN (Figure 2.1A). No dose of RAN alone caused a significant increase in ALT activity, and doses above 30 mg/kg resulted in significant mortality (data not shown). A similar dose-response study was performed for LPS at 30 mg/kg RAN (Figure 2.1B). Rats were given various doses LPS (0, 7.4, 14.8, 22.2 or 44.4 X 10^6 EU/kg) 2 h before 30 mg/kg RAN and ALT activity

was evaluated 24 h after RAN. A significant increase in ALT activity was observed in animals cotreated with RAN and an LPS dose as low as 14.8 X 10^6 EU/kg. Based on these results, doses of RAN and LPS were selected to be 30 mg/kg and 44.4 X 10^6 EU/kg, respectively, for the remaining studies.

2.4.2 Development of hepatotoxicity after LPS/RAN cotreatment.

Rats were given LPS or its vehicle 2 h before RAN or its vehicle. RAN or LPS given alone was without significant effect on ALT (Figure 2.2A) activity compared to control at any time evaluated. Treatment with RAN caused a slight but statistically significant increase in AST activity at 12 h but was without effect at other times (Figure 2.2B). Since AST is not specific for liver injury and RAN caused no change in serum ALT activity or liver histopathologic change (see below), the small increase in AST activity likely arose from an extrahepatic source. Cotreatment of rats with LPS/RAN resulted in a 6-10-fold increase in ALT (Figure 2.2A) and a 7-14-fold increase in AST (Figure 2.2B) activities that were significant as early as 6 h after RAN treatment and remained elevated through 24 h. Biliary injury, as reflected in serum GGT activity, increased by 6 h after administration of LPS/RAN and remained elevated by at least 1.5-fold through 24 h. RAN or LPS treatment alone had no effect (Figure 2.2C).

2.4.3 Histopathological examination.

Acute, multifocal, midzonal hepatic necrosis developed in LPS/RAN-cotreated rats as early as 3 h and increasing in severity and incidence through 24 h (Figure 2.3). Necrotic foci varied in size and number and were characterized by hepatocellular cytoplasmic

Figure 2.1: Dose-ranging studies with RAN and LPS. (A) Rats were treated with 44.4 X 10^6 EU/kg LPS, (iv), then two hours later with various doses of RAN (0, 10, 20, 25 or 30 mg/kg) (iv). n= 3-10 animals per group. (B) Rats were treated with various doses of LPS (0, 7.4, 14.8, 22.2 or 44.4 X 10^6 EU/kg), (iv), then two hours later with 30 mg/kg RAN, (iv). n= 4-7 animals per group. Hepatic parenchymal cell injury was estimated 24 h after RAN administration from increases in serum ALT activity. Data are expressed as mean ± SEM. *Significantly different from respective control group ($p<0.05$).

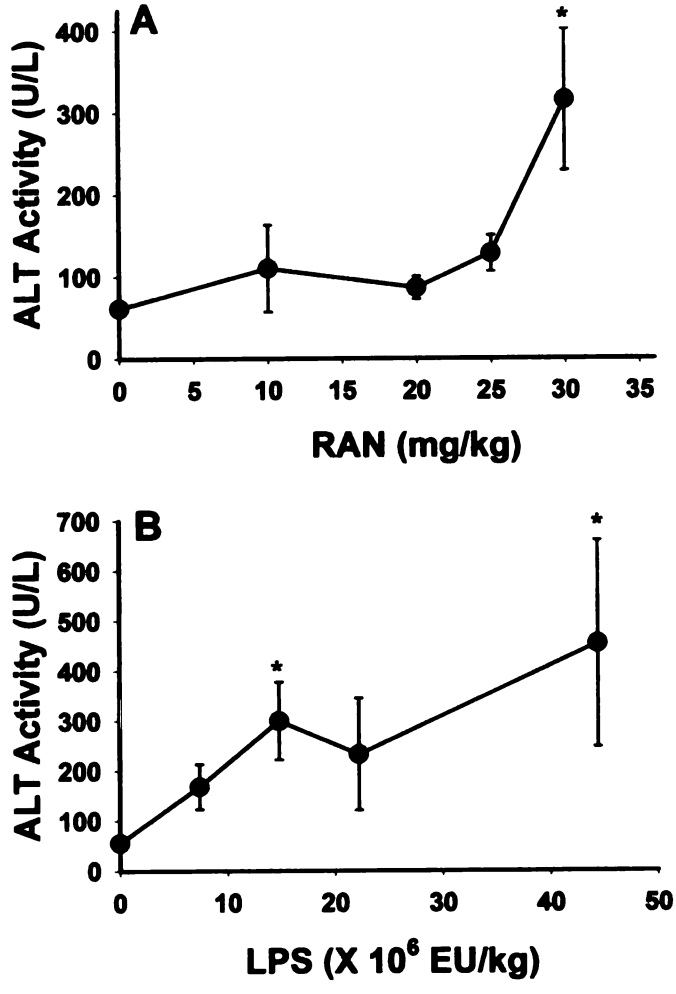

A
ALT Activity (U/L)
400
300
200
100
0
0
5
10
15
20
25
30
35
RAN (mg/kg)
*
B
ALT Activity (U/L)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
LPS (X 10^6 EU/kg)
*
*

Figure 2.2: Hepatotoxicity from LPS/RAN cotreatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Hepatic parenchymal cell injury was estimated 3, 6, 12 or 24 h after RAN administration by increases in serum (A) ALT and (B) AST activities. Cholestatic injury was estimated from increases in serum (C) GGT activity. n= 6-17 rats per group. Data are expressed as mean ± SEM. *Significantly different from all other groups at the same time. #Significantly different from Veh/Veh-treated rats at that time ($p<0.05$).

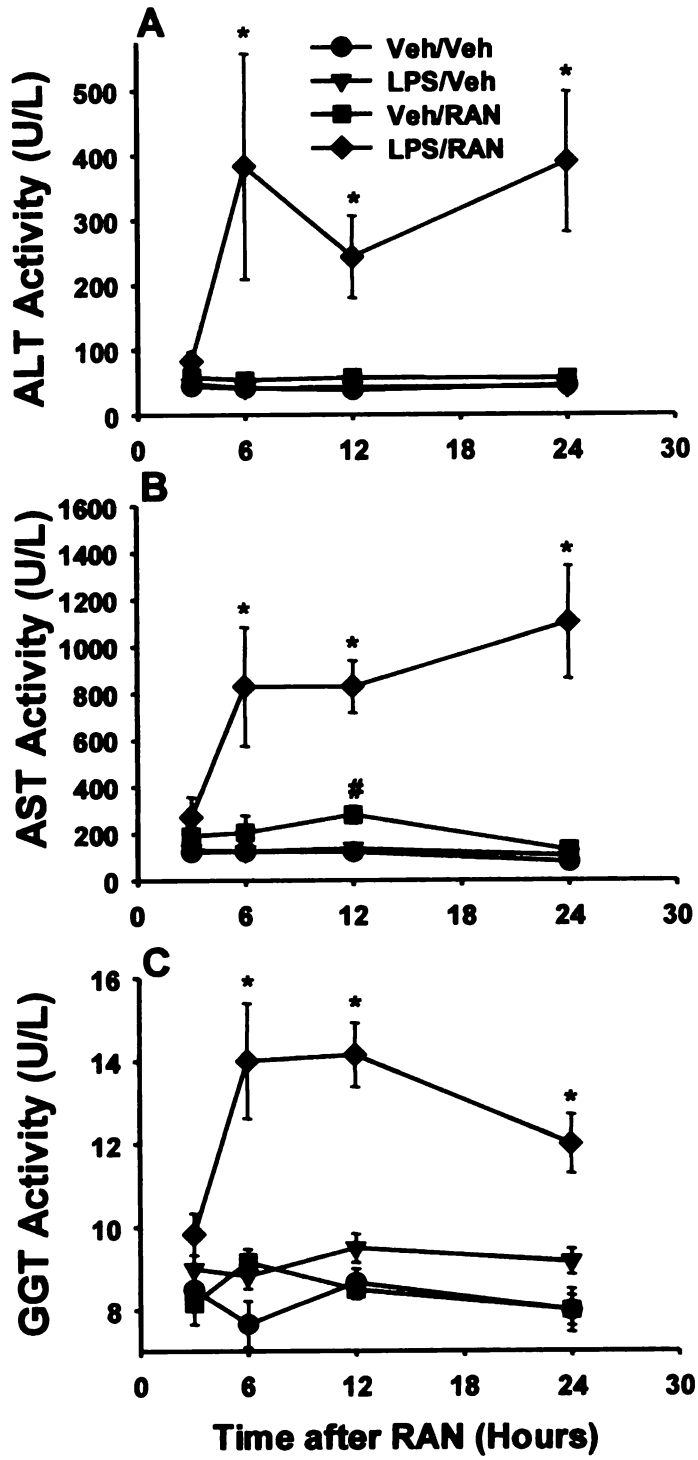
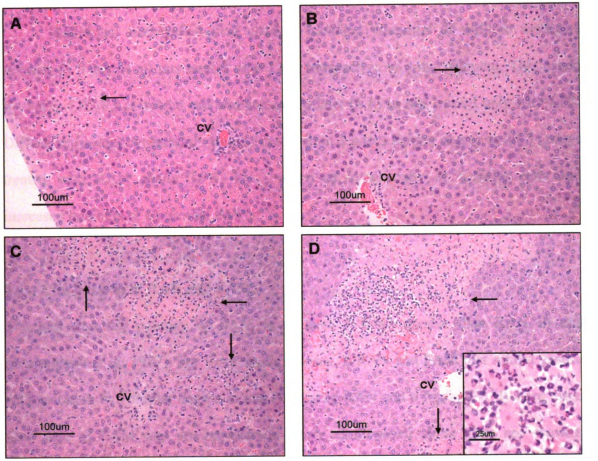

A
Veh/Veh
LPS/Veh
Veh/RAN
LPS/RAN
ALT Activity (U/L)
0
100
200
300
400
500
0
6
12
18
24
30
B
AST Activity (U/L)
0
200
400
600
800
1000
1200
1400
1600
C
GGT Activity (U/L)
8
10
12
14
16
Time after RAN (Hours)

Figure 2.3: Representative photomicrographs of liver after LPS/RAN cotreatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Livers were removed 3 (A), 6 (B), 12 (C) or 24 h (D) after RAN administration, fixed in 10% neutral-buffered formalin, stained for H&E, and examined by light microscopy. Acute, multifocal, necropurulent hepatitis (arrows) was present at each time period, and increased in severity and frequency from 3 to 24 h. The inset in panel D shows infiltrating PMNs, many of which are necrotic themselves. CV indicates central vein. Images kindly provided by Dr. Gary Cockerell.


A
CV
100um
B
CV
100um
C
CV
100um
D
CV
100um
25um

eosinophilia and nuclear pyknosis. Variable numbers of infiltrating PMNs, many of which were necrotic, were consistently present within hepatocellular necrotic foci. Qualitatively similar lesions developed in the same time frame in LPS/Veh-treated rats, but with much less severity and frequency as compared to LPS/RAN-cotreated rats. This lesion was not present in any other treatment group. Table 2.1 presents a summary of the severity of the liver lesion in rats treated with LPS and or RAN over 24 h.

Additional histopathological changes included vasculitis of mild to moderate severity in livers of all rats treated with LPS, irrespective of drug treatment. This began by 3 h in LPS/RAN-treated rats and somewhat later (6 h) in rats treated only with LPS. Diffuse sinusoidal hypercellularity occurred within 3 h to a similar degree in all 3 groups treated with RAN and/or LPS. This comprised hypertrophy of Kupffer cells and increased numbers of sinusoidal PMNs.

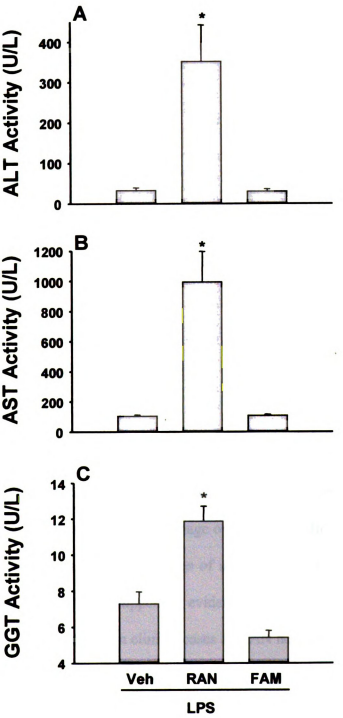
2.4.4 Comparison of RAN and FAM.

The anti-secretory potency of FAM is at least 5 times greater than RAN in rats (Scarpignato *et al,* 1987). For these studies, doses of RAN and FAM that inhibit gastric acid secretion to a similar degree in rats (30 mg/kg RAN and 6 mg/kg FAM) were used. This dose of FAM was not hepatotoxic when administered alone (data not shown). Significant increases in markers of hepatic parenchymal cell injury were not observed in animals cotreated with LPS/FAM after 24 h, whereas marked elevations were observed in animals given LPS/RAN (Figure 2.4A and 2.4B). Similar results were observed for GGT activity (Figure 2.4C). Histopathologic evaluation revealed midzonal hepatocellular necrosis in livers of rats treated with LPS/RAN, but this was absent in LPS/FAM-treated

Table 2.1: Midzonal hepatic necrosis after LPS/RAN cotreatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Livers were removed 3, 6, 12 or 24 h after RAN treatment, fixed in 10% neutral buffered formalin, and evaluated by light microscopy. Lesions characterized by midzonal hepatocellular necrosis were assigned a histopathology score based on the following scale: 0, no significant lesion; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. n= 6-17 rats per group. Data are expressed as mean and range of scores for each group. *Significantly different from Veh/Veh-treated rats at that time ($p<0.05$). Livers were evaluated and scored by Dr. Gary Cockerell.

Treatment	Time after RAN (h)			
	3	6	12	24
Veh/Veh	0	0	0	0
LPS/Veh	0	0.2 (0-1)	0.3 (0-2)	0.5 (0-2)
Veh/RAN	0	0	0	0
LPS/RAN	0.2 (0-1)	0.7 (0-3)	1.6 (0-3)*	2.0 (0-4)*

Figure 2.4: Comparison of LPS/RAN and LPS/FAM cotreatments. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with either 30 mg/kg RAN, a pharmacologically equipotent dose of FAM (6 mg/kg) or vehicle (iv). Hepatic parenchymal cell injury was estimated 24 h after H2 antagonist administration as increases in serum (A) ALT and (B) AST activities. Cholestatic injury was estimated as increases in serum (C) GGT activity. Data are expressed as mean ± SEM. n=5-21 rats per group. *Significantly different from LPS/Veh-treated rats ($p<0.05$).


A
ALT Activity (U/L)
400
300
200
100
0
*
B
AST Activity (U/L)
1200
1000
800
600
400
200
0
*
C
GGT Activity (U/L)
14
12
10
8
6
4
*
Veh
RAN
FAM
LPS

rats. Compared to rats treated with LPS alone, no significant increase in ALT activity was observed in rats cotreated with LPS and a dose of FAM that was equimolar to that of RAN (data not shown).

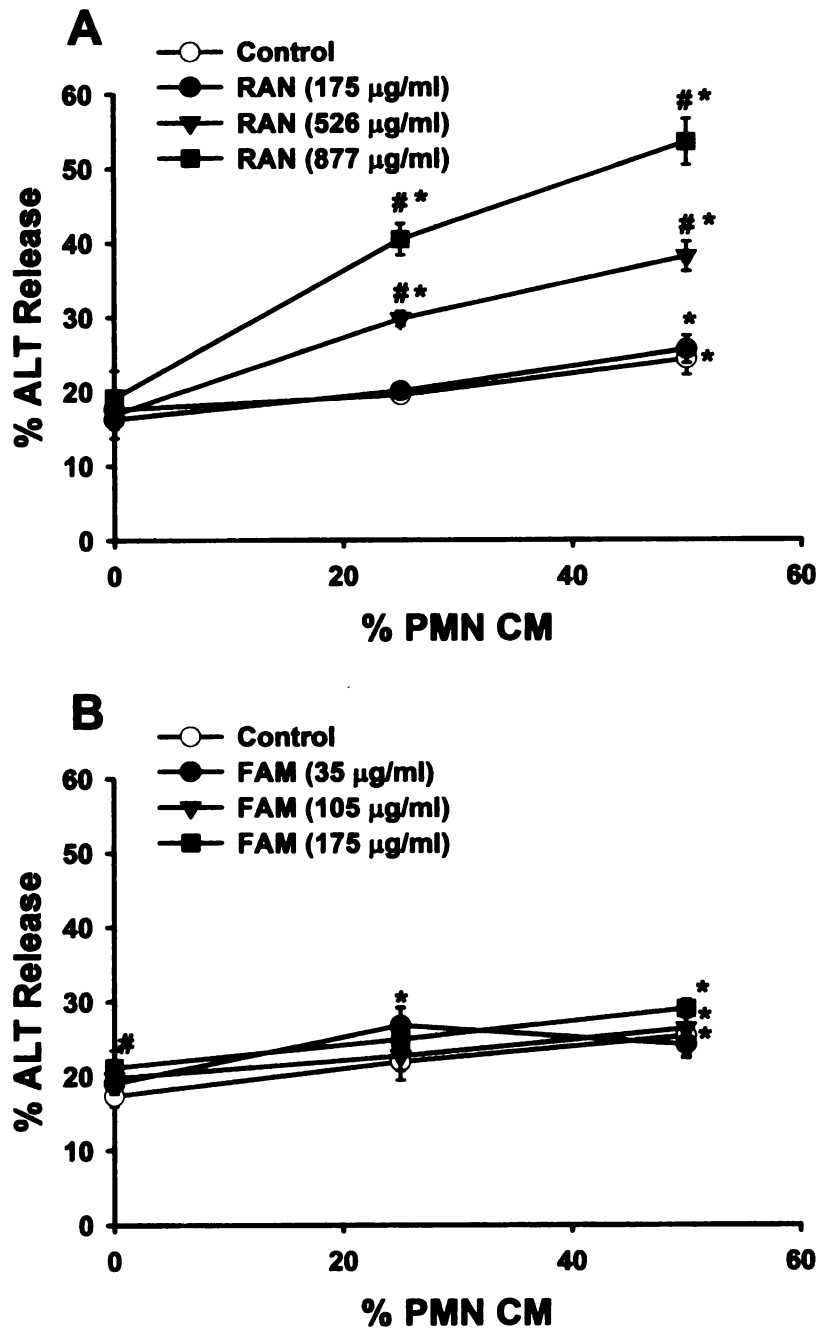
2.4.5 Effect of RAN on killing of hepatocytes by PMN-CM.

Cytotoxicity was evaluated by release of ALT into culture medium after 16 h of incubation with RAN/PMN-CM. PMN-CM alone caused a concentration-dependent increase in ALT release (Figure 2.5), as reported previously (Ho *et al,* 1996). By itself, RAN did not cause significant ALT release at any concentration used (Figure 2.5A). However, it enhanced the hepatocellular killing activity of PMN-CM in a concentration-dependent manner. Pharmacologically equipotent concentrations of FAM were tested for comparison. FAM alone at the largest concentration used caused a very slight, but statistically significant increase in ALT release (Figure 2.5B). The cytotoxicity of PMN-CM was unaffected by FAM at any concentration of the drug tested.

2.5 Discussion

It is widely accepted that idiosyncratic drug reactions arise from production of reactive drug metabolites capable of causing tissue damage or from a specific immune response to drug or drug metabolite haptens. These modes of action have been proposed for RAN idiosyncrasy (Vial et al., 1991), but supporting evidence is lacking, and neither appears to explain easily all features observed in clinical cases of RAN hepatotoxicity. For example, time of onset of hepatotoxicity relative to the initiation of RAN therapy varies greatly: some episodes appear as early as one week, whereas others do not occur until months

Figure 2.5: Effect of RAN and FAM on killing of hepatocytes by PMN-CM. Rat hepatocytes were cultured at a cell density of 2.5 X 10^5 cells/ml in Williams' Medium E containing 10% FBS. Four h later the medium was changed to serum-free medium containing conditioned medium from activated PMNs (PMN-CM) at a concentration of 0, 25 or 50%, and either (A) RAN or (B) FAM at the concentrations indicated. Cytotoxicity was evaluated as ALT released into culture medium 16 h later. Data are expressed as mean ± SEM. For RAN treatment, n=6 separate hepatocyte isolations. For FAM treatment, n=3 separate hepatocyte isolations. *Significantly different from respective treatment in the absence of PMN-CM. # Significantly different from the value at the same % PMN-CM in absence of drug. ($p<0.05$).


A
Control
RAN (175 μg/ml)
RAN (526 μg/ml)
RAN (877 μg/ml)
% ALT Release
% PMN CM
B
Control
FAM (35 μg/ml)
FAM (105 μg/ml)
FAM (175 μg/ml)
% ALT Release
% PMN CM

after the start of maintenance therapy (Ramrakhiani et al., 1998;Hiesse et al., 1985; Halparin, 1984). Furthermore, elevations in markers of hepatocellular damage resolve despite continued RAN therapy (Barr and Piper, 1981), which seems unlikely to occur if accumulation of reactive metabolites is necessary. A role for a specific immune response in RAN idiosyncrasy is equally unsupported. Clinical signs of allergic responses such as eosinophilia have been observed in some cases (Souza Lima, 1984; Devuyst et al., 1993) but is not a consistent finding (Hiesse et al., 1985; Barr and Piper, 1981). Anti-RAN antibodies have not been identified. Additionally, an autoimmune component of RAN idiosyncrasy has not been identified conclusively. Of 14 cases for which the presence of autoantibodies was assessed, only one described the presence of anti-smooth muscle antibodies, albeit at a very low concentration (Barr and Piper, 1981). Additionally, rechallenge with RAN does not always result in a recurrence of toxicity (Graham et al., 1985), as might be expected with drug allergy. Thus, current hypotheses regarding mechanisms of RAN-induced liver injury are not consistent with all of the clinical features of these reactions.

Interestingly, prodromal indicators consistent with inflammation and endotoxemia are observed in many cases of RAN idiosyncrasy. Evaluation of 34 cases of RAN-related liver injury revealed accounts of diarrhea, fever, nausea/vomiting and/or abdominal pain in nearly 60% of the cases. Exposure of people to inflammagens such as LPS is episodic and commonplace (Roth et al., 1997). Indeed, health care providers have noted associations between transient illness characterized by signs consistent with inflammation/endotoxemia and increases in liver enzymes in serum (Barr and Piper, 1981; Halparin, 1984). In addition, factors known to cause translocation of endogenous

LPS across the gastrointestinal lumen such as excessive alcohol consumption and surgery have been noted in some cases of RAN idiosyncrasy (Halparin, 1984; Hiesse et al., 1985). Thus, although no definitive studies in humans have been reported, it appears that hepatotoxic responses to RAN are often accompanied by signs consistent with inflammation.

Modest inflammation can markedly increase sensitivity to hepatotoxic effects of xenobiotic agents (Ganey and Roth, 2001; Luyendyk et al., 2002). Thus, it is likely that the liver may emerge as a target organ if an inflammatory episode occurs during RAN treatment. The studies presented herein showed that a normally nonhepatotoxic dose of RAN is rendered hepatotoxic when administered to rats undergoing acute inflammation triggered by LPS. Rats cotreated with LPS/RAN showed a larger change in serum markers of parenchymal cell injury (e.g., ALT, AST) as compared to cholestasis (e.g., GGT). These data are consistent with observations made in clinical cases of human RAN idiosyncrasy, in which increases in serum markers of hepatocellular injury were usually greater than markers of cholestatic injury. Thus, the nature of alterations in serum markers of liver damage after treatment of rats with LPS/RAN is similar to RAN idiosyncrasy in people. It should be borne in mind, however, that this model involves acute administration of RAN and may represent one of several mechanisms of idiosyncratic liver injury. Case reports of severe RAN idiosyncrasy describe marked acute inflammatory changes such as intra-acinar accumulation of plasma cells, macrophages, eosinophils and PMNs, accompanied by bridging hepatocellular necrosis (Lauritsen et al., 1984; Ribeiro et al., 2000). Consistent with the elevated serum ALT and AST activities, lesions in LPS/RAN-treated rats were characterized by acute midzonal

hepatocellular necrosis accompanied by large numbers of infiltrating PMNs. The elevation in GGT activity did not have an obvious histological correlate. Although several confounding factors impinge on comparing lesions observed in severe cases of RAN idiosyncrasy in humans and LPS/RAN-treated rats (e.g., time of liver sampling), similar features such as marked inflammatory cell infiltrates and severe hepatocellular necrosis are found in both.

To our knowledge, there have only been 3 published reports linking FAM administration to hepatotoxicity as compared to the 34 published reports for RAN. One report described a hepatotoxic response that occurred greater than 2 months after FAM therapy was discontinued, leading the authors to question whether FAM was responsible (Jimenez-Saenz et al., 2000). The link between FAM and idiosyncratic hepatotoxicity in another case was confounded by earlier RAN treatment, and the authors noted that a RAN contribution could not be ruled out (Ament et al., 1994). FAM-associated hepatotoxicity has been observed in a third patient who also developed hepatotoxicity after treatment with the H2-antagonist (CIM) cimetidine (Hashimoto et al., 1994), suggesting a general sensitivity to H2-receptor antagonists. Accordingly, FAM has been associated with few published reports of idiosyncratic hepatotoxicity, and in those cases the contribution of FAM to hepatotoxic responses described was not clear. Thus, unlike RAN, FAM has little propensity to cause idiosyncratic hepatotoxicity.

We tested the hypothesis that FAM would not have the same hepatotoxic interaction with LPS that occurs with RAN. For people the recommended dose of FAM is less than that for RAN, since FAM is a more potent H2 receptor blocker (Lin, 1991). Accordingly, pharmacologically equipotent doses of the two drugs were selected based

on their relative antisecretory effect and pharmacologic potencies in rats (Scarpignato et al., 1987). Cotreatment of rats with LPS and RAN resulted in the expected hepatocellular damage as marked by increases in serum ALT and AST activities, whereas cotreatment of rats with FAM and LPS was without significant effect. Furthermore, LPS/FAM did not cause an elevation in GGT activity. These results suggest that the ability of inflammation to cause a drug in this class to produce liver injury may be selective for those drugs that have a propensity to cause idiosyncratic reactions in humans.

Rats treated with large doses of LPS develop acute liver injury characterized by midzonal necrosis accompanied by Kupffer cell swelling and marked PMN accumulation (Hewett et al., 1992). Liver lesions resulting from chemical-LPS synergy can resemble those produced by hepatotoxic doses of the chemical or LPS or both (Barton et al., 2000b; Yee et al., 2000). LPS/RAN treatment caused an acute, midzonal, suppurative, necrotizing hepatitis that resembled lesions in animals treated with a hepatotoxic dose of LPS. This result suggests that RAN may increase hepatic parenchymal cell sensitivity to an LPS-like hepatotoxic response. In livers of rats treated with LPS/RAN, inflammatory infiltrates comprised predominately PMNs, suggesting the possibility of a role for these cells in LPS/RAN liver injury. In other models of interaction between xenobiotic agents and LPS, PMNs are present in the liver lesions and contribute to the hepatotoxic response (Barton et al., 2000a; Yee et al., 2003c). PMNs are also critically involved in the hepatotoxic response to large, toxic doses of LPS and probably act through the release of cytotoxic factors when these cells are activated (Hewett et al., 1992; Ho et al., 1996).

The exact role of PMNs in LPS/RAN liver injury has not been evaluated. Interestingly, RAN attenuates liver injury after ischemia-reperfusion, probably by

inhibiting release of cytotoxic factors by PMNs (Okajima et al., 2002). Previous studies demonstrated that RAN was nontoxic to hepatocytes even at high (e.g., 5mM) concentrations (Zimmerman et al., 1986), and our results confirmed these previous findings (Figure 2.5). However, hepatocytes treated with RAN were rendered more sensitive to killing by cytotoxic factors released by activated PMNs. In the context of observations in LPS/RAN-treated rats, these results suggest that RAN may act by increasing hepatocellular sensitivity to PMN-derived factors. In contrast, FAM did not increase the sensitivity of hepatocytes to killing by PMN-derived cytotoxic factors. Since pharmacologically equipotent concentrations were used, this suggests that the sensitizing effect of RAN on hepatocytes is independent of H2 receptor blockade. Further studies are needed to understand the mechanism by which RAN alters hepatocyte sensitivity to PMN-derived products.

In summary, RAN was rendered hepatotoxic in rats undergoing a mild inflammatory response triggered by LPS. LPS/RAN-cotreated animals developed midzonal necrosuppurative hepatitis, and a liver-related clinical chemistry pattern resembling human cases of RAN idiosyncrasy. In contrast, animals cotreated with LPS and a pharmacologically equipotent or equimolar dose of FAM did not develop liver damage, a result consistent with the far lesser (and debatable) propensity of FAM to cause idiosyncrasy in people. Treatment of hepatocytes *in vitro* with RAN, but not with FAM, increased hepatocellular sensitivity to cytotoxicity from PMN-derived factors. Overall, our demonstration that modest inflammation causes the emergence of liver as a target for RAN toxicity in rats suggests a role for inflammation in idiosyncratic reactions to this H2-antagonist. In addition, the results raise the possibility of developing animal

and cell-based models for predicting which drug candidates are more or less likely to cause idiosyncratic reactions in people and for studying the underlying mechanisms by which these reactions occur.

CHAPTER 3

Luyendyk, J.P., Mattes, W.B., Burgoon, L.D., Zacharewski, T.R., Maddox, J.F., Cosma, G.N., Ganey, P.E., and Roth, R.A. (2004). Gene expression analysis points to hemostasis in livers of rats cotreated with lipopolysaccharide and ranitidine. Toxicol Sci. **80**:203-13.

3.1 Abstract

Studies in rats have demonstrated that modest underlying inflammation can precipitate idiosyncrasy-like liver injury from the histamine 2-receptor antagonist ranitidine (RAN). Coadministration to rats of nonhepatotoxic doses of RAN and the inflammagen, bacterial lipopolysaccharide (LPS), results in hepatocellular injury. We tested the hypothesis that hepatic gene expression changes could distinguish Vehicle-, LPS-, RAN- and LPS/RAN-treated rats before the onset of significant liver injury in LPS/RAN-treated rats (i.e., 3 h post-treatment). Rats were treated with LPS (44 x 10^6 EU/kg, iv) or its vehicle, then two hours later with RAN (30 mg/kg, iv) or its vehicle. They were killed 3 h after RAN treatment, and liver samples were taken for evaluation of liver injury and RNA isolation. Hepatic parenchymal cell injury, as estimated by increases in serum alanine aminotransferase (ALT) activity, was not significant at this time. Hierarchical clustering of gene expression data from Affymetrix U34A rat genome arrays grouped animals according to treatment. Relative to treatment with vehicle alone, treatment with RAN and/or LPS altered hepatic expression of numerous genes, including ones encoding for products involved in inflammation, hypoxia, and cell death. Some of them were enhanced synergistically by LPS/RAN cotreatment. Real-time PCR confirmed robust changes in expression of B-cell translocation gene 2, early growth response-1, and plasminogen activator inhibitor-1 (PAI-1) in cotreated rats. The increase in PAI-1 mRNA was reflected in an increase in serum PAI-1 protein concentration in LPS/RAN-treated rats. Consistent with the antifibrinolytic activity of PAI-1, significant fibrin deposition occurred only in livers of LPS/RAN-treated rats. The results suggest the possibility that

expression of PAI-1 promotes fibrin deposition in liver sinusoids of LPS/RAN-treated rats and are consistent with the development of local ischemia and consequent tissue hypoxia.

3.2 Introduction

LPS recognition by toll-like receptors on Kupffer cells and other inflammatory cells activates signal transduction pathways, leading to cell activation and elaboration of inflammatory mediators (Beutler, 2002). An important component of LPS activity is transcriptional activation of numerous genes (Gao et al., 2002) in macrophages and other inflammatory cells. Many of these gene products, such as tumor necrosis factor-α (TNF), can further activate transcription of other cytokines, adhesion molecules, and neutrophil (PMN) chemokines in other cell types, such as endothelial cells (Zhao et al., 2003). Increased TNF-α mRNA can be detected in livers of rats treated with LPS, and serum TNF-α concentration is markedly increased after LPS exposure (Barton et al., 2001) (Hewett et al., 1993). Interestingly, TNF-α is important for liver injury from large doses of LPS in a mechanism dependent on PMNs (Hewett et al., 1993). Thus, enhanced expression of certain genes after LPS exposure is important for liver injury, and understanding these changes could help to elucidate mechanisms of inflammatory tissue injury.

Investigation of gene expression patterns might also identify mechanisms of pathogenesis in models in which modest, noninjurious inflammation potentiates xenobiotic-induced liver injury. For example, cotreatment of rats with a nonhepatotoxic

dose of LPS potentiates allyl alcohol (AA)-induced liver injury and results in greater expression of hepatic cyclooxygenase-2 (COX-2) compared to treatment with either agent alone (Ganey et al., 2001). In this model, COX-2 inhibition afforded partial protection from liver injury, suggesting that augmented COX-2 gene expression is important for AA/LPS-induced liver injury (Ganey et al., 2001). In rats cotreated with nonhepatotoxic doses of aflatoxin B_1 (AFB_1) and LPS, TNF-α mRNA is increased in liver to a level similar to rats treated with LPS alone. However, the serum concentration of TNF-α is significantly greater in AFB_1/LPS-treated rats, and this cytokine is causally involved in the potentiation of hepatocellular injury (Barton et al., 2001). Thus, in other models of LPS-potentiation, a difference in magnitude of gene expression in LPS and LPS/xenobiotic-treated rats may or may not be sufficient to cause liver injury, and post-transcriptional increases in protein or interaction between two genes expressed at otherwise noninjurious levels might contribute to liver injury. The use of gene array technology can facilitate investigation of such interactions. For example, in rats treated with galactosamine and LPS, gene arrays were used to identify changes in gene expression related to inflammation and oxidative stress, among others (Li et al., 2003).

The purpose of this study was to test the hypothesis that hepatic gene expression changes could distinguish rats treated with LPS, RAN or LPS/RAN before the onset of significant liver injury in LPS/RAN-treated rats. Genes were segregated based on their patterns of expression and classified based on the nature of their respective gene products. Real-time PCR and ELISA were used to confirm enhanced expression of one of these genes, plasminogen activator inhibitor-1 (PAI-1), and hepatic fibrin deposition was

evaluated to determine if the enhanced PAI-1 expression was associated with tissue fibrin deposition as a functional consequence.

3.3 Materials and Methods

3.3.1. Materials

For information on this topic please refer to Chapter 2 Materials and Methods.

3.3.2 Animals

For information on this topic please refer to Chapter 2 Materials and Methods.

3.3.3 Experimental protocol

Rats fasted for 24 hours were given 44.4 X 10^6 EU/kg LPS or its saline vehicle, iv Two hours later 30 mg/kg RAN or sterile phosphate-buffered saline (PBS) vehicle was administered iv. RAN solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. Accordingly, rats were treated with either saline and PBS (Veh group), LPS and PBS (LPS group), saline and RAN (RAN group), or LPS and RAN (LPS/RAN group). Three hours later, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and killed by exsanguination. Blood was allowed to clot at room temperature, and serum was collected and stored at -80° C until use. Slices (3-4 mm) of the left lateral liver lobe were collected and fixed in 10% neutral buffered formalin. Three 100 mg midlobe pieces of the right medial lobe were flash frozen in liquid nitrogen for RNA

isolation. Groups treated with either Veh, LPS, or RAN contained 3 rats, whereas 4 rats comprised the LPS/RAN group.

3.3.4 Hepatotoxicity assessment

Sinusoidal endothelial cell (SEC) function was estimated using a commercially available, enzyme-linked immunosorbent assay (ELISA) for hyaluronic acid (Corgenix Medical Corporation, Westminster, CO). Otherwise, for more information on this section please refer to Chapter 2 Materials and Methods.

3.3.5 RNA isolation

Total RNA was isolated from snap-frozen liver samples (approximately 100 mg) in accordance with protocols recommended by Affymetrix Inc. (Santa Clara, CA) for GeneChip experiments. Total RNA was isolated with Trizol reagent (Invitrogen Corp, Carlsbad, CA) according to manufacturer's instructions. Samples were subsequently passed over a Qiagen RNeasy column (Qiagen Corp., Valencia, CA) for further purification. RNA quality and concentration were assessed by absorbance at 280 and 260 nm and by analysis with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

3.3.6 Affymetrix GeneChip® analysis

RNA isolated from each rat was processed and analyzed as described below using separate Affymetrix RG_ U34A arrays. Synthesis of double-stranded cDNA from total RNA, synthesis of biotin-labeled cRNA, fragmentation of the cRNA for target preparation, eukaryotic target hybridization, washing, staining and scanning of the

RG_U34A arrays were carried out according to the Affymetrix GeneChip® Expression Analysis Technical Manual (701021 rev 1). Scan analysis was carried out with both the scaling and normalization factors set to 1. For data normalization, the array was treated as a XYZ-dimensional vector, and normalized by dividing each data point by the cartesian length (magnitude) of the vector, then multiplied by the average of the magnitudes of the XX arrays in the data set. XYZ=number of data points on the array XX=number of arrays. Normalized signal data were imported into the JMP® (Release 5.0.1.2, SAS Institute Inc.) software for principal component analysis.

3.3.7 Data analysis and determination of gene activity

To determine which probesets changed after treatment compared to Veh-treated rats, the following steps were performed. Preliminary filtering was performed using the transcript detection call as described in the Affymetrix Statistical Reference Guide. Probesets that did not have at least 2 samples in any treatment group with "present" or "marginal" detection calls were eliminated from further analysis. Assessment of gene activity was made by the Student's t-test using R software (version 1.7.0., www.r-project.org). Adjustment for multiple comparisons was made using a false discovery rate (FDR) criterion (Benjamini, 1995). The FDR provided an approach capable of decreasing, to a user-determined level, the likelihood of committing a type I error, at the same time as providing a manageable number of probesets for continued analysis. For this study, the 1000 most active probesets compared to the Veh-treated group were identified for each treatment (i.e., LPS, RAN, or LPS/RAN) by a FDR criterion with α=0.05. Each probeset that changed relative to its expression in Veh-treated rats was then

assigned to a set defined by the treatment or treatments that produced a change in its activity (i.e., LPS/RAN [LR], L, R, L∩R, LR∩L, LR∩R, LR∩L∩R, where "∩" indicates intersection of sets). The resulting sets were visualized using a Venn diagram (Figure 3.3). Student's t-test was used to compare the expression of FDR-active probesets after LPS/RAN treatment with their expression after treatment with either agent alone. Genes with greater expression in LPS/RAN-treated rats compared with either agent given alone were identified in the LR, LR∩L, LR∩R and LR∩L∩R sets (see below). Genes with similar expression in LPS/RAN- and LPS-treated rats, or in LPS/RAN- and RAN-treated rats were identified in the LR∩L and LR∩L∩R or in the LR∩R and LR∩L∩R sets, respectively. Genes expressed to a greater degree in rats treated with LPS alone as compared to rats cotreated with LPS/RAN were identified in the L and LR∩L sets.

Hierarchical clustering was performed using Spotfire Decision Site for Functional Genomics (Spotfire Inc., Somerville, MA) on all unique probesets showing a significant treatment effect. Two-way agglomerative hierarchical clustering was performed using an unweighted average and Euclidean distance as the similarity measure. Probeset annotation was completed as described previously (Mattes, 2004). A cosine correlation similarity measure in the profile search tool in Spotfire Decision Site for Functional Genomics (Spotfire Inc., Somerville, MA) was used to identify genes with patterns of expression similar to increases in serum HA concentration.

3.3.8 Reverse transcription and real-time PCR

RNA quantification was performed on the same samples from the gene array experiment using a Spectramax Microplate Spectrophotometer (Molecular Devices,

Sunnyvale, CA). Random priming was performed in a final volume of 12.5ml using 500ng of total RNA, 7.5 mM Random Hexamers (Amersham Biosciences, Piscataway, NJ), and 1mM dNTPs (Invitrogen, Carlsbad, California). RNA was denatured at 65°C for 5 minutes and chilled on ice. Reverse Transcription (RT) master mix was prepared in a final volume of 12.5 ml with a final concentration of 20 U/ml of Superscript II Rnase H- Reverse Transcriptase, 4 Units/ml RNAseOut, 2mM dithiothreitol, and 1X 1st Strand RT buffer (Invitrogen, Carlsbad, California). Denatured RNA and RT master mix were combined in total volume of 25 μl. The reverse transcriptase reaction was performed at room temperature for 10 minutes, at 42°C for 60 minutes, and then at 70°C for 15 minutes in a MJ Research Thermocycler (MJ Research Inc., Reno, NV).

The following oligonucleotide primers, designed using Oligo 6 program software (Molecular Biology Insights, Cascade, CO), were used to quantify mRNA levels of the following genes by real-time PCR analyses. Early growth response-1 (egr-1): upper primer- 5' TGA ACG CAA GAG GCA TAC CA 3'; lower primer- 5' GAG CCC GGA GAG GAG TAA GAG 3'. B-cell translocation gene-2 (btg-2): upper primer- 5' CCA GCC AGT CAC CCT TAG TG 3'; lower primer- 5' CGG GCA GAG TGT TTG GTA AGT 3'. Plasminogen activator inhibitor-1 (PAI-1): upper primer- 5' AAC CCA GGC CGA CTT CA 3'; lower primer- 5' CAT GCG GGC TGA GAC TAG AAT 3'. Ribosomal protein L19 (Rpl19): upper primer- 5' CTC GAT GCC GGA AGA ACA C 3'; lower primer- 5' CGA GCG TTG GCA GTA CCC 3'. A 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was used to analyze purities of RNA and PCR products.

Real-time PCR reactions using SYBR Green dye methodology were prepared in a final volume of 25 μl per reaction with 1 ng of cDNA and 1X SYBR Green PCR Master

Mix (PE Applied Biosystems, Foster City, CA). Primer mixture was prepared in 5 µl per reaction with a final concentration of 0.3 µM per primer. SYBR Green real-time PCR was performed using an ABI 7900 (PE Applied Biosystems, Foster City, CA). Relative amounts of target were calculated using the comparative C_T method with ribosomal protein L19 (RPL19, RefSeq Accession # NM_031103) as an endogenous reference and a calibrator consisting of RNA pooled from all livers of Veh-treated rats.

3.3.9 Immunohistochemistry

Liver samples evaluated for fibrin immunohistochemistry were from the 3 h post-treatment timepoint in a previous study (Luyendyk et al., 2003b). A $1cm^3$ block of liver cut from the left medial lobe was frozen for 8 minutes in liquid nitrogen-chilled isopentane then stored at –80°C until processing. 8 µm-thick sections of frozen liver were fixed in 10% buffered formalin containing 2% acetic acid for 30 minutes at room temperature. This fixation protocol solubilizes all fibrinogen and fibrin except for cross-linked fibrin; therefore, only cross-linked fibrin remains in sections of liver (Schnitt et al., 1993). Sections were blocked with PBS containing 10% horse serum (i.e., blocking solution; Vector Laboratories) for 30 minutes, and this was followed by incubation overnight at 4°C with goat anti-rat fibrinogen antibody diluted (1:1000, ICN Pharmaceuticals, Aurora, OH) in blocking solution. Next, sections were incubated for three hours with donkey anti-goat secondary antibody conjugated to Alexa 594 (1:1000, Molecular Probes, Eugene, OR) in blocking solution for 3 hours. Sections were washed three times, 5 minutes each, with PBS and visualized using a fluorescent microscope. No staining was observed in controls for which the primary or secondary antibody was

eliminated from the staining protocol. Liver sections from all treatment groups that were compared morphometrically were stained at the same time.

3.3.10 Quantification of fibrin staining

Fluorescent staining in sections of liver was visualized with an Olympus AX-80T microscope (Olympus, Lake Success, NY). Ten randomly chosen digital images (100X magnification) were captured using a SPOT II camera and SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI). Samples were coded such that the evaluator was not aware of treatment. Each digital image encompassed a total area of 1.4 mm^2 and contained several centrilobular and periportal regions. Quantification of immunostaining was performed with Scion Image Beta 4.0.2 (Scion Corporation, Frederick, MD) using the method described by (Copple et al., 2002a). Ten random fields analyzed for each liver section were averaged and counted as a replicate, i.e., each replicate represents a different rat.

3.3.11 Evaluation of serum PAI-1 concentration

Serum total PAI-1 concentration (i.e., inactive, active, and bound to plasminogen activator) was evaluated using a commercially available ELISA purchased from American Diagnostica Inc. (Greenwich, CT.).

3.3.12 Statistical analysis

Two-way analysis of variance with Tukey's test for multiple comparisons was used for analysis of clinical chemistry, immunohistochemistry, and ELISA. The criterion for significance was $p<0.05$.

3.4 Results

3.4.1 Development of hepatic parenchymal cell injury.

Given alone, the doses of LPS and RAN are not hepatotoxic up to 24 h after administration (Luyendyk et al., 2003b). Confirming earlier results (Luyendyk et al., 2003b), no significant change in ALT was observed in rats given RAN or LPS (Figure 3.1), and LPS/RAN cotreatment did not cause a statistically significant increase in ALT by 3 h (Figure 3.1). However, one of the LPS/RAN-cotreated animals had a serum ALT activity (454 U/L) that was considerably greater than the others (103, 119, 167 U/L).

3.4.2 Cluster analysis.

Affymetrix U34A probesets defined as active after treatment with LPS and/or RAN (see *Materials and Methods*) were subjected to hierarchical clustering. The resulting dendrogram is displayed in Figure 3.2. Four clusters resolved from this analysis, segregating animals by their specific treatment (Veh, LPS, RAN, or LPS/RAN).

Figure 3.1: Evaluation of hepatic parenchymal cell injury after LPS/RAN-cotreatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Hepatic parenchymal cell injury was estimated 3 h after RAN administration by increases in serum ALT activity. n=3 for rats given Veh/Veh, LPS/Veh, or Veh/RAN. n=4 for rats given LPS/RAN. Data are expressed as mean ± SEM. No treatment was found to be significantly different from Veh-treated rats.

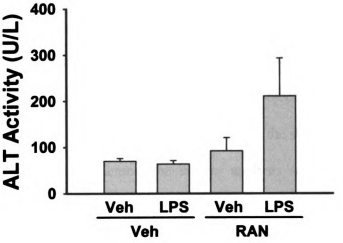
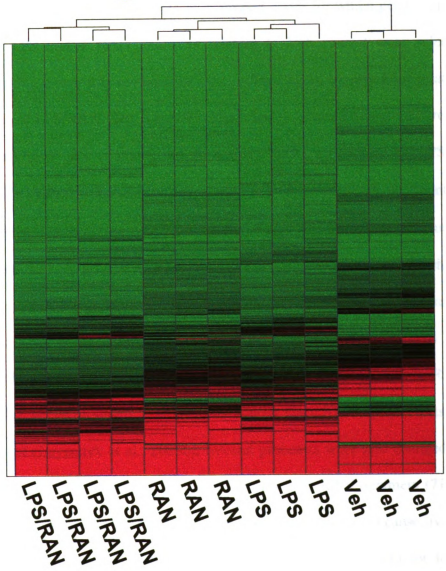

ALT Activity (U/L)
400
300
200
100
0
Veh
LPS
Veh
LPS
Veh
RAN

Figure 3.2: Hierarchical clustering of hepatic gene expression after LPS/RAN-cotreatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Three hours after RAN administration, RNA was isolated from liver, and gene expression was evaluated by Affymetrix U34A Rat Genome Array. RNA from each rat was analyzed using a separate array. Affymetrix probesets passing a false discovery rate statistical filter were subjected to hierarchical clustering using an unweighted average and Euclidean distance as the similarity measure. As the dendrogram (top of figure) indicates, the cluster analysis segregated rats by treatment.


LPS/RAN
LPS/RAN
LPS/RAN
LPS/RAN
LPS/RAN
RAN
RAN
RAN
LPS
LPS
LPS
Veh
Veh
Veh

Additionally, animals treated with LPS, RAN or LPS/RAN clustered separately from Veh-treated animals.

3.4.3 Gene expression changes after LPS, RAN, or LPS/RAN treatment.

From the population of probesets examined, those for which gene expression was altered by LPS and/or RAN treatment relative to Veh control were selected. Each probeset in this group was assigned to a set defined by its change after treatment with LPS, RAN, or LPS/RAN. Sets were also identified for those probesets altered by more than one treatment. For example, the set defined by the intersection of LPS/RAN and LPS sets (i.e., LR$\cap$L) contains probesets changed after LPS/RAN treatment and after LPS treatment. The resulting sets are summarized as a Venn diagram in Figure 3.3. The genes represented by probesets defining each set were identified and are shown along with gene symbol, Unigene identification (Rn build 117), locuslink identification, signal intensities relative to Veh treatment, and standard deviations in Supplemental Tables 1-7. Several probesets were either increased (27) or decreased (381) only in LPS/RAN-treated animals. This set of probesets is of obvious interest since liver injury results only from this treatment (Luyendyk et al., 2003b). Several probesets were also changed only after LPS treatment (163 increased; 46 decreased) or only after RAN treatment (71 increased; 20 decreased). Changes in these probesets are likely not sufficient to cause liver injury by themselves since rats treated with LPS or RAN alone at these doses do not develop liver injury (Luyendyk et al., 2003b); however, the potential exists for interaction of one of these gene products with another, resulting in liver injury. Probesets changing after LPS/RAN treatment and after either agent given alone (i.e., LR$\cap$L, LR$\cap$R) are

Figure 3.3: Venn diagram depiction of probeset activity relative to Veh/Veh-treated rats. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Three hours after RAN administration, RNA was isolated from liver and gene expression evaluated by Affymetrix U34A Rat Genome Array. The number of Affymetrix probesets increased (↑) or decreased (↓) relative to Veh-treated rats in a given treatment set is shown. Probesets with activities altered by more than one treatment are indicated by an intersection symbol (∩). LR: probesets changed only after treatment with LPS/RAN (Supplemental Table 1); L: probesets changed only after treatment with LPS (Supplemental Table 2); R: probesets changed only after treatment with RAN (Supplemental Table 3); L∩R: probesets changed after treatment both with LPS alone and with RAN alone (Supplemental Table 4); LR∩L: probesets changed after treatment both with LPS/RAN and with LPS alone (Supplemental Table 5); LR∩R: probesets changed after treatment both with LPS/RAN and with RAN alone (Supplemental Table 6); LR∩L∩R: probesets changed after treatment with LPS/RAN, LPS alone, and RAN alone (Supplemental Table 7).

potentially important, but injury would likely require a different magnitude of expression in LPS/RAN-treated rats, since liver injury only occurs in this group.

Genes with specific treatment-related expression patterns were identified in the sets described above and further categorized into groups using a secondary statistical filter as described in *Materials and Methods*. Genes with greater expression in LPS/RAN-treated rats compared with either agent given alone were identified in the LR, LR∩L, LR∩R and LR∩L∩R sets (Table 3.1). Overexpression of one or more genes in this group might be a determinant of liver injury in rats treated with LPS/RAN. In addition, groups of genes with similar expression in LPS/RAN- and LPS-treated rats (from LR∩L and LR∩L∩R sets) or in LPS/RAN- and RAN-treated rats (from LR∩R and LR∩L∩R sets) were identified. Since liver injury does not occur after treatment of rats with LPS or RAN alone, expression of these genes might be important if an interaction occurs between two or more gene products. Lastly, a group of genes expressed to a greater degree in rats treated with LPS alone as compared to rats cotreated with LPS/RAN was identified in the L and LR∩L sets. The importance of this group lies in the possibility that RAN might interfere with the upregulation by LPS of a gene that protects against liver injury.

Genes were grouped based on these 4 expression patterns, and their gene products were classified into one or more categories, including inflammation, acute phase, hypoxia-inducible, oxidative stress, cell death signaling, cell cycle control, and repair (Table 3.1). This classification revealed that several genes with greater expression in LPS/RAN-treated rats compared to other treatments were related to inflammation and/or

	Gene Product Classification							
Expression Pattern	Inflammation	Acute Phase	Hypoxia-inducible	Oxidative Stress	Cell Death Signalling	Cell Cycle Control	Repair	Unknown/ ESTs
LPS/RAN >LPS and >RAN	9	5	10	4	4	2	4	5
LPS/RAN ≈ LPS	25	6	7	3	10	5	3	34
LPS/RAN ≈ RAN	7	4	2	1	4	3	3	16
LPS/RAN < LPS	22	4	0	8	22	2	1	51

Table 3.1: Expression pattern groups and gene product classification.Genes with four different expression patterns were identified from the sets of genes shown in Figure 3 as described in Materials and Methods. Four groups were identified defined by genes expressed to a greater degree in LPS/RAN-cotreated rats compared to either agent given alone (LPS/RAN > LPS and > RAN), similar expression after treatment with LPS/RAN or LPS (LPS/RAN∩LPS), similar expression after treatment with LPS/RAN or RAN (LPS/RAN∩RAN), and genes expressed to a lesser degree after LPS/RAN cotreatment compared to treatment with LPS alone (LPS/RAN∩LPS). Genes in these groups were classified based on the relationship of their gene product to one or more functions. A gene may be classified to more than one group. Supplemental tables 8-11 list the specific gene products within each group. Genes with unknown identity or with unknown function are also shown.

were hypoxia-inducible (Table 3.1). For example, hypoxia-inducible genes, including early growth response-1 (egr-1), glucose transporter-1 (GLUT-1), insulin-like growth factor binding protein-1 (igfbp-1), and plasminogen activator inhibitor-1 (PAI-1), had greater expression after LPS/RAN treatment as compared to expression after treatment with LPS or RAN alone (Table 3.2). Furthermore, genes involved in inflammation such as the chemokine (C-X-C motif) ligand 10 (CxCl10) and the cell cycle regulator B-cell translocation gene-2 (btg-2) segregated into this group (Table 3.2).

Table 3.1 shows that groups of genes expressed to a similar degree after treatment with LPS or LPS/RAN had gene products largely related to inflammation. Likewise, several genes expressed similarly after LPS/RAN or RAN treatment fell into this group. A group of genes with attenuated expression in LPS/RAN-treated rats compared to rats treated with LPS alone was also identified. Gene products in this group were related to inflammation, cell death signaling, and oxidative stress (Table 3.1). Specific genes comprising each of these groups are available online in Supplemental Tables 8-11.

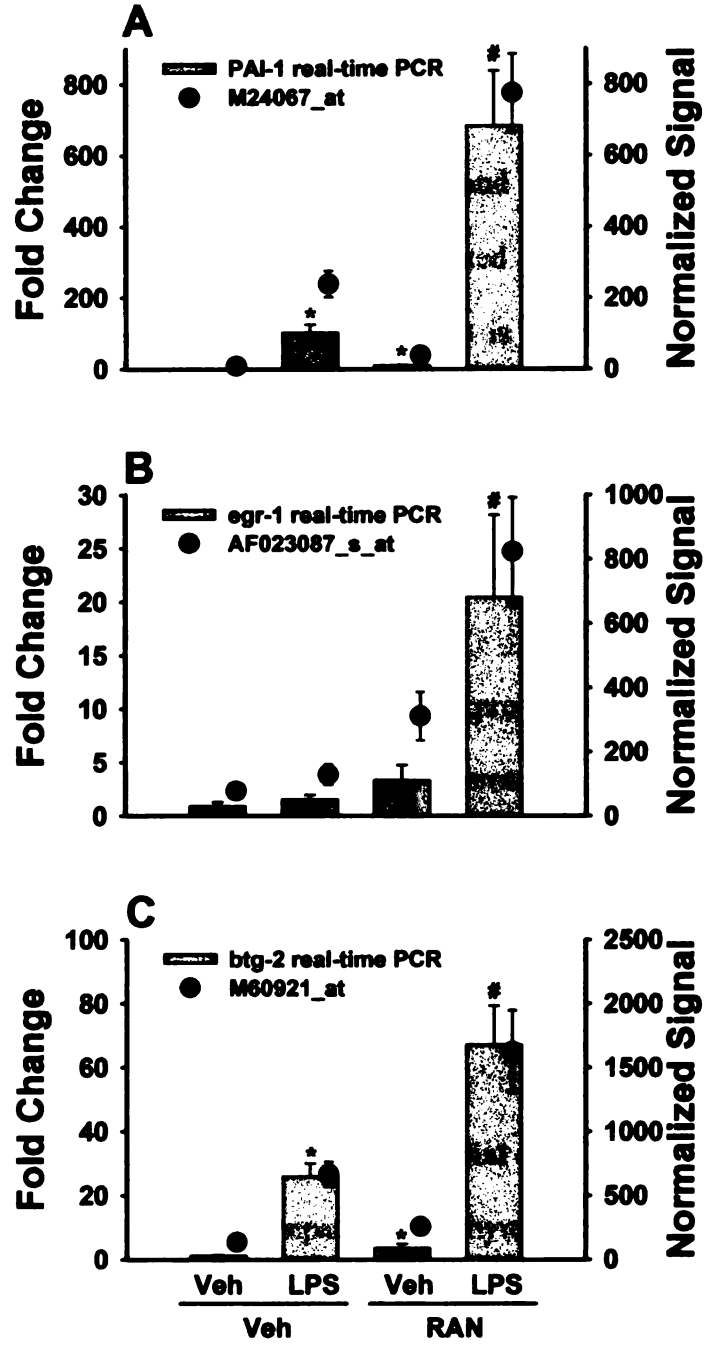
3.4.4 Real-time PCR.

Genes were selected for real-time PCR analysis based on the FDR filter results in addition to treatment comparisons using Spotfire Decision Site for Funtional Genomics. Real-time PCR was performed for three of the genes with increased expression in LPS/RAN-treated rats: PAI-1, egr-1, and btg-2. PAI-1 expression was significantly increased in LPS-treated and RAN-treated rats by 102- and 10-fold, respectively, but by nearly 700-fold in LPS/RAN-treated rats (bars, Figure 3.4A). This pattern of expression was consistent with signal intensities for the corresponding Affymetrix probeset

Gene Name	Set	Gene Product Classification						
		Inflammation	Acute Phase	Hypoxia-Inducible	Oxidative Stress	Cell Death Signalling	Cell Cycle Control	Repair
B-cell translocation gene 2	LR∩L			X	X	X	X	X
chemokine (C-X-C motif) ligand 10	LR∩L∩R	X		X				
early growth response 1	LR	X		X		X		
glucose transporter 1	LR∩L			X				
hemopexin	LR∩R	X	X		X			
hexokinase 2	LR∩L			X				
insulin-like growth factor binding protein 1	LR	X		X				
jun B proto-oncogene	LR∩R	X	X	X	X	X	X	X
macrophage galactose N-acetyl-galactosamine specific lectin	LR	X						
metallothionein	LR∩L∩R	X	X	X	X	X		X
pancreatitis-associated protein	LR∩R		X					
rat VL30 element	LR			X				
plasminogen activator inhibitor-1	LR∩L∩R	X		X				X
T-kininogen	LR∩R	X	X					

Table 3.2: Genes with greater signal in livers from LPS/RAN-treated rats compared to other treatments. Genes with greater expression in LPS/RAN-treated rats compared with expression after administration of LPS or RAN alone were identified in the LR, LR∩L, LR∩R, and LR∩L∩R sets of probesets (Figure 3) as described in Materials and Methods. Each gene in this group was classified based on the relationship of its gene product to one or more functions. Genes are listed alphabetically. Genes in this group that could not be classified or were ESTs are listed in Supplemental Table 8.

Figure 3.4: Real-time PCR confirmation of gene array data. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Three hours after RAN administration, RNA was isolated from whole liver, and SYBR Green real-time PCR was performed for (A) plasminogen activator inhibitor-1 (PAI-1), (B) early growth response-1 (egr-1), and (C) B-cell translocation gene-2 (btg-2). Ribosomal protein L19 was used as a housekeeping gene. Results are shown as fold change relative to average expression in Veh-treated rats as determined by the comparative Ct ($\Delta\Delta$Ct) method (bars). Normalized signal intensities for corresponding Affymetrix probesets are graphed for comparison (circles). Although a single probeset for btg-2 (M60921_at) is shown for comparison, all 3 probesets for this gene determined to be active by the FDR filter had a similar expression pattern (M60921_at, M60921_g_at, rc_AA944156_s_at). Data are expressed as mean ± SEM. n=3-4. *Significantly different from Veh/Veh-treated rats ($p<0.05$). #Significantly different from all other treatments ($p<0.05$).


A
PAI-1 real-time PCR
M24067_at
Fold Change
Normalized Signal
800
600
400
200
0
B
egr-1 real-time PCR
AF023087_s_at
30
25
20
15
10
5
1000
C
btg-2 real-time PCR
M60921_at
100
80
60
40
2500
2000
1500
1000
500
Veh
LPS
RAN

(circles, Figure 3.4A) identified as active by the FDR filter. For egr-1 (Figure 3.4B), a significant increase in mRNA was observed in livers from LPS/RAN-cotreated rats, but not in livers of rats treated with LPS or RAN alone. This was consistent with signal intensities for the associated Affymetrix probeset (circles) identified by the FDR as active only in LPS/RAN-cotreated rats (Figure 3.4B). Btg-2 expression was significantly increased in rats given LPS or RAN alone by 25- and 4-fold, respectively, but a much greater increase (67-fold) occurred in rats cotreated with LPS/RAN (Figure 3.4C). Although a single probeset for btg-2 (M60921_at) is shown for comparison (circles, Figure 3.4C), all 3 probesets for this gene determined to be active by the FDR filter had a similar expression pattern (M60921_at, M60921_g_at, rc_AA944156_s_at).

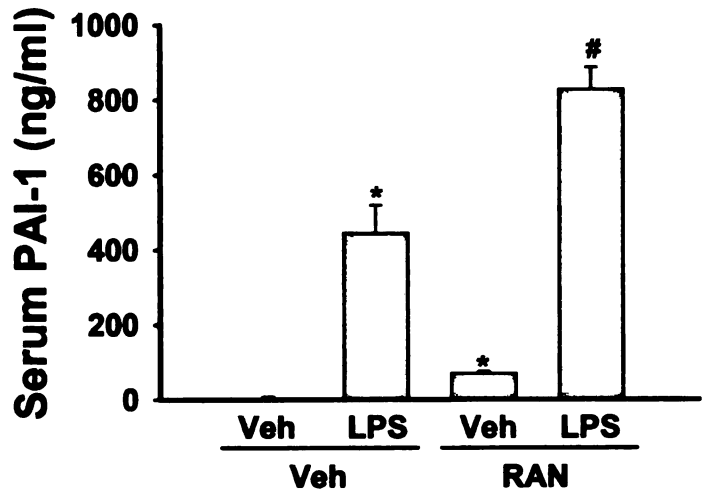
3.4.5 Evaluation of serum PAI-1.

To determine if the change in hepatic gene expression of PAI-1 resulted in altered serum concentration of PAI-1 protein, total PAI-1 protein was measured. Serum PAI-1 was significantly increased after either LPS or RAN treatment by 450- and 70-fold, respectively. Serum PAI-1 in LPS/RAN-treated rats was significantly greater than that of rats treated with either agent given alone (Figure 3.5).

3.4.6 LPS/RAN treatment and sinusoidal endothelial cells (SECs).

In addition to expression in hepatic parenchymal cells, PAI-1 is expressed by endothelial cells exposed to factors such as LPS and inflammatory cytokines (Colman, 1994). To investigate alteration of sinusoidal endothelial cell (SEC) function in livers of LPS/RAN-treated rats, serum hyaluronic acid (HA) was measured. Ordinarily, 90% of

Figure 3.5: Serum plasminogen activator inhibitor-1 (PAI-1) concentration after LPS/RAN treatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Serum concentration of PAI-1 in its active, latent and complexed forms was evaluated three hours later using an ELISA. n= 3-4 rats per group. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats ($p<0.05$). #Significantly different from all other treatments ($p<0.05$).


Serum PAI-1 (ng/ml)
1000
800
600
400
200
0
*
*
#
Veh
LPS
Veh
LPS
Veh
RAN

HA in the blood is cleared by SECs in the liver (Kobayashi et al., 1999). Accordingly, increased plasma HA concentration suggests altered SEC function, and this has been used as a biomarker after toxic insult (Copple et al., 2002a; Deaciuc et al., 1993). A modest, but significant elevation in serum HA concentration was observed in rats treated with either LPS or RAN alone, whereas serum HA was elevated more than 8-fold in rats cotreated with LPS/RAN (Figure 3.6). PAI-1 expression, as determined by either gene array or real-time PCR, correlated significantly (r^2=0.93) with changes in serum HA concentration (data not shown).

3.4.7 Hepatic fibrin deposition.

Enhanced PAI-1 expression and serum HA concentration in LPS/RAN-cotreated rats suggested altered SEC function consistent with a procoagulant environment in the liver, raising the possibility of enhanced fibrin deposition. Accordingly, livers were removed 3 h after RAN treatment, as in the gene array experiment, and processed for fibrin immunohistochemistry. Figure 3.7 shows representative images of hepatic fibrin staining in livers. Fibrin staining in the intima of the larger vessels of Veh-treated rats (Figure 3.7A) occurs post-mortem (i.e., artifactually) and can be prevented by perfusing the liver with heparin prior to organ removal (data not shown). Minimal staining was observed in the sinusoids of Veh-treated rats. Similarly, no sinusoidal staining was observed in rats given RAN alone (Figure 3.7C). Slight fibrin staining was observed in livers of rats treated with LPS alone (Figure 3.7B). In LPS/RAN-treated rats (Figure 3.7D), a much more pronounced, panlobular fibrin staining occurred in sinusoids.

Morphometry revealed a statistically significant increase in fibrin staining only in livers from animals cotreated with LPS/RAN (Figure 3.7E).

3.5 Discussion

The work presented approached the study of drug-inflammation interaction by examining gene expression in an animal model of RAN idiosyncratic liver injury. In animals given a nonhepatotoxic dose of LPS followed two hours later by a nonhepatotoxic dose of RAN, we showed previously that livers were normal at 3 h post RAN treatment but became injured by 6 h. We chose to examine gene expression changes in liver at a time (3 h) just before the onset of liver injury in LPS/RAN-treated animals (Luyendyk et al., 2003b). At this time, hierarchical cluster analysis of hepatic gene expression changes was able to segregate rats by treatment group (Figure 3.2). To identify changes in gene expression related to initiation of liver injury in this model, the activity of genes after treatment with LPS and/or RAN was compared to activity in Veh-treated rats (Figure 3.3). Increases in pro-inflammatory gene products or altered hepatocellular homeostasis might precipitate liver injury in LPS/RAN-treated rats, therefore, we identified genes within these sets that followed four expression profiles consistent with potential involvement in the pathogenesis (Table 3.1). The most obvious genes to examine were those with greater or attenuated expression in LPS/RAN-treated rats compared to treatment with LPS or RAN alone, since only rats in this group develop liver injury.

Figure 3.6: Effect of LPS/RAN cotreatment on serum hyaluronic acid (HA) concentration. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Altered sinusoidal endothelial cell function was estimated 3 h after RAN administration by increases in serum HA activity. n= 3-4 rats per group. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats ($p<0.05$). #Significantly different from all other treatments ($p<0.05$).

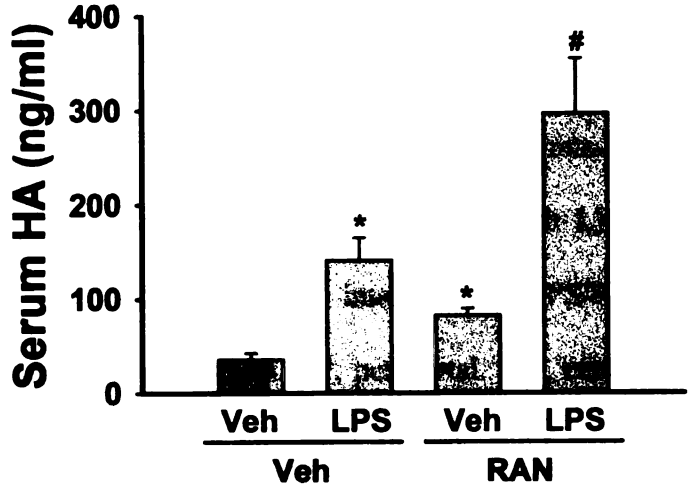
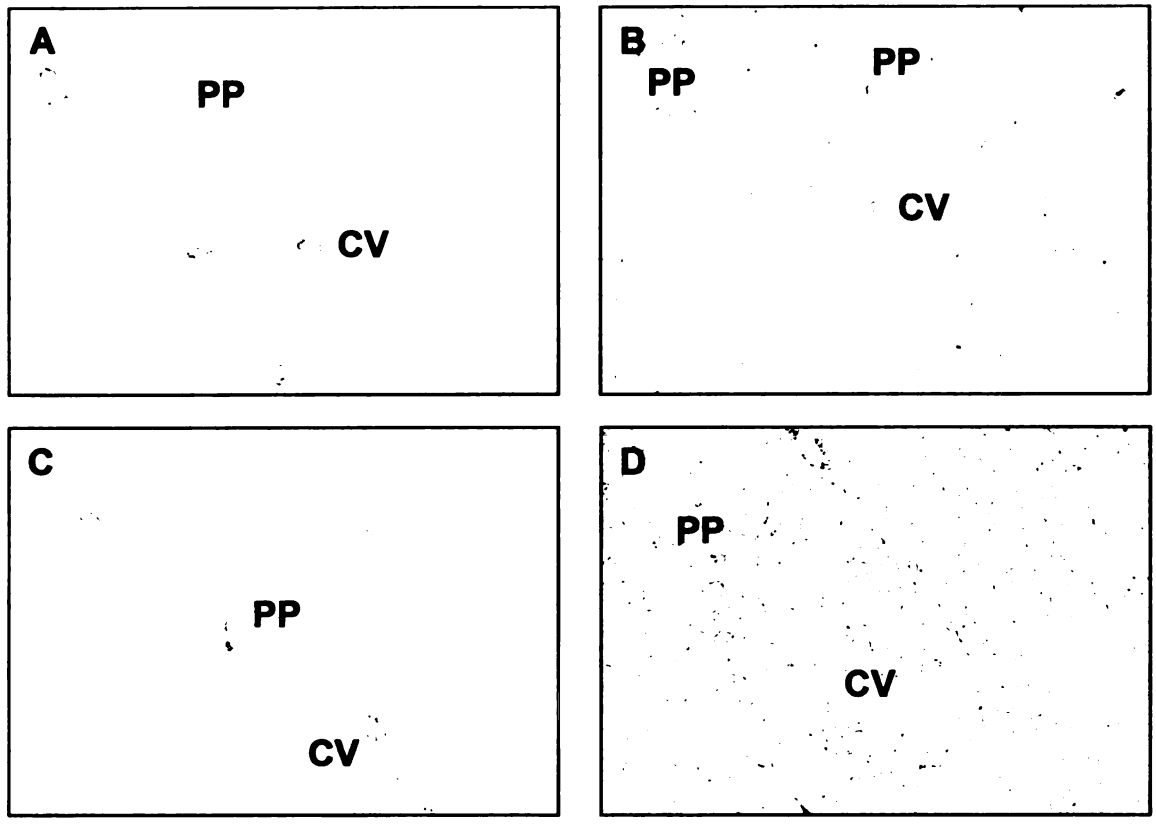
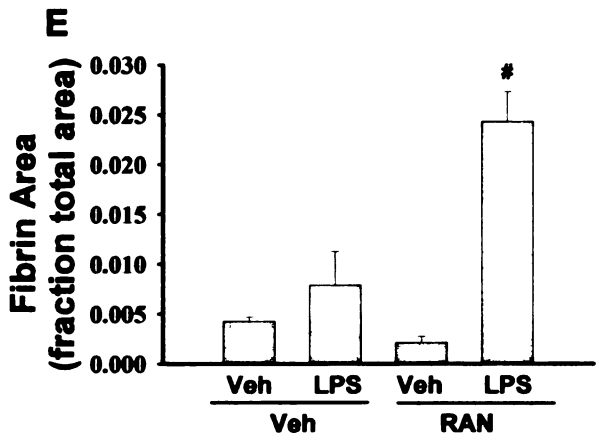

Serum HA (ng/ml)
400
300
200
100
0
*
*
#
Veh
LPS
Veh
LPS
Veh
RAN

Figure 3.7: Effect of LPS/RAN cotreatment on hepatic fibrin deposition. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle (iv), then two hours later with 30 mg/kg RAN or its vehicle (iv). Livers were removed 3 h after RAN treatment and processed for immunohistochemistry as described in *Materials and Methods*. Representative images of fibrin staining in livers from (A) Veh- and (C) RAN- treated rats showing minimal staining (black). (B) Representative image from rat treated with LPS showing slight panlobular fibrin staining. (D) Representative image from LPS/RAN-cotreated rat showing marked panlobular fibrin deposition. PP, periportal. CV, central vein. For (E), the area of positive fibrin staining in 10 randomly chosen, 100X fields per tissue was determined morphometrically as described in *Materials and Methods*. Data are expressed as mean ± SEM. n=3. $^{\#}$Significantly different from all other treatments ($p<0.05$).


A
PP
CV
B
PP
PP
CV
C
PP
CV
D
PP
CV


E
Fibrin Area
(fraction total area)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
#
Veh
LPS
Veh
LPS
Veh
RAN

A less obvious possibility is that genes expressed to a similar degree after LPS or LPS/RAN treatment or to a similar degree after RAN or LPS/RAN treatment are important for liver injury in LPS/RAN- treated rats. Since liver injury does not develop in rats treated with LPS or RAN alone, induction of such a gene is probably not sufficient to cause liver injury by itself but may be involved in the pathogenesis of liver injury if it interacts with one or more gene products. Several genes were identified with similar expression in livers after treatment with LPS compared to treatment with LPS/RAN (see LPS/RAN≈LPS in Table 3.1, Supplemental Table 9). Genes in this group were related to inflammation or could be identified as LPS-inducible (Supplemental Table 9). For example, genes encoding inflammatory cytokines, including TNF-α and interleukin-1β as well as inducible nitric oxide synthase, showed similar expression after LPS-treatment or LPS/RAN-cotreatment. Furthermore, cell surface molecules including the adhesion molecules lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), CD14 and CD38, the transcription factors CCAAT/enhancer binding protein (C/EBP)-delta and –beta, and products involved in signal transduction such as Janus kinase 2 (Jak2) and phosphodiesterase 4B (PDE4B) fit this pattern of expression.

PDE4B is an important regulator of inflammatory responses, including expression of cytokines and activation of inflammatory cells such as neutrophils (PMNs; Jin and Conti, 2002; Essayan, 1999). In this regard, it is of interest that hepatic lesions which develop in LPS/RAN-treated rats are laden with PMNs (Luyendyk et al., 2003b). Regulation of inflammation by PDE4B or other gene products may be essential for LPS/RAN-induced liver injury but not sufficient to produce liver injury in the absence of RAN cotreatment. Indeed, RAN can sensitize hepatocytes to the cytotoxic effects of

PMN-derived factors (Luyendyk et al., 2003b). It therefore seems possible that RAN sensitizes hepatocytes to become injured from otherwise noninjurious upregulation of pro-inflammatory genes, leading to idiosyncratic hepatotoxicity.

Whereas some genes were induced after LPS- and LPS/RAN-treatment to a similar degree, the expression of others was increased after LPS treatment but showed an attenuated response after cotreatment with RAN (see LPS/RAN>LPS in Table 3.1, Supplemental Table 11). This pattern is of interest because RAN might prevent expression of gene products that downregulate inflammation or cell death signals, thereby enhancing inflammation to tissue-damaging levels and/or activating cell death pathways. Indeed, within this group, several members of signal transduction pathways were identified, including protein kinase C (PKC)-epsilon. Interestingly, PKC-epsilon and another gene with this expression pattern, oxygen regulated protein (150kD) (ORP150), have been implicated in protection against ischemic stress (Gray et al., 1997; Ozawa et al., 1999). This suggests the possibility that livers from LPS/RAN-cotreated rats are more sensitive to harmful effects of local ischemia due to loss of protective gene products. In addition, expression of the proteasome subunits LMP2 and LMP7 was observed in LPS-treated rats, but this increase was attenuated after LPS/RAN treatment. Cells lacking LMP2 and LMP7 have defective NF-kB translocation and are sensitive to TNF-α-induced apoptosis (Hayashi and Faustman, 2000). Overall, these and other genes expressed to a greater degree after LPS treatment (heat shock 70kD protein 1A, heme oxygenase-2, dnaJ homolog subfamily b member 9) compared to LPS/RAN cotreatment may confer some degree of cytoprotection, perhaps by decreasing sensitivity to hypoxia, inflammatory mediators, or oxidative stress.

Genes expressed to a greater degree in LPS/RAN-cotreated rats compared to treatment with either agent alone could represent a population with mechanistic importance if the gene product became expressed at or above the level required to participate in liver injury. Our analysis revealed that genes characterized by this expression pattern were primarily hypoxia-inducible or involved in inflammation (see LPS/RAN>LPS>RAN in Table 3.1, Supplemental Table 8, Table 3.2). Several of the hypoxia-inducible genes in this group can also participate in inflammatory responses. For example, CxCl10 (interferon-inducible cytokine IP-10) is induced under hypoxic conditions and modulates recruitment and retention of inflammatory cells (Neville et al., 1997). The hypoxia-inducible transcription factor egr-1 is involved in cell death signaling (Thiel and Cibelli, 2002), but it can also influence inflammatory responses by altering cytokine expression (Yan et al., 1999; Shi et al., 2002). The observation that numerous hypoxia-inducible genes are expressed in LPS/RAN-cotreated rats suggests the possibility that hypoxia is involved in liver injury in this idiosyncrasy model. However, further studies are required to confirm tissue hypoxia in livers of LPS/RAN-cotreated rats and its role in pathogenesis.

Another gene product expressed to a larger degree in LPS/RAN-cotreated rats compared to rats treated with LPS or RAN alone is PAI-1. PAI-1 is induced by various stimuli, including LPS, inflammatory cytokines, and hypoxia and is expressed by several cells in the liver, including parenchymal and endothelial cells (Binder et al., 2002; Kietzmann et al., 1999; Hamaguchi et al., 2003). Although the cell source and mechanism of enhanced PAI-1 expression in livers of LPS/RAN-cotreated rats is not known, one possibility is that RAN may indirectly augment expression by increasing

levels of cytokines known to induce PAI-1, such as TNF-α or IL-1. Interestingly, although hepatocellular liver injury was not observed at this early time after LPS/RAN cotreatment, serum HA concentration was significantly increased, suggesting altered SEC homeostasis. This elevation in serum HA concentration supports SECs as a potential source of PAI-1. Perturbation of SECs by hypoxia (Kietzmann et al., 1999) or altered signal transduction may be responsible for augmented PAI-1 expression in endothelial cells in LPS/RAN-treated rats. P38 mitogen-activated protein kinase is involved in induction of PAI-1 during hypoxia but does not appear to be important in induction of PAI-1 by TNF-α (Kietzmann et al., 2003; Hamaguchi et al., 2003). Another possibility is that cotreatment with RAN influences PAI-1 expression at the transcriptional level. (Gruber et al., 2003) demonstrated that the PAI-1 promoter contains a response element for the orphan receptor Nur77 (NGFI-B, TR3). Furthermore, Nur77 overexpression in human umbilical vein endothelial cells activates a luciferase reporter gene controlled by a PAI-1 promoter, and Nur77 is necessary for induction of PAI-1 expression by TNF-α (Gruber et al., 2003). Since Nur77 is also a hypoxia-inducible gene (Choi et al., 2004), its expression might be expected to be enhanced in LPS/RAN-treated rats, since numerous other hypoxia-inducible genes, including PAI-1, were identified in this group (Table 3.2). Although probesets for Nur77 did not emerge as active after LPS/RAN-cotreatment, the expression of these was more than 10-fold greater than Veh-control in 3 of 4 LPS/RAN-treated rats, whereas less than 2-fold changes occurred in livers of rats given LPS or RAN alone. This result raises the possibility that Nur77 is important for enhanced expression of PAI-1 in LPS/RAN-treated rats. However, additional experiments are necessary to prove such a connection.

The greater expression of the PAI-1 gene in livers of LPS/RAN-treated rats (Figure 3.4A) was reflected in enhanced concentration of PAI-1 protein in serum (Figure 3.5), suggesting a functional consequence to its induction. PAI-1 has many physiological roles including inhibition of fibrinolysis and modulation of inflammatory cell migration (Binder et al., 2002; Marshall et al., 2003). Consistent with its antifibrinolytic activity and pattern of expression, significant fibrin deposits occur only in livers of rats given LPS/RAN. Concurrently elevated serum concentrations of both HA and PAI-1 suggest the possibility that altered SEC homeostasis might favor activation of the hemostatic system and fibrin deposition in liver. Fibrin deposition could cause local ischemia, and resultant hypoxia might contribute to the development of liver injury. This hypothesis is consistent with the observation that numerous hypoxia-inducible genes were expressed in livers of LPS/RAN-cotreated rats (Table 3.3). Inasmuch as the PAI-1 gene is hypoxia-inducible, the presence of hypoxia might further enhance its expression, triggering a cascade of hemostatic dysregulation. Additionally, hypoxic upregulation of egr-1 could facilitate coagulation system activation by upregulating tissue factor on liver cell membranes (Pawlinski et al., 2003). Overall, the data suggest that enhanced PAI-1 expression in LPS/RAN-cotreated rats encourages formation of fibrin clots, possibly resulting in disrupted liver blood flow and hepatocellular hypoxia that could contribute to the development of necrosis. Preliminary studies showing protection from liver injury by fibrinolytic or anticoagulant drugs in LPS/RAN-treated rats support this hypothesis (Luyendyk, 2004a).

In summary, rats were treated with either a nonhepatotoxic dose of LPS or its Veh and with either RAN or its Veh. Of the four treatments, only LPS/RAN treatment results

in liver injury (Luyendyk et al., 2003b). At a time before the onset of significant hepatotoxicity in LPS/RAN-treated rats, hierarchical clustering of hepatic gene expression segregated animals by treatment. Hypoxia-inducible genes, including PAI-1 and egr-1, were expressed to a greater degree in livers after LPS/RAN treatment compared to either agent given alone. The enhanced PAI-1 gene expression was reflected in increased PAI-1 protein in serum. Significant fibrin deposits in liver sinusoids were observed only in LPS/RAN-cotreated rats, consistent with the antifibrinolytic activity of PAI-1. Overall, the results suggest that altered expression of genes promoting hemostasis might contribute to liver injury in LPS/RAN-cotreated rats. The studies presented represent a snapshot in time at one dose; examination of gene expression at other times after treatment and other doses of LPS and RAN will illuminate this connection further. The association of hypoxia-inducible gene expression with hepatic fibrin deposition in LPS/RAN-cotreated rats is consistent with the development of tissue ischemia/hypoxia as a contributing factor to liver pathogenesis in this model of RAN idiosyncrasy.

3.6 Supplemental data

The results summarized as a Venn diagram in Figure 3 are available for download in Microsoft Excel format. The genes represented by probesets defining each set were identified and are shown along with gene symbol, Unigene identification (Rn build 117), locus-link identification (hyperlink available), signal intensities relative to vehicle treatment, and standard deviations in Supplemental Tables 1–7. Supplemental text describing the Venn diagram is also available for download in Microsoft Word format. Genes expressed with the specific patterns summarized in Table 1 are available as Tables

8–11 in Microsoft Word format. Following each table are selected references for each gene product. In some cases, probesets for the same gene followed the same expression pattern but were segregated to different patterns by statistical analysis. Supplemental data are available at www.toxsci.oupjournals.org.

CHAPTER 4

Luyendyk, J.P., Maddox, J.F., Green C.D., Ganey, P.E., and Roth, R.A. (2004). Augmentation of lipopolysaccharide-induced fibrin deposition by ranitidine and its connection to idiosyncrasy-like liver injury in rats. Hepatology. **In press**.

4.1 Abstract

Coadministration of nonhepatotoxic doses of the histamine 2-receptor antagonist ranitidine (RAN) and bacterial lipopolysaccharide (LPS) results in hepatocellular injury in rats, the onset of which occurs in 3-6 h. This reaction resembles RAN idiosyncratic hepatotoxicity in humans. Early fibrin deposition occurs in livers of rats cotreated with LPS/RAN. Accordingly, we tested the hypothesis that the hemostatic system contributes to liver injury in LPS/RAN-treated rats. Rats were given either LPS (44.4 X 10^6 EU/kg) or its vehicle, then RAN (30 mg/kg) or its vehicle 2 h later. They were killed 2, 3, 6, 12, or 24 h after RAN treatment, and liver injury was estimated from serum alanine aminotransferase (ALT) activity and liver histopathology. A modest elevation in serum hyaluronic acid, which was most pronounced in LPS/RAN-cotreated rats, suggested altered sinusoidal endothelial cell function. A decrease in plasma fibrinogen and increases in thrombin-antithrombin (TAT) dimers and in serum concentration of plasminogen activator inhibitor-1 (PAI-1) occurred before the onset of liver injury. Hepatic fibrin deposition was observed in livers from LPS/RAN-cotreated rats 3 and 6 h after RAN. Liver injury was abolished by the anticoagulant, heparin, and was significantly attenuated by the fibrinolytic agent, streptokinase. Hypoxia, one potential consequence of sinusoidal fibrin deposition, was observed in livers of LPS/RAN-treated rats. Taken together, the results suggest that the hemostatic system is activated after LPS/RAN cotreatment and that fibrin deposition in liver is important for the genesis of hepatic parenchymal cell injury in this model.

4.2 Introduction

Exposure of rats to large doses of LPS results in midzonal hepatic necrosis that requires several factors including cytokines, inflammatory cells, an activated hemostatic system, and platelets (Hewett and Roth, 1995; Hewett et al., 1993; Hewett et al., 1992; Pearson et al., 1995). The involvement of similar factors in LPS/RAN-induced hepatotoxicity has not been investigated. The hemostatic system is composed of two branches (Figure 3.1), the coagulation and fibrinolytic pathways. In the liver, SECs play a critical role in regulation of coagulation and fibrinolysis. Under resting (normal) conditions, SECs express factors including thrombomodulin and protein C that inhibit coagulation, thereby suppressing fibrin formation (Schultze and Roth, 1998). However, when activated or damaged the anticoagulant properties of these cells are lost, and expression of procoagulant factors including tissue factor (TF) can facilitate coagulation system activation. In a healthy vasculature, the accumulation of fibrin is also prevented by the process of fibrinolysis. SECs produce both profibrinolytic and antifibrinolytic factors. Accordingly, altered SEC homeostasis is a potential predictor of disturbances in the hemostatic system.

Coagulation system activation occurs through both the intrinsic and extrinsic pathways, ultimately resulting in conversion of prothrombin to thrombin. Thrombin can enzymatically cleave circulating fibrinogen to fibrin monomers, which are then cross-linked by factor XIIIa, resulting in formation of insoluble fibrin clots (Figure 4.1). Thrombin is rapidly inactivated by antithrombin III, resulting in formation of thrombin-antithrombin III dimers (TAT). The formation of fibrin clots is counteracted via their lysis by the enzyme plasmin, a key member of the fibrinolytic system. Conversion of

plasminogen to plasmin is controlled by the plasminogen activators, urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). In turn, plasmin activity is controlled by α2-antiplasmin, and tPA and uPA activities are inhibited by plasminogen activator inhibitor-1 (PAI-1), and important physiological regulator of fibrinolysis (Figure 4.1).

Several biomarkers can be measured to evaluate coagulation system activation and fibrinolysis. An increase in plasma TAT concentration, decrease in plasma fibrinogen concentration, and/or tissue fibrin deposition are indicative of an activated coagulation system. Pharmacologic inhibition of coagulation system activation (see Figure 4.1) is accomplished by inhibition of thrombin activation (e.g., heparin) and by agents (e.g., warfarin) that interfere with synthesis of vitamin K-dependent coagulation factors such as fibrinogen. Profibrinolytic agents include streptokinase (SK), tPA, and urokinase, which increase plasmin-mediated fibrinolysis. Elevated plasma concentrations of PAI-1 can impair fibrinolysis.

Recent results suggested the possibility that the hemostatic system is important in this model (Luyendyk et al., 2004c). For example, sinusoidal endothelial cell (SEC) homeostasis is altered before the onset of significant hepatic parenchymal cell injury in LPS/RAN-treated rats (Luyendyk et al., 2004c). Additionally, significant hepatic fibrin deposition resulted from LPS/RAN-cotreatment but not from treatment with either agent alone (Luyendyk et al., 2004c). Also, consistent with impaired fibrinolysis, hepatic expression of PAI-1 was augmented in LPS/RAN-treated rats (Luyendyk et al., 2004c). Such suppression would be expected to enhance fibrin deposition. However, whether or

not the coagulation system is activated after LPS/RAN-treatment and the consequences of hemostatic dysregulation in LPS/RAN-induced liver injury have not been determined. The studies presented here tested the hypothesis that the hemostatic system is activated before the onset of liver injury and is critical for HPC injury in LPS/RAN-treated rats. Toward this end, biomarkers of thrombin activation, hepatic fibrin deposition, and serum concentration of PAI-1 were evaluated at times before and after the development of parenchymal cell injury. Rats were treated with the anticoagulant heparin or the fibrinolytic agent streptokinase (SK) to investigate the importance of the hemostatic system and fibrin deposition in LPS/RAN-induced parenchymal cell injury. In addition, we examined whether LPS/RAN cotreatment caused liver hypoxia as one consequence of fibrin deposition that could promote hepatocellular injury.

4.3 Materials and Methods

4.3.1 Materials

For information on this topic please refer to Chapter 2 Materials and Methods.

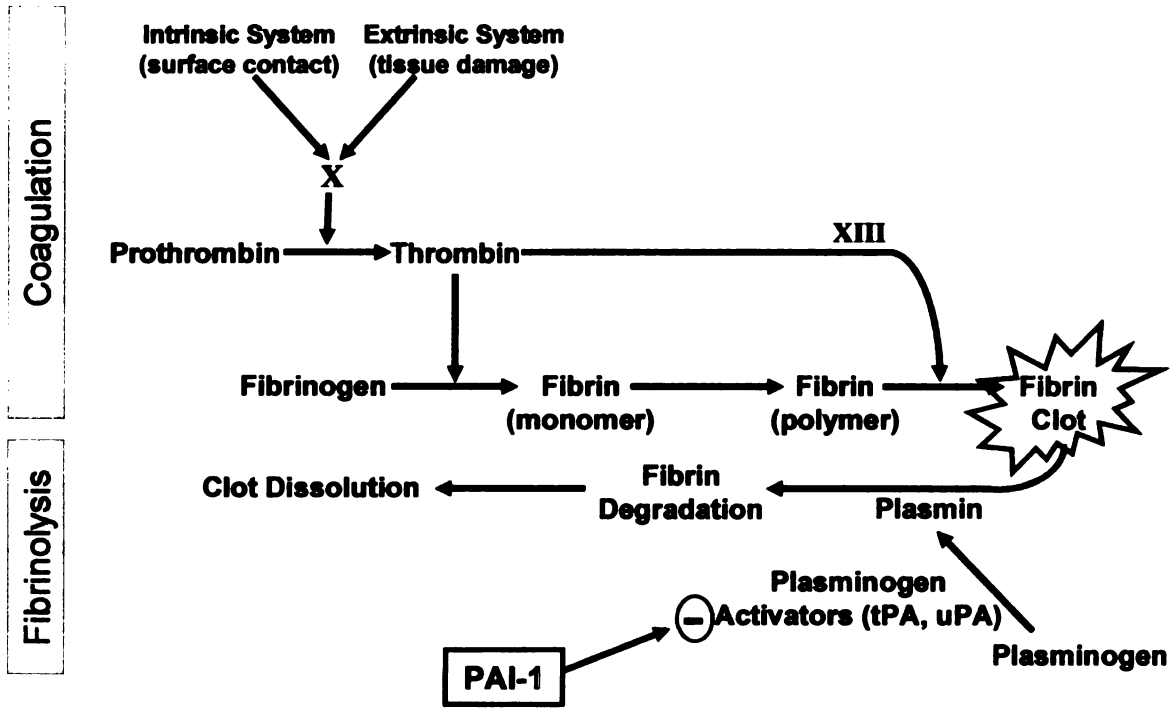
4.3.2 Animals

For information on this topic please refer to Chapter 2 Materials and Methods.

4.3.3 Experimental Protocol

Plasma was collected by drawing blood from the vena cava into a syringe containing sodium citrate (final concentration, 0.38%), and rats were then killed

Figure 4.1 Balancing of coagulation and fibrinolysis by the coagulation system. Formation of fibrin clots is regulated by both the procoagulant and fibrinolytic arm of the hemostatic system. Coagulation activation via either the intrinsic or extrinsic pathways results in conversion of prothrombin to thrombin, conversion of soluble fibrinogen to insoluble fibrin monomers, ultimately producing fibrin clots. The process of fibrinolysis balances this system through degredation of fibrin clots by the enzyme plasmin. Plasminogen activators (i.e., uPA and tPA) are important for activation of plasmin and can be negatively regulated by plasminogen activator inhibitor-1. Figure developed by Robert Roth.


Coagulation
Fibrinolysis
Intrinsic System
(surface contact)
Extrinsic System
(tissue damage)
X
Prothrombin
Thrombin
XIII
Fibrinogen
Fibrin
(monomer)
Fibrin
(polymer)
Fibrin
Clot
Clot Dissolution
Fibrin
Degradation
Plasmin
Plasminogen
Activators (tPA, uPA)
Plasminogen
PAI-1

by exsanguination from the dorsal aorta. Blood was allowed to clot at 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. For more information on this section please refer to Chapter 2 Materials and Methods.

Inhibition of coagulation system activation was achieved by administration of heparin. Rats were treated with LPS/RAN as above, but 1 h before RAN treatment, heparin (3000 U/kg, s.c.) or sterile saline was administered. Rats were killed 6 h after RAN administration, and serum and liver samples were taken. For studies with SK, rats were treated with LPS and RAN, then two h later they were given SK (25,000 U/kg) or sterile saline (i.p.). Three h later, a second administration of SK (20,000 U/kg) was given. Rats were killed 6 h after treatment with RAN, and serum and liver samples were collected as described above.

4.3.4 Hepatotoxicity assessment

For information on this topic please refer to Chapter 3 Materials and Methods.

4.3.5 Determination of serum PAI-1, plasma fibrinogen and plasma thrombin-antithrombin dimer (TAT) concentrations

Total serum PAI-1 concentration was evaluated using a commercially available ELISA purchased from American Diagnostica Inc. (Greenwich, CT.). The concentration of functionally active PAI-1 in serum was assessed using a commercially available ELISA purchased from Molecular Innovations Inc. (Southfield, MI). 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 TAT concentration was determined using kit #OWMG15 from Dade-Behring.

4.3.6 Fibrin and RECA-1 immunohistochemistry

A $1cm^3$ block of liver cut from the left medial lobe was frozen for 8 minutes in liquid nitrogen-chilled isopentane. For liver endothelial cell immunostaining, 8 μm-thick sections of frozen liver were fixed in acetone (4°C) for 5 minutes. Sections were incubated in a blocking solution consisting of PBS with 5% goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes, then overnight at 4°C in blocking solution containing diluted (1:20) mouse anti-rat RECA-1 (rat endothelial cell antigen-1) antibody (Serotec, Inc., Raleigh, NC). The RECA-1 antibody binds to rat endothelium but not to other cell types (Duijvestijn et al., 1992). In the liver, this antibody stains both SECs and endothelial cells of larger vessels (Copple et al., 2002a). After incubation with the RECA-1 antibody, sections were washed three times, 5 minutes each, with PBS then incubated for 3 hours at room temperature with goat anti-mouse secondary antibody conjugated to Alexa 594 (1:1000, Molecular Probes, Eugene, OR) in blocking solution containing 2% rat serum. Sections were washed three times, 5 minutes each, with PBS and visualized using a fluorescent microscope. For more information on fibrin immunohistochemistry, please refer to Chapter 3 Materials and Methods. For both protocols, no staining was observed in controls in which the primary or secondary antibody was eliminated from the staining protocol. All treatment groups that were compared morphometrically were stained immunohistochemically at the same time.

4.3.7 Morphometric evaluation

For more information on this section please refer to Chapter 3 Materials and Methods.

4.3.8 Evaluation of liver hypoxia.

Liver hypoxia was evaluated by two methods. First, hypoxic areas of liver were identified by injection of pimonidazole (PIM) and immunostaining for PIM-modified proteins. PIM is a 2-nitroimidazole marker of hypoxia and has been used to identify regions of hypoxia in liver (Arteel et al., 1998;Arteel et al., 1995). Rats were given 120 mg/kg Hypoxyprobe™-1 (PIM hydrochloride; Chemicon International Inc., Temecula, CA) i.p. two hours before they were killed. PIM-adduct immunostaining was performed on formalin-fixed liver samples sectioned at 5 μm. Tissues were deparaffinized at room temperature by 3 X 5 minute incubations in xylene, 2 X 5 min incubations in 100% ethanol, 1 X 5 min incubation in 95% ethanol, 1 X 5 min incubation in 75% ethanol, 1 X 5 min incubation in distilled water, 1 X 2 min incubation in distilled water + 0.2% Brij 35 (Fisher Scientific, Pittsburgh, PA), and 1 X 2 min incubation in PBS + 0.2% Brij 35. Sections were washed 3 times with PBS then incubated in BioMeda pronase reagent (Biomeda Corp., Foster City, CA) at 40° C for 40 min. They were then washed with PBS + 0.2% Brij 35 for 2 min and blocked for 5 min at room temperature in undiluted DAKO serum-free protein block solution (DakoCytomation, Carpinteria, CA). Sections were incubated for 40 min at room temperature with the monoclonal hypoxyprobe antibody (1:50, Chemicon International Inc., Temecula, CA) in PBS containing 0.2% Brij 35 and 1

drop DAKO block solution/ml. Sections were washed 3X with PBS + 0.2% Brij 35, then incubated for 3 hours at room temperature with rabbit anti-mouse secondary antibody conjugated to Alexa 594 (1:500, Molecular Probes, Eugene, OR) in PBS containing 0.2% Brij 35 and 1 drop DAKO block solution/ml. Sections were washed 3X with PBS containing 0.2% Brij 35 and 3X with PBS and visualized using a fluorescent microscope. Quantification of PIM immunostaining was performed using Scion Image Beta 4.0.2 as for fibrin staining (above). Background was estimated to be the average pixel intensity identified in periportal regions of Veh/Veh-treated livers (i.e., an area where no hypoxia occurs (Arteel et al., 1995). An increase in positive immunostaining for PIM-modified proteins indicates hypoxia in the liver tissue.

Second, immunostaining for hypoxia-inducible factor-1α (HIF-1α) was performed. HIF-1α is a key regulator of responses to hypoxia (Semenza, 1999) and stabilization of HIF-1α protein can be detected immunohistochemically in hepatocyte nuclei in hypoxic liver (Stroka et al., 2001). For HIF-1α immunostaining, 8 μm-thick sections of frozen liver were fixed in 4% neutral-buffered formalin at room temperature for 10 minutes. Sections were blocked with PBS containing 5% goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes, and this was followed by incubation overnight at 4°C with mouse anti- HIF-1α antibody (NB 100-123, Novus Biologicals, Littleton, CO) diluted (1:100) in PBS containing 5% goat serum. After incubation with the HIF-1α antibody, sections were washed three times, 5 minutes each, with PBS then incubated for 3 hours at room temperature with goat anti-mouse secondary antibody conjugated to Alexa 594 (1:500, Molecular Probes, Eugene, OR) in PBS containing 5% goat serum and 2% rat serum. Sections were then washed three times, 5 minutes each,

with PBS and visualized using a fluorescent microscope. Quantification of liver HIF-1α staining was performed using Scion Image Beta 4.0.2 as with fibrin staining. An increase in nuclear staining of HIF-1α indicates liver hypoxia. For each of these procedures all slides were stained and visualized on the same day.

4.3.9 Statistical Analysis

Two-way analysis of variance with Tukey's test for multiple comparisons was used for analysis of clinical chemistry in the streptokinase study, immunohistochemistry, ELISA, and fibrinogen measurements. Student's t-test was used to compare PAI-1 ELISA data in the same treatment group between times. One-way analysis of variance with Tukey's test for multiple comparisons was used in the heparin study. The criterion for significance for all studies was $p<0.05$.

4.4 Results

4.4.1 Coagulation system activation after LPS/RAN treatment

Coagulation system activation was evaluated in rats treated with LPS/RAN at a time prior to the onset of significant liver injury (i.e., 2 h). Consistent with previous results (Luyendyk et al., 2003b), serum ALT activity was not changed in any treatment group by 2 h (Figure 4.2A). Relative to Veh/Veh-treated rats, plasma fibrinogen concentration was not significantly changed by LPS/Veh-treatment, but it was decreased slightly in rats treated with Veh/RAN (Figure 4.2B). In contrast, a marked decrease (~85%) was observed in LPS/RAN-treated rats (Figure 4.2B). Thrombin was estimated by measuring the plasma concentration of TAT. TAT concentration was not significantly

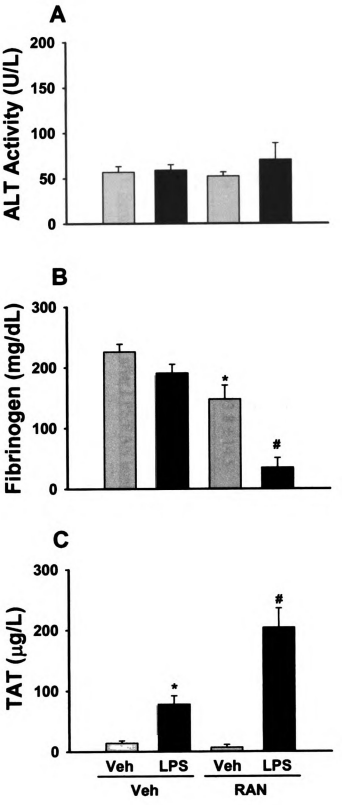
changed in Veh/RAN-treated rats. However, treatment with LPS/Veh resulted in a significant increase in TAT concentration (~5-fold), whereas a more pronounced increase (~14-fold) was observed after LPS/RAN treatment (Figure 4.2C).

4.4.2 Altered sinusoidal endothelial cell (SEC) function after treatment with LPS/RAN

Since hepatic SECs remove circulating HA, an increase in serum HA concentration has been used as a biomarker of altered SEC function. We reported previously that HA concentration was slightly increased in Veh/RAN-treated rats 3h after treatment (Luyendyk et al., 2004c). Results presented in Table 4.1 confirm these findings and demonstrate that this increase is transient: HA concentration in Veh/RAN-treated rats was not different from Veh/Veh-treated rats from 6-24 h. In rats treated with LPS/Veh, serum HA concentration increased by 3 h and continued to increase until 12 h, then leveled off thereafter. (Table 4.1). Rats treated with LPS/RAN had a serum HA concentration significantly greater than rats treated with either LPS/Veh or Veh/RAN at 3, 6, and 24 h.

To evaluate whether overt SEC injury occurred after LPS/RAN treatment, livers were stained immunohistochemically for RECA-1. Decreased RECA-1 staining intensity is associated with endothelial cell loss in other models of hepatotoxicity.(Yee et al., 2003b) No treatment caused a significant change in RECA-1 staining intensity at either 3 or 6 h (Figure 4.3).

Figure 4.2: Coagulation system activation after LPS/RAN treatment. Rats were given LPS (44.4 X 10^6 EU/kg, iv) or its Veh 2 h prior to administration of RAN (30 mg/kg) or its Veh. Hepatic parenchymal cell injury was estimated 2 h after RAN administration by increases in serum ALT activity (A). Coagulation system activation was evaluated by measuring plasma fibrinogen concentration (B) and plasma TAT concentration (C). n=4-6. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats. #Significantly different from all other treatments. ($p<0.05$)


A
ALT Activity (U/L)
200
150
100
50
0
B
Fibrinogen (mg/dL)
300
200
100
0
*
#
C
TAT (μg/L)
300
200
100
0
*
#
Veh
LPS
Veh
LPS
Veh
RAN

	Time after RAN (h)			
Treatment	**3**	**6**	**12**	**24**
Veh/Veh	40.9 ± 5.4	65.2 ± 9.4	40.8 ± 4.2	61.4 ± 8.3
LPS/Veh	104.5 ± 8.5*	143.4 ± 16.9*	272.7 ± 37.9*	261.3 ± 25.1*
Veh/RAN	89.4 ± 13.3*	93.8 ± 14.5	40.8 ± 2.6	72.0 ± 16.1
LPS/RAN	156.1 ± 24.7#	200.1 ± 26.8#	313.8 ± 23.1*	651.3 ± 69.4#

Table 4.1: Serum HA concentration after LPS/RAN treatment. Serum HA was measured as an estimation of SEC dysfunction in LPS and/or RAN-treated rats 3, 6, 12, and 24 h after RAN treatment. n=6-17 rats per group. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats. #Significantly different from all other treatments at that time. ($p<0.05$).

4.4.3 Effect of LPS/RAN treatment on hepatic fibrin deposition.

Minimal fibrin staining was associated with the larger vessels but not in the sinusoids in livers from Veh/Veh-treated rats (Figure 4.4A). This staining occurs post mortem and can be prevented by perfusing the liver with heparin before its removal (data not shown). Marked panlobular staining occurred in livers of LPS/RAN-treated rats (Figure 4.4B and C). Morphometric analysis revealed no significant increase in hepatic fibrin deposits in RAN-treated rats at either 3 or 6 h (Figure 4.4D), confirming earlier results (Luyendyk et al., 2004c). A slight increase in fibrin staining occurred in LPS/Veh-treated rats that became significant at 6 h (Figure 4.4D). Staining in LPS/RAN-treated rats was significantly greater than in all other treatment groups at 3 and 6 h (Figure 4.4D).

4.4.4 Effect of LPS/RAN treatment on serum PAI-1 concentration

The concentrations of total PAI-1 protein (Figure 4.5A) and active PAI-1 (Figure 4.5B) were evaluated in rats 3 and 6 h after RAN treatment. Treatment with Veh/RAN caused a slight increase in total serum PAI-1 concentration at 3 and 6 h but was without effect on the concentration of active PAI-1. The serum concentrations of both total and active PAI-1 were significantly increased in LPS/Veh-treated rats at 3 h, but these increases waned by 6 h. The serum concentrations of both total and active PAI-1 were increased in LPS/RAN-treated rats at 3 and 6 h to a greater degree than after treatment with LPS/Veh or Veh/RAN. In contrast to rats treated with LPS/Veh, PAI-1 concentration in LPS/RAN-treated rats did not decrease significantly between these times.

Figure 4.3: Quantification of RECA-1 staining in livers after LPS/RAN treatment. Rats were given LPS (44.4 X 10^6 EU/kg, iv) or its Veh 2 h prior to administration of RAN (30 mg/kg) or its Veh. Three or 6 h after RAN treatment, livers were removed and stained immunohistochemically for RECA-1 as described in *Materials and Methods*. The total area of RECA-1 staining was evaluated in 10 randomly chosen fields (100X) per liver section and analyzed morphometrically as described in *Materials and Methods*. n=3-7 rats per group at each time. None of the treatments caused a significant change in RECA-1 staining intensity relative to Veh/Veh-treated rats at either 3 or 6 h. ($p<0.05$)

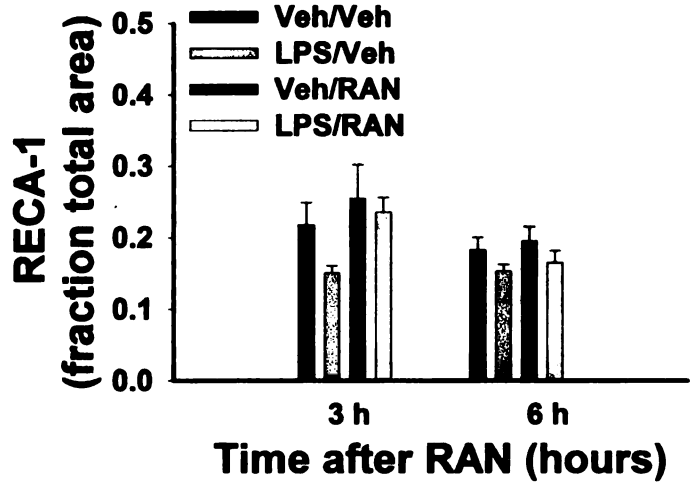
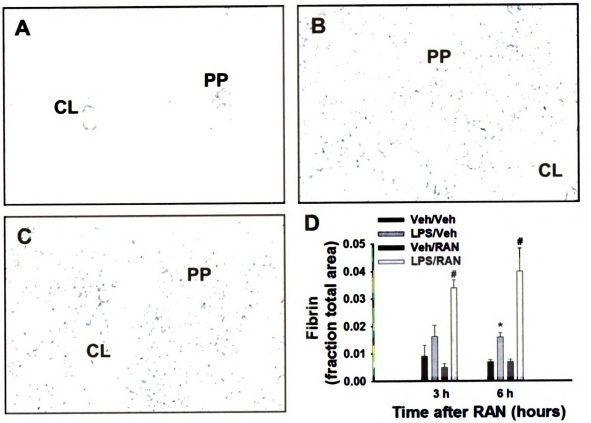

Veh/Veh
LPS/Veh
Veh/RAN
LPS/RAN
RECA-1
(fraction total area)
0.5
0.4
0.3
0.2
0.1
0.0
3 h
6 h
Time after RAN (hours)

Figure 4.4: Liver fibrin deposition in LPS/RAN-treated rats. Rats were given LPS (44.4 X 10^6 EU/kg, iv) or its Veh 2 h prior to administration of RAN (30 mg/kg) or its Veh. Three or 6 h after RAN treatment, livers were removed and stained immunohistochemically for fibrin as described in *Materials and Methods*. (A) Representative photomicrograph (100X magnification) of fibrin staining in liver of a Veh/Veh-treated rat showing minimal staining (black) in the intima of larger vessels. (B) Representative photomicrograph showing panlobular fibrin staining characteristic of livers taken from LPS/RAN-treated rats 3 h after RAN. (C) Representative photomicrograph showing panlobular sinusoidal fibrin staining characteristic of livers taken from LPS/RAN-treated rats 6 h after RAN. PP, periportal region. CL, centrilobular region. (D) Quantification of liver fibrin staining in rats treated with LPS and/or RAN. n=3-6. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats at that time. #Significantly different from all other treatments at that time. ($p<0.05$)


A
PP
CL
B
PP
CL
C
PP
CL
D
Veh/Veh
LPS/Veh
Veh/RAN
LPS/RAN
Fibrin
(fraction total area)
0.05
0.04
0.03
0.02
0.01
0.00
#
*
#
3 h
6 h
Time after RAN (hours)

4.4.5 Effect of SK on LPS/RAN-induced liver injury

To investigate the role of fibrin clots in LPS/RAN-induced liver injury, the fibrinolytic agent SK was used. SK treatment caused a slight but statistically significant decrease in liver fibrin staining in Veh/Veh-treated rats (Figure 4.6A). Fibrin deposition was elevated by LPS/RAN treatment, and this effect was markedly reduced (60%) by SK (Figure 4.6A), confirming the effectiveness of the SK treatment. The effect of SK treatment on hepatic parenchymal cell injury was estimated 6 h after LPS/RAN treatment by changes in serum activities of ALT (Figure 4.6B) and AST (Figure 4.6C). SK was without effect in Veh/Veh-treated rats but significantly reduced the serum elevations in ALT (~50%) and AST (~35%) in LPS/RAN-treated rats.

4.4.6 Effect of heparin on LPS/RAN-induced liver injury

The importance of an activated coagulation system in LPS/RAN-induced liver injury was evaluated by treating rats with the anticoagulant, heparin. Activation of the coagulation system was evaluated by changes in plasma fibrinogen concentration. The concentration of plasma fibrinogen was significantly decreased in rats treated with LPS/RAN, and this decrease was prevented by coadministration of heparin (Figure 4.7A). LPS/RAN treatment significantly increased serum ALT and AST activities, and this increase was prevented by coadministration of heparin (Figure 4.7B, C).

4.4.7 LPS/RAN treatment and liver hypoxia

Figure 4.5: Serum concentration of PAI-1 after treatment with LPS/RAN. The concentrations of (A) total PAI-1 and (B) active PAI-1 were evaluated at 3 h and 6 h in serum taken from rats treated with LPS and/or RAN. n=6-7. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats at that time. #Significantly different from all other treatments at that time. [a]Significantly different from the same treatment at 3 h. ($p<0.05$)

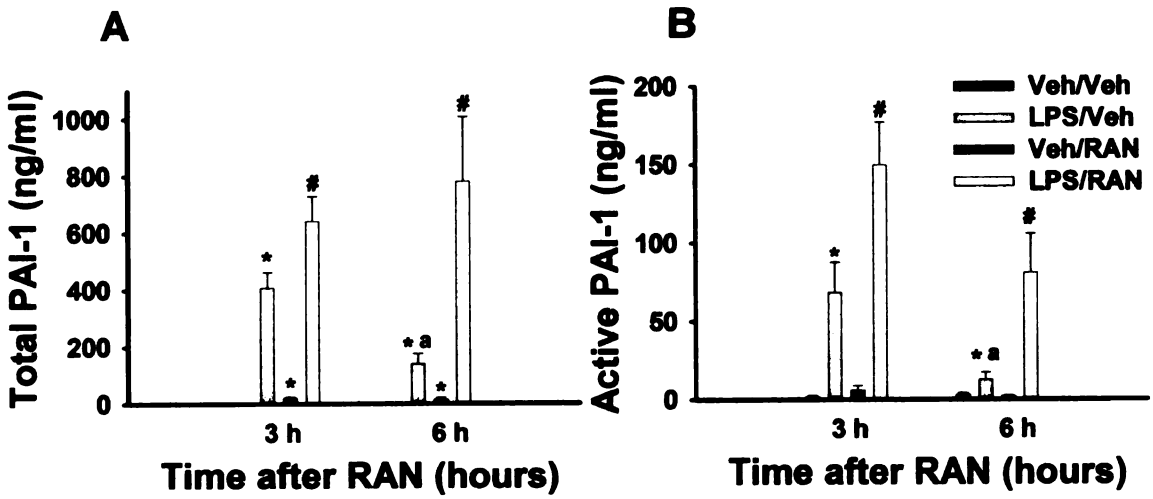
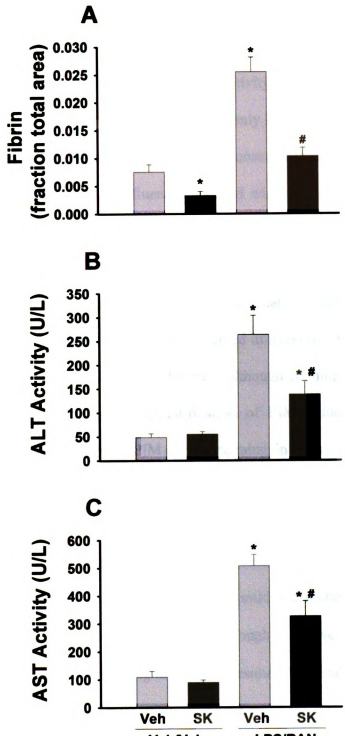

A
B
Total PAI-1 (ng/ml)
Active PAI-1 (ng/ml)
1000
800
600
400
200
0
200
150
100
50
0
3 h
6 h
Time after RAN (hours)
Veh/Veh
LPS/Veh
Veh/RAN
LPS/RAN

Figure 4.6: Effect of streptokinase (SK) on LPS/RAN-induced liver injury. Rats were given SK or saline 1 h and 4 h after treatment with either Veh/Veh or LPS/RAN. Livers were removed at 6 h and stained immunohistochemically for fibrin, which was quantified as described in *Materials and Methods* (A). Hepatic parenchymal cell injury was estimated at 6 h by increases in serum ALT (B) and AST (C) activities. n=6-8. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh-treated rats. #Significantly different from LPS/RAN/Veh and Veh/Veh/SK-treated rats. ($p<0.05$).


A
Fibrin (fraction total area)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
*
*
#
B
ALT Activity (U/L)
350
300
250
200
150
100
50
0
*
* #
C
AST Activity (U/L)
600
500
400
300
200
100
0
*
* #
Veh
SK
Veh
SK
Veh/Veh
LPS/RAN

Rats were treated with LPS and/or RAN and immunostaining for PIM-adducts and HIF-1α protein was evaluated at a time near the onset of hepatotoxicity in LPS/RAN-treated rats (i.e., 3 h after RAN administration). In contrast to previous experiments (Luyendyk et al., 2003b), serum ALT activity was significantly increased at 3 h in LPS/RAN-treated rats compared to rats given only LPS or RAN (data not shown), suggesting that 3 h marks the approximate time of onset of liver injury in this model. LPS/RAN hepatotoxicity was not influenced by PIM administration (data not shown). Little PIM-adduct staining was observed in livers of Veh/Veh-treated rats (Figure 4.8A). PIM-adduct staining increased slightly in livers of rats treated with LPS/Veh (Figure 4.8B) or Veh/RAN (Figure 4.8C). By contrast, a dramatic increase in the area and intensity of positive PIM-adduct staining was observed in livers of LPS/RAN-treated rats (Figure 4.8D). No zonal specificity was observed, although staining appeared darker in midzonal and centrilobular regions. Quantification of PIM-adduct staining revealed statistically significant increases in PIM-adduct staining in livers of rats treated with LPS/Veh or Veh/RAN (Figure 4.8E). PIM-adduct staining in livers of LPS/RAN-treated rats was markedly greater (~10 times) than staining in livers of rats treated with LPS or RAN alone (Figure 4.8E).

Immunostaining for HIF-1α protein revealed mild and scattered nuclear staining in livers of Veh/Veh-treated rats (Figure 4.9A). Although livers were removed from the animals and frozen rapidly, this staining might have resulted from stabilization of HIF-1α during tissue removal. HIF-1α staining in livers of LPS/Veh-treated (Figure 4.9B) and Veh/RAN-treated (Figure 4.9C) rats also appeared as mild nuclear staining that was not significantly different from Veh/Veh-treated rats (Figure 4.9E). In livers from LPS/RAN-

treated rats, HIF-1α nuclear staining was significantly greater than in livers from rats treated with either agent alone.

4.5 Discussion

Previous studies demonstrated that LPS/RAN-treated rats develop liver injury characterized by midzonal hepatocellular necrosis and elevations in ALT and AST activities by 6 h (Luyendyk et al., 2003b). The studies presented here tested the hypothesis that the coagulation system is activated after LPS/RAN treatment. At a time before the onset of liver injury in LPS/RAN-treated rats (i.e., 2 h, Figure 4.2A), a pronounced decrease in plasma fibrinogen concentration (Figure 4.2B) was associated with a significant increase in plasma TAT concentration (Figure 4.2C). This result suggests that thrombin activation (i.e., activation of the coagulation system) occurred prior to liver injury. Treatment with LPS/Veh, which at this dose was not hepatotoxic within 24 h (Luyendyk et al., 2003b), caused a significant increase in plasma TAT concentration (Figure 4.2C), suggesting activation of thrombin; however, the degree of activation was insufficient to cause a decrease in plasma fibrinogen (Figure 4.2B). Interestingly, a slight but statistically significant decrease in plasma fibrinogen occurred without an increase in TAT concentration in Veh/RAN-treated rats (Figure 4.2B and C), suggesting consumption of fibrinogen that was not related to the action of thrombin. Other proteases, such as matrix metalloproteinases, can also degrade fibrinogen without coagulation system activation (Bini et al., 1996), but the cause for this decrease in Veh/RAN-treated rats is not understood. Overall, the data indicate that the coagulation

system is markedly activated after treatment with LPS/RAN before the onset of hepatic parenchymal cell injury.

One contributor to coagulation system activation in LPS/RAN-treated rats could be endothelial cell activation (Hewett and Roth, 1993; Colman, 1994). Confirming earlier results (Luyendyk et al., 2004c), the concentration of serum HA was elevated (~2.5-fold) in LPS/Veh-treated rats (Table 4.1) at 3 h. Veh/RAN treatment also increased serum HA. In LPS/RAN-cotreated rats, the effects of the two agents appeared to be additive at this time. No additional effect of Veh/RAN treatment occurred after 3 h. The rate of increase from 3-12 h was similar in both LPS-treated groups irrespective of RAN cotreatment (LPS/Veh, 19.1 ng/ml/h, r^2=0.99; LPS/RAN, 17.7 ng/ml/h, r^2=0.99). Taken together, these results suggest the occurrence of an early, transient effect of RAN and a sustained LPS effect on SEC function. The early changes in serum HA concentration were not accompanied by altered RECA-1 staining, suggesting the absence of SEC destruction. Between 12-24 h, the concentration of HA increased markedly in the cotreated rats. The latter change may be a consequence of overt liver injury (Luyendyk et al., 2003b). Overall, these results suggest that SEC dysfunction in LPS/RAN-treated rats occurred at a time prior to hepatocellular injury and to a greater degree than in LPS/Veh-treated rats. A modest increase in HA concentration has been reported previously at doses of LPS that do not cause hepatocellular injury (Yee et al., 2003b;Luyendyk et al., 2003a). Although its contribution is not fully understood, this perturbation of SEC function might be important for liver injury after LPS/RAN treatment. For example, HA has been reported to enhance expression of PAI-1 (Horton et al., 2000), increasing the likelihood of sustained fibrin clotting.

Figure 4.7: Effect of heparin on LPS/RAN-induced liver injury. LPS/RAN-treated rats were given heparin or saline 1 h after treatment with LPS. Plasma fibrinogen (A) and hepatic parenchymal cell injury were evaluated 6 h after RAN treatment. Hepatic parenchymal cell injury was estimated by increases in serum ALT (B) and AST (C) activities. n=3-9. Data are expressed as mean ± SEM. *Significantly different from Veh/Veh/Veh-treated rats. # Significantly different from LPS/Veh/RAN-treated rats. ($p<0.05$)

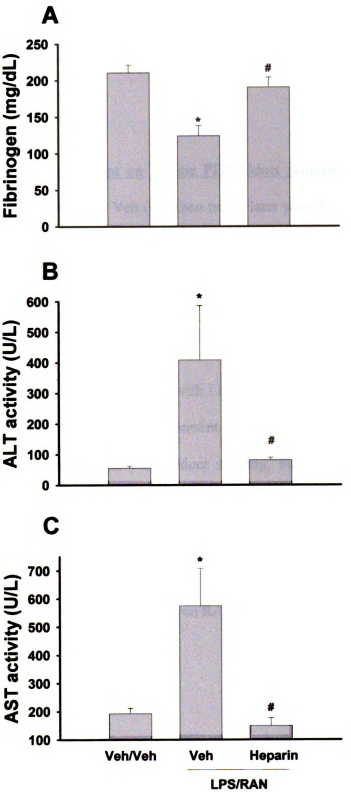
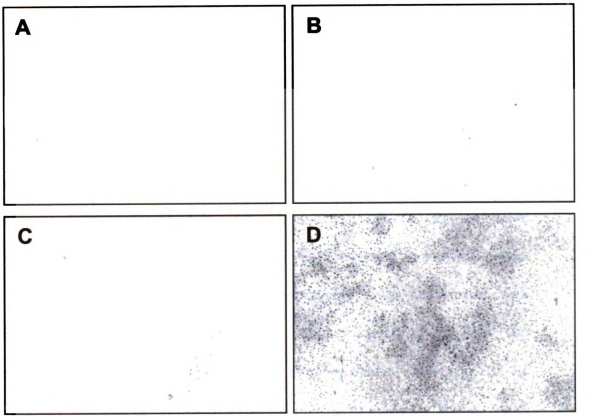

A
Fibrinogen (mg/dL)
250
200
150
100
50
0
*
#
B
ALT activity (U/L)
600
500
400
300
200
100
0
*
#
C
AST activity (U/L)
700
600
500
400
300
200
100
*
#
Veh/Veh
Veh
Heparin
LPS/RAN

Figure 4.8: Effect of LPS/RAN treatment on hepatic PIM-adduct staining. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then two h later with 30 mg/kg RAN or its Veh (iv). PIM (120 mg/kg, ip) was injected 2 h after RAN treatment. Livers were removed 3 h after RAN treatment and processed for PIM-adduct immunohistochemistry as described in *Materials and Methods*. (A) Representative photomicrograph of PIM-adduct staining in liver from a Veh/Veh-treated rat showing minimal staining (black). Representative photomicrographs from rats treated with LPS/Veh (B) and Veh/RAN (C) show a slight increase in PIM staining. (D) Representative image from LPS/RAN-cotreated rat showing marked, panlobular PIM-adduct staining. For (E), the area of positive PIM staining in 10 randomly chosen, 100X fields per tissue was determined morphometrically as described in *Materials and Methods*. Data are expressed as mean ± SEM. n=5-8 rats. *Significantly different from the respective group not given LPS; #Significantly different from respective group not given RAN ($p<0.05$)


A
B
C
D

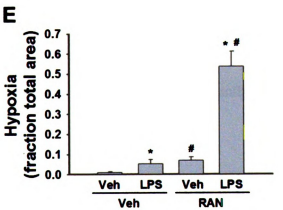
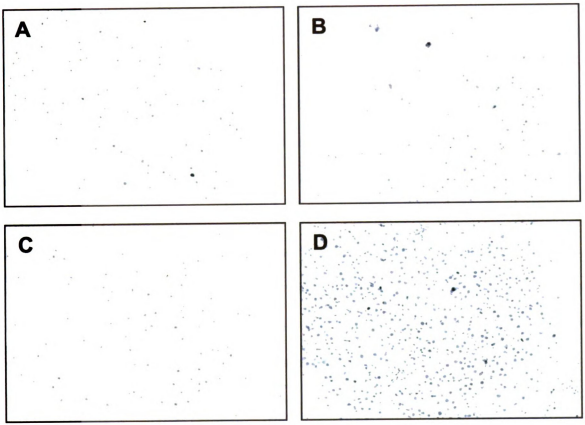
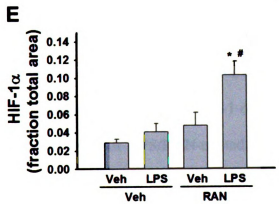

E
Hypoxia
(fraction total area)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*#
#
Veh
LPS
Veh
LPS
Veh
RAN

Figure 4.9: Effect of LPS/RAN treatment on hepatic HIF-1α staining. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then two h later with 30 mg/kg RAN or its Veh (iv). Livers were removed 3 h after RAN treatment and processed for HIF-1α immunohistochemistry as described in *Materials and Methods*. (A) Representative photomicrograph of HIF-1α staining in liver from a Veh/Veh-treated rat showing modest nuclear staining (black). Representative photomicrographs from rats treated with LPS/Veh (B) and Veh/RAN (C) show HIF-1α staining similar to Veh/Veh. (D) Representative image from LPS/RAN-cotreated rat showing marked panlobular HIF-1α staining. For (E), the area of positive HIF-1α staining in 10 randomly chosen, 100X fields per tissue was determined morphometrically as described in *Materials and Methods*. Data are expressed as mean ± SEM. n=5-8 rats *Significantly different from the respective group not given LPS; #Significantly different from respective group not given RAN ($p<0.05$)


A
B
C
D


E
HIF-1α
(fraction total area)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*,#
Veh
LPS
Veh
LPS
Veh
RAN

One interpretation of the results is that activation of the hemostatic system by LPS is magnified by RAN cotreatment. Treatment with LPS/Veh caused slight thrombin activation and the appearance of fibrin in the liver; both of these were more pronounced in LPS/RAN-treated rats (Figure 4.2, 4.3). Thus, activation of the coagulation system is associated with increased fibrin deposition in livers of LPS/RAN-treated rats. An impaired fibrinolytic system could also contribute to microvascular fibrin deposits by decreasing plasmin's capacity to degrade fibrin (Colman, 1994). PAI-1 is an important downregulator of plasmin activation and is expressed in animals and in cells after exposure to numerous agents, including cytokines and LPS (Binder et al., 2002; Hamaguchi et al., 2003; Sawdey and Loskutoff, 1991). In animal models of endotoxemia, antibody-mediated inhibition or genetic knockout of PAI-1 significantly attenuated fibrin deposition in tissues, suggesting that PAI-1 activity is important for this effect of LPS exposure (Montes et al., 2000; Abrahamsson et al., 1996; Savov et al., 2003). Interestingly, hepatic expression of the gene encoding PAI-1 is enhanced in LPS/RAN-treated rats (Luyendyk et al., 2004c), and this expression is mirrored by an augmented concentration of PAI-1 protein in serum (Figure 4.5). Moreover, the increase in serum PAI-1 persisted in LPS/RAN-treated rats, whereas it waned by 6 h in rats exposed only to LPS. Accordingly, persistent PAI-1 overexpression might contribute to stabilizing fibrin clots in livers of LPS/RAN-treated rats in the face of ongoing coagulation system activation.

Hepatic fibrin deposition occurs in numerous models of hepatotoxicity, and anticoagulants afford protection against hepatocellular injury in some of them (Luyendyk et al., 2003a; Fujiwara et al., 1988; Copple et al., 2002b; Yee et al., 2003d; Kinser et al.,

2002; Pearson et al., 1996b). In LPS/RAN-treated rats, anti-coagulation by heparin prevented the development of hepatocellular injury (Figure 4.7). In addition, SK treatment significantly reduced hepatic fibrin deposition as well as hepatic parenchymal cell injury (Figure 4.6). These results suggest that fibrin deposition is important for LPS/RAN-induced hepatotoxicity.

The mechanism by which fibrin clots cause toxicity in this model is not understood. One potential consequence of fibrin deposition is disruption of sinusoidal hepatic blood flow leading to hypoxia. For example, fibrin deposition and hypoxia precede centrilobular oncotic necrosis in rats treated with monocrotaline (MCT) (Copple et al., 2004a). The anticoagulant warfarin significantly reduces fibrin deposition, hypoxia, and hepatocellular injury after MCT exposure, suggesting a causal role for fibrin and hypoxia in the injury (Copple et al., 2002b; Copple et al., 2004a). Exposure to hypoxia alone is sufficient to cause hepatic parenchymal cell injury in isolated, perfused livers (Marotto et al., 1988; Lemasters et al., 1981). Interestingly, the severity of lesions caused by LPS is enhanced by exposing rats to a hypoxic atmosphere (Shibayama, 1987). Indeed, enhanced hepatic expression of hypoxia-regulated genes occurred in LPS/RAN-treated rats (Luyendyk et al., 2004c), and two markers of hypoxia were observed in livers of LPS/RAN-treated rats (Figure 4.8 and Figure 4.9,). Taken together, these results suggest that LPS/RAN-treatment results in liver hypoxia with a timeframe similar to that of hepatic fibrin deposition and hepatocellular injury.

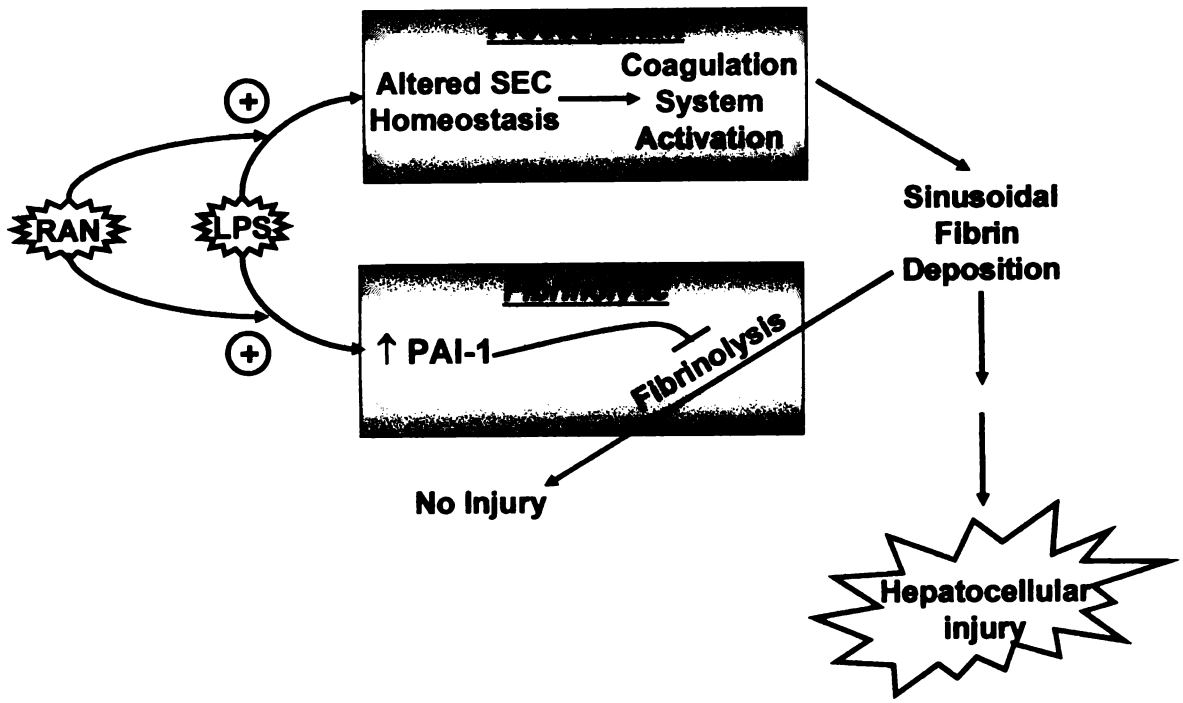
Appreciation of mechanisms by which the hemostatic system causes hepatotoxicity in the LPS/RAN rat model might yield insight into sensitivity of people to idiosyncratic RAN hepatotoxicity. A well-defined association between idiosyncratic

hepatotoxicity and hemostatic dysregulation has not been previously proposed, and epidemiological studies examining this relationship are lacking. As in the rat, RAN administration alone does not appear to enhance coagulation in people (Stadnicki, 1984). The results presented herein suggest, however, that RAN augments activation of the hemostatic system caused by exposure to inflammagens (e.g., LPS). Interestingly, a recent case report described RAN hepatotoxicity in a patient with a deficiency in the vitamin-K dependent anticoagulant factor, Protein S (Valois et al., 2003), suggesting that genetic predisposition favoring coagulation might be a susceptibility factor for RAN idiosyncrasy. Polymorphisms in several components of the hemostatic system, including PAI-1, have been identified (for review, see Lane and Grant, 2000) and could represent a potential interaction between genetic and environmental (i.e., LPS exposure) factors in causing idiosyncratic reactions. Furthermore, it seems reasonable to speculate that consequences of fibrin deposition such as tissue hypoxia might be important in drug idiosyncrasy. In this regard, LPS treatment results in liver injury in hypoxic rats exposed to halothane, an inhalation anesthetic associated with idiosyncratic hepatotoxicity (Lind et al., 1984). Another drug associated with infrequent hepatotoxicity during its clinical trials is the quinoxalinone anxiolytic, panadiplon (Ulrich et al., 2001), and interestingly, treatment of hepatocytes with panadiplon rendered them more sensitive to hypoxia-induced cell death (Bacon et al., 1996). Despite these associations, much remains to be understood about the interplay of inflammation, hypoxia, and the hemostatic system in idiosyncratic hepatotoxicity.

Figure 4.10 summarizes RAN's influence on LPS-induced activation of both the procoagulant and fibrinolytic arms of the hemostatic system and the relationship of this

action to liver injury. At a time prior to liver injury, RAN enhanced LPS-induced SEC dysfunction. Furthermore, a decrease in plasma fibrinogen, increase in TAT concentration and increase in hepatic fibrin deposition all occurred in livers of LPS/RAN-treated rats before the onset of liver injury. RAN cotreatment also caused a persistent increase in serum PAI-1 triggered by LPS beginning at a time before liver injury, suggesting that RAN impairs activity of the fibrinolytic arm of the hemostatic system. Liver injury from LPS/RAN treatment was significantly attenuated by activation of fibrinolysis with SK and abolished by inhibition of coagulation system activation by heparin. Overall, the results show that RAN can augment LPS-induced hepatic fibrin deposition in rats, leading to marked fibrin accumulation and tissue hypoxia as a functional consequence. Furthermore, the data suggest that this procoagulant state is critical for liver injury by a mechanism dependent on hepatic fibrin deposition.

Figure 4.10: Effect of RAN on LPS-induced fibrin deposition and the connection to liver injury. Nonhepatotoxic doses of LPS alter SEC homeostasis leading to a procoagulant state and modest fibrin deposition (see Figure 1, Table 1, and Fig 3). Additionally, LPS exposure increases expression of PAI-1 (see Figure 4). This response alone is not sufficient to cause parenchymal cell injury. RAN magnifies the effect of LPS on both activation of the coagulation arm of the hemostatic system and on inhibition of fibrinolysis by PAI-1, resulting in marked hepatic fibrin deposition (see Figure 4). Anticoagulation or activation of fibrinolysis decreases hepatocellular injury from LPS/RAN, indicating that fibrin clots are critical factors in the injury (see Figure 5 and Figure 6).


Altered SEC Homeostasis
Coagulation System Activation
RAN
LPS
↑ PAI-1
Fibrinolysis
No Injury
Sinusoidal Fibrin Deposition
Hepatocellular injury

Chapter 5

Role of coagulation system activation and liver PMN accumulation in LPS/RAN-treated rats

5.1 Abstract

Rats cotreated with normally noninjurious doses of bacterial lipopolysaccharide (LPS) and the histamine-2 (H2)-receptor antagonist ranitidine (RAN) develop idiosyncrasy-like hepatocellular injury characterized by midzonal necrosis and neutrophil (PMN) infiltration. Activation of the coagulation system and liver hypoxia occur in LPS/RAN-treated rats with a timecourse similar to the development of liver injury, and the anticoagulant, heparin, significantly reduces LPS/RAN-induced liver injury. We tested the hypothesis that an activated coagulation system is required for liver hypoxia in LPS/RAN-treated rats. Furthermore, the effect of anticoagulation on liver PMN accumulation was evaluated. Rats were given LPS (44.4 X 10^6 EU/kg) or its Veh, then two h later they were given RAN (30 mg/kg) or its Veh. LPS treatment caused an increase in the serum concentration of cytokine-induced chemoattractant-1 (CINC-1) and accumulation of PMNs in liver, neither of which were affected by RAN cotreatment. Confirming previous results, heparin treatment significantly attenuated hepatocellular injury in LPS/RAN-treated rats, as estimated by serum alanine aminotransferase (ALT) activity 3 and 6 h after drug administration. Heparin also reduced liver hypoxia in LPS/RAN-treated rats but was without effect on serum CINC-1 or liver PMN accumulation. *In vitro*, exposure to hypoxia rendered hepatocytes sensitive to killing by PMN elastase (PMN-E). Overall, the results suggest that the coagulation system is important for the generation of liver hypoxia after LPS/RAN-cotreatment and that hypoxia increases the sensitivity of HPCs to PMN-mediated killing.

5.2 Introduction

One possible consequence of hepatic fibrin deposition is disruption of blood flow and liver hypoxia. For example, rats treated with a hepatotoxic dose of monocrotaline (MCT) develop centrilobular liver injury that is preceded by hepatic fibrin deposition and hypoxia (Copple et al., 2002a; Copple et al., 2004b). The anticoagulant warfarin significantly reduced fibrin clot formation and liver hypoxia, and this was associated with decreased parenchymal cell injury (Copple et al., 2004a). Liver hypoxia was also observed in LPS/RAN-treated rats (Figure 4.8) at a time near the onset of hepatotoxicity (Luyendyk, 2004b), but the role of thrombin generated by LPS/RAN-cotreatment in causing hypoxia is not known.

Hypoxia/anoxia is sufficient to cause injury to isolated-perfused livers and cultured hepatocytes (Lemasters et al., 1981; Marotto et al., 1988; Khan and O'Brien, 1997). Hypoxia can also increase susceptibility to hepatotoxicity from certain agents. For example, LPS-induced liver injury is magnified in hypoxic rats (Shibayama, 1987), suggesting an increased susceptibility to liver injury from inflammatory mediators. The role of hypoxia and interactions between hypoxia and inflammation in LPS/RAN-treated rats is not known. It is possible that hypoxia caused by LPS/RAN magnifies the ability of inflammatory mediators to cause liver injury. As mentioned in chapter 1, several inflammatory mediators contribute to liver injury from large doses of LPS, including neutrophils (PMNs) and the mediators (e.g., ROS, elastase, cathepsin G) they release. In accordance, PMNs accumulated in liver lesions caused by LPS/RAN-treatment in rats (Luyendyk et al., 2003b; See chapter 2). Inasmuch as hypoxia and PMN accumulation occur concurrently, the potential exists for the two to interact. Although the release of

inflammatory mediators by isolated PMNs was blunted under hypoxic conditions *in vitro* (Derevianko et al., 1996), PMNs harvested from blood of people exposed to modest hypoxia showed enhanced release of cytotoxic mediators and delayed apoptosis *in vitro* (Tamura et al., 2002), suggesting an indirect priming of PMN function under conditions of hypoxia *in vivo*. In addition, hypoxia might alter sensitivity of hepatocytes to the toxic effects of PMN-derived cytotoxic factors. In one study, hypoxia/reoxygenation rendered cardiac myocytes sensitive to killing by PMN elastase during reoxygention (Buerke et al., 1994). However, the effect of hypoxia on PMN accumulation/activation has not been investigated in the LPS/RAN model, and the effect of hypoxia on sensitivity of hepatocytes to the toxic effects of PMN-derived products is not understood.

The purpose of this study was to test the hypothesis that LPS/RAN-treatment causes liver hypoxia by a mechanism dependent on thrombin activation. Furthermore, the effect of anticoagulation on hepatic PMN accumulation was evaluated. To this end, the effect of heparin treatment on markers of hepatocellular injury, fibrin deposition, and liver hypoxia was evaluated after LPS/RAN treatment. The plasma concentration of the PMN chemokine cytokine-induced chemoattractant-1 (CINC-1) and hepatic PMN accumulation were evaluated at a time near the onset of injury in LPS/RAN-treated rats, and the effect of heparin coadministration on each was assessed. In addition, an *in vitro* system was developed to test the hypothesis that hypoxia renders hepatocytes sensitive to killing by PMN elastase. The implications of these results and ongoing experiments are discussed.

5.3 Materials and Methods

5.3.1 Materials

For information on this topic please refer to Chapter 2 Materials and Methods.

5.3.2 Animals

For information on this topic please refer to Chapter 2 Materials and Methods.

5.3.3 Experimental Protocol

Rats fasted for 24 hours were given 44.4 X 10^6 EU/kg LPS or its saline vehicle (Veh) iv and food was then returned. Two hours later, 30 mg/kg RAN or sterile phosphate-buffered saline (PBS) Veh was administered iv. RAN solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. To simplify treatment nomenclature for the four groups, the following designations will be applied: Saline/PBS (Veh/Veh), LPS/PBS (LPS/Veh), Saline/RAN (Veh/RAN), and LPS/RAN. Three or 6 h after RAN treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Plasma was collected by drawing blood from the vena cava into a syringe containing sodium citrate (final concentration, 0.38%), and rats were then killed by exsanguination from the dorsal aorta. This blood was allowed to clot at 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.

Inhibition of coagulation system activation was achieved by administration of heparin. Rats were treated with LPS/RAN as above, but 1 h before RAN treatment,

heparin (3000 U/kg, s.c.) or sterile saline was administered. Rats were killed 3 or 6 h after RAN administration, and serum and liver samples were taken.

5.3.4 Hepatotoxicity Assessment

For information on this topic please refer to Chapter 4 Materials and Methods.

5.3.5 Fibrin immunohistochemistry and quantification

For information on this topic please refer to Chapter 3 Materials and Methods.

5.3.6 Evaluation of liver hypoxia

For information on this topic please refer to Chapter 4 Materials and Methods.

5.3.7 Evaluation of hepatic PMN accumulation and serum CINC-1 concentration

PMN immunohistochemistry was performed on formalin-fixed liver sections as described previously (Yee et al., 2003c). Hepatic PMN accumulation was evaluated by identifying the average number of PMNs counted in 20 randomly selected, high-powered fields (HPF, 400X). Slides were coded and the evaluator was unaware of treatment. The serum concentration of CINC-1 was determined using a commercially available, enzyme-linked immunosorbent assay (ELISA) purchased from Assay Designs, Inc. (Ann Arbor, MI).

5.3.8 Hepatocyte isolation

Hepatocytes were allowed to attach to plates in 5% Cosmic Calf Serum (Hyclone, Logan, UT). Otherwise, for information on this topic please refer to Chapter 3 Materials and Methods.

5.3.9 Effect of hypoxia on elastase-induced cytotoxicity

Serum free Williams' Medium E containing various concentrations (0, 0.7, 1.3, 2.2, 3.3, 4.4, 6.6, 8.8 units (U) of activity/ml) of human PMN elastase (PMN-E, Molecular Innovations, Southfield, MI) was added to hepatocytes. PMN-E activity was determined using a colorometric PMN-E substrate MeOSuc-Ala-Ala-Pro-Val-pNA (Calbiochem, San Diego, CA). One unit of PMN-E activity was defined as the amount of PMN-E (i.e. μg) of enzyme required to cause a change of 1.0 absorbance unit at 410 nm in 10 minutes at 37 degrees C. PMN-E-treated cells were immediately transferred to incubators containing either 20% or 5% oxygen (O_2) (balanced with nitrogen (N_2)), with carbon dioxide (CO_2) controlled at 5%. Two or 8 h later, the medium was collected, and the remaining attached cells were lysed immediately with 1% triton X-100 followed by brief sonication. Media and lysates were centrifuged at 600xg for 5 min. Cytotoxicity was assessed by measuring ALT release into the medium using Infinity-ALT reagent from Thermo Electron Corp. (Louisville, CO). ALT activity in the medium was expressed as a percent of total ALT activity (i.e., medium activity plus lysate activity).

5.3.10 Statistical Analysis

Two-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was used for the heparin study and for CINC-1 and PMN accumulation

studies. Repeated measures two-way ANOVA was applied for *in vitro* experiments. The criterion for significance for all studies was $p<0.05$.

5.4 Results

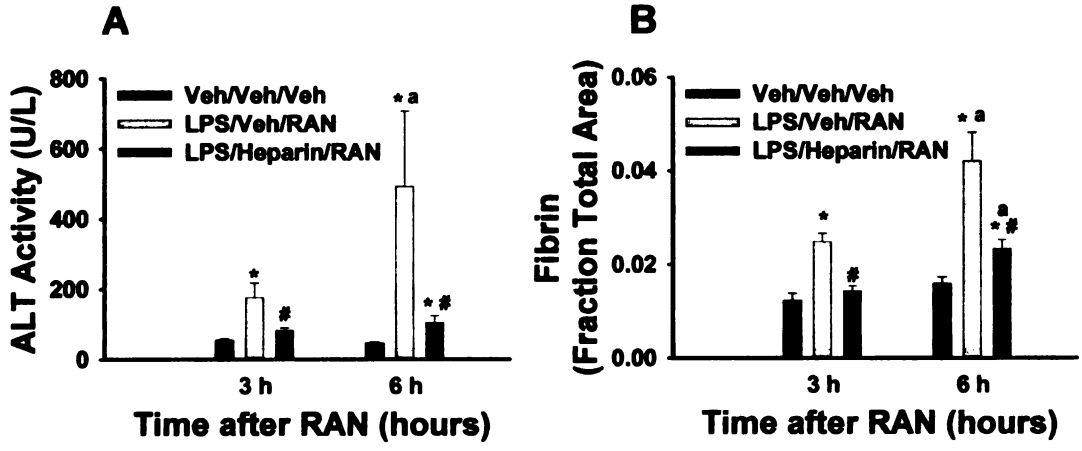
5.4.1 Effect of heparin on hepatotoxicity after LPS/RAN treatment

Given alone, the doses of LPS or RAN are not hepatotoxic up to 24 h after administration (Luyendyk et al., 2003b). Previous studies have shown that the coagulation system is a critical mediator of LPS/RAN-induced liver injury (Luyendyk, 2004b). Confirming these results, LPS/RAN-cotreatment caused a significant increase in serum ALT activity, and this was greatly attenuated by heparin at 6 h (Figure 5.1A), a time associated with maximal injury in LPS/RAN-treated rats (Luyendyk et al., 2003b). Complementing this result, heparin completely prevented the slight increase in ALT activity observed at 3 h in this study (Figure 5.1A), a time near the onset of LPS/RAN-induced liver injury (Luyendyk et al., 2004b; Luyendyk et al., 2003b). Heparin efficacy was confirmed by immunohistochemical staining for hepatic fibrin deposition. Heparin prevented or significantly attenuated LPS/RAN-induced hepatic fibrin deposition at 3 and 6 h, respectively (Figure 5.1B).

5.4.2 Effect of heparin on liver hypoxia after LPS/RAN treatment

Previous studies demonstrated that hepatic fibrin deposition is important for LPS/RAN-induced liver injury, and that one consequence of fibrin deposition, i.e., liver hypoxia, occurs at time near the onset of liver injury (Luyendyk, 2004b). Accordingly, the effect of the anticoagulant heparin on liver hypoxia was evaluated using PIM

Figure 5.1: Effect of heparin on hepatotoxicity after LPS/RAN treatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then one h later with 3000 U/kg heparin or its Veh (s.c.). One h later, 30 mg/kg RAN or its Veh was administered (iv). For (A), hepatic parenchymal cell injury was estimated 3 or 6 h after RAN administration by increases in serum ALT activity. For (B), livers were removed 3 or 6 h after RAN treatment and processed for fibrin immunohistochemistry, and the area of positive fibrin staining in 10 randomly chosen, 100X fields per tissue was determined morphometrically. Data are expressed as mean ± SEM. n=6-9 in each group. *Significantly different from Veh/Veh/Veh-treated rats at that time. #Significantly different from LPS/Veh/RAN-treated rats at that time. [a]Significantly different from the same treatment at 3 h. ($p<0.05$).


A
ALT Activity (U/L)
800
600
400
200
0
Veh/Veh/Veh
LPS/Veh/RAN
LPS/Heparin/RAN
*a
*
#
*#
3 h
6 h
Time after RAN (hours)
B
Fibrin
(Fraction Total Area)
0.06
0.04
0.02
0.00
Veh/Veh/Veh
LPS/Veh/RAN
LPS/Heparin/RAN
*a
*
#
a
*#
3 h
6 h
Time after RAN (hours)

immunohistochemical staining. Little PIM-adduct staining was noted in livers of Veh/Veh-treated rats (Figure 5.2A). As described previously (Luyendyk, 2004b), PIM-adduct staining was significantly increased in livers of LPS/RAN-treated rats at 3 h, and this staining persisted to 6 h (Figure 5.2D). Heparin significantly attenuated the increase in PIM-adduct staining at both 3 and 6 h (Figure 5.2D).

5.4.3 Serum CINC-1 concentration and PMN accumulation in livers of LPS/RAN-treated rats

Accumulation of PMNs in liver and serum CINC-1 concentration were evaluated at a time near the onset of injury in LPS/RAN-treated rats (i.e., 3 h). In this study, serum ALT activity was not significantly increased in LPS/RAN-treated rats compared to Veh/Veh- treated rats at this time (data not shown). CINC-1, the rat homologue of human interleukin 8, is important for PMN accumulation in liver and lungs after exposure to LPS (Zhang et al., 1995;Yamasawa et al., 1999). Treatment with RAN alone caused a 10-fold increase in serum CINC-1 concentration (Figure 5.3A). CINC-1 was increased much more dramatically (~900-fold) in rats treated with LPS alone, and was increased similarly in LPS/RAN-cotreated rats. PMNs accumulated in livers of LPS-treated rats and to a lesser degree in LPS/RAN-treated rats at 3 h (Figure 5.3B). Treatment with RAN alone appeared to cause a small increase in PMN accumulation that did not reach statistical significance compared to Veh/Veh-treated rats (Figure 5.3B). Histological evaluation revealed that PMNs were distributed evenly across the liver lobule in LPS/Veh-treated rats. A similar distribution was observed in LPS/RAN-treated rats, although infrequent midzonal and subserosal clusters of PMNs were noted in some rats.

Figure 5.2: Effect of heparin on liver hypoxia in LPS/RAN-treated rats. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then one h later with 3000 U/kg heparin or its Veh (s.c.). One h later, 30 mg/kg RAN or its Veh was administered (iv). Livers were removed 3 or 6 h after RAN treatment and processed for PIM-adduct immunohistochemistry as described in *Materials and Methods*. (A) Representative photomicrograph (100X) showing little PIM-adduct staining in a liver from a Veh/Veh/Veh-treated rats. (B) Representative photomicrograph (100X) showing marked panlobular PIM-adduct staining in a liver from a LPS/Veh/RAN-treated rat. (C) Representative photomicrograph (100X) showing reduced staining intensity compared to LPS/Veh/RAN as a result of heparin administration. The area of positive PIM-adduct staining in 10 randomly chosen, 100X fields per tissue was determined morphometrically as described in *Materials and Methods*. Data are expressed as mean ± SEM. n=6-9 rats per group. *Significantly different from Veh/Veh/Veh-treated rats at that time. #Significantly different from LPS/Veh/RAN-treated rats.

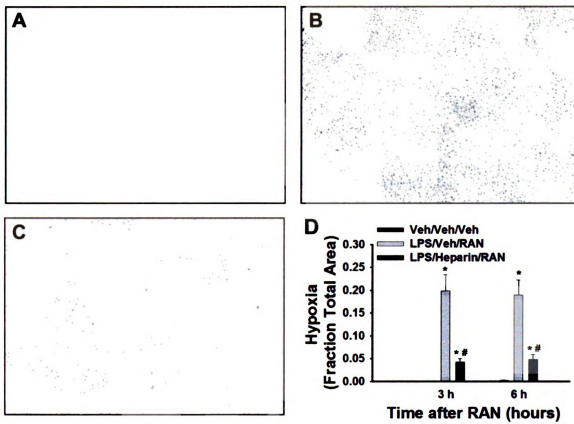
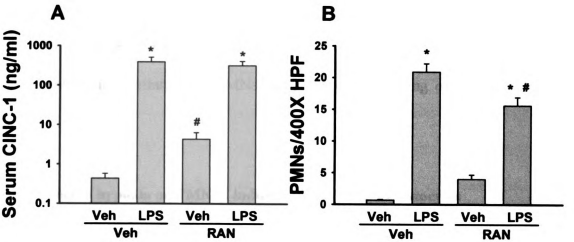

A
B
C
D
Veh/Veh/Veh
LPS/Veh/RAN
LPS/Heparin/RAN
Hypoxia
(Fraction Total Area)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
*#
*
*#
3 h
6 h
Time after RAN (hours)

Figure 5.3: Serum CINC-1 concentration and PMN accumulation in livers of LPS/RAN-treated rats. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then two h later with 30 mg/kg RAN or its Veh (iv). The serum concentration of CINC-1 (A) and hepatic PMN accumulation (B) were evaluated 3 h after drug treatment. Data are expressed as mean ± SEM. n=3-4 rats in each group. *Significantly different from respective group not given LPS #Significantly different from respective group not given RAN ($p<0.05$).


A
Serum CINC-1 (ng/ml)
1000
100
10
1
0.1
*
*
#
Veh
LPS
Veh
LPS
Veh
RAN
B
PMNs/400X HPF
25
20
15
10
5
0
*
* #
Veh
LPS
Veh
LPS
Veh
RAN

5.4.4 Effect of heparin on serum CINC-1 concentration and PMN accumulation in livers of LPS/RAN-treated rats

LPS/Veh/RAN-treatment caused a significant increase in serum CINC-1 at 3 h that persisted at 6 h (Figure 5.4A), and this increase was unaffected by heparin cotreatment at either time. Consistent with the change in serum CINC-1, PMNs accumulated in livers of LPS/RAN-treated rats at 3 h and their presence persisted at 6 h (Figure 5.4B). PMNs were distributed across the hepatic lobule in LPS/RAN-treated rats, but more midzonal and subserosal clusters were observed in lesioned areas at 6 h. PMN accumulation in LPS/RAN-treated rats was unaffected by heparin cotreatment at both times. However, the reduction in hepatocellular injury by heparin was associated with a more panlobular distribution of PMNs as opposed to clustering of PMNs in lesioned areas.

5.4.5 Effect of hypoxia on PMN-E induced killing of rat hepatocytes

Preliminary experiments were conducted to identify a reduced O_2 exposure not associated with significant cytotoxicity, as estimated by release of ALT into culture medium 8 h after exposure. Incubation of hepatocytes for 8 h in 2% O_2 but not in 5% O_2 caused significant ALT release relative to exposure to an oxygen replete atmosphere (i.e., 20% O_2) (data not shown). Accordingly, 5% O_2 was chosen as an hypoxic chamber concentration that did not by itself cause cytotoxicity. At 8 h, 8.8 U/ml elastase caused significant ALT release from hepatocytes cultured in 20% O_2, consistent with the timecourse of PMN-CM-mediated cytotoxicity (Ganey et al., 1994). However, exposure to 5% O2 resulted in a leftward shift in PMN-E-induced cytotoxicity at this time

Figure 5.4: Effect of heparin on serum CINC-1 concentration and hepatic PMN accumulation in LPS/RAN-treated rats. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then one h later with 3000 U/kg heparin or its Veh (s.c.). One h later, 30 mg/kg RAN or its Veh was administered (iv). Serum CINC-1 concentration (A) and hepatic PMN accumulation (B) were evaluated 3 or 6 h after drug administration. Data are expressed as mean ± SEM. n=4-9 rats in each group. *Significantly different from Veh/Veh/Veh-treated rats at that time. #Significantly different from LPS/Veh/RAN-treated rats. &Significantly different from the same treatment at 3 h. ($p<0.05$).

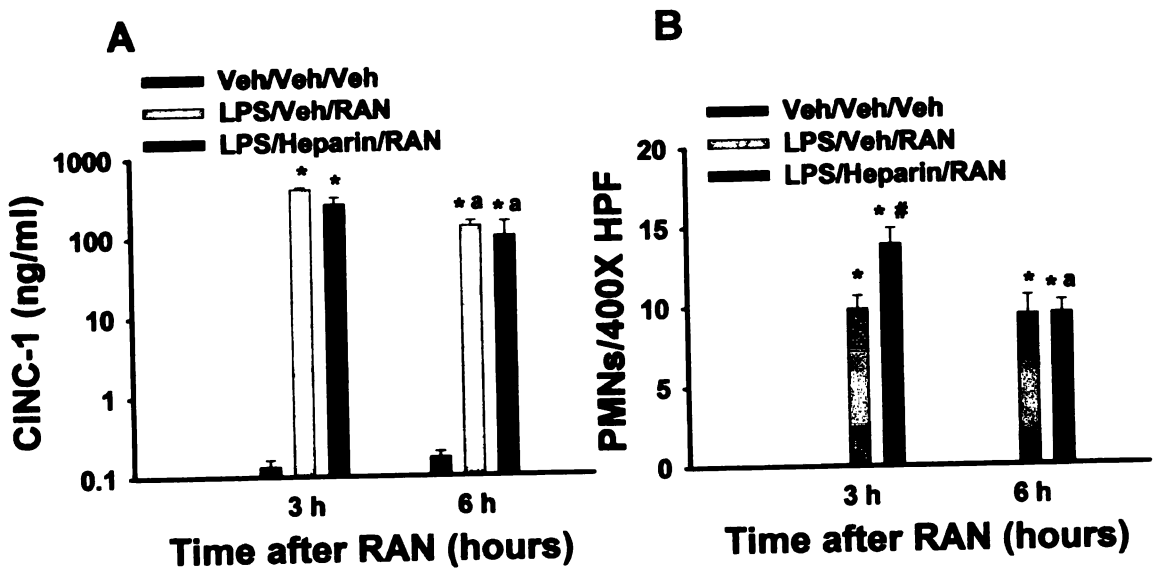
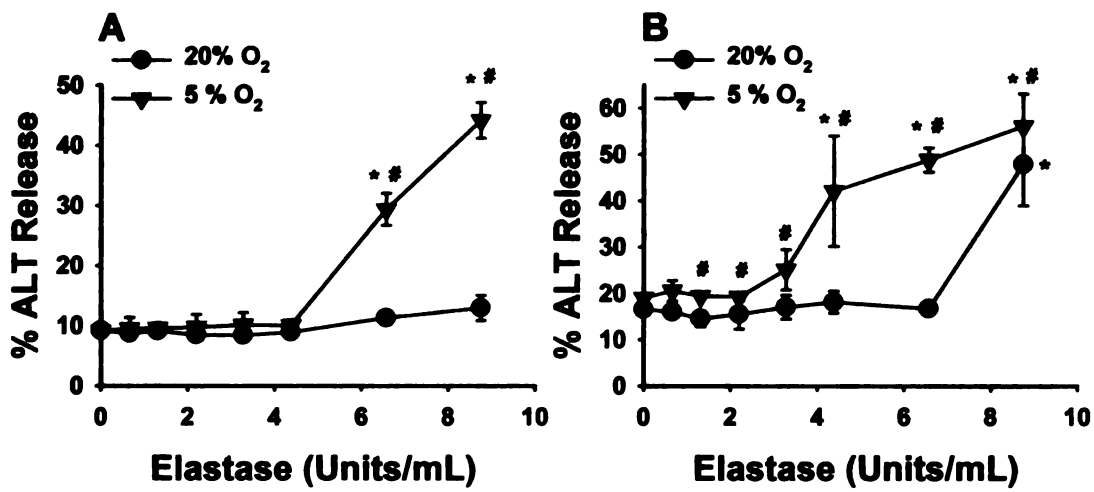

A
Veh/Veh/Veh
LPS/Veh/RAN
LPS/Heparin/RAN
CINC-1 (ng/ml)
1000
100
10
1
0.1
3 h
6 h
Time after RAN (hours)
B
Veh/Veh/Veh
LPS/Veh/RAN
LPS/Heparin/RAN
PMNs/400X HPF
20
15
10
5
0
3 h
6 h
Time after RAN (hours)

Figure 5.5: Effect of hypoxia on PMN-E-induced killing of rat hepatocytes. Rat hepatocytes were cultured at a cell density of 2.5 X 10^5 cells/ml in Williams' Medium E containing 5% CCS. Two h later, the medium was changed to serum-free medium containing human PMN elastase (PMN-E) at a concentration of 0, 0.7, 1.3, 2.2, 3.3, 4.4, 6.6, or 8.8 U/ml, and incubated in either 5% or 20% O_2. Cytotoxicity was evaluated as ALT released into culture medium 2 h (A) or 8 h (B) later. Data are expressed as mean ± SEM. n=3 separate hepatocyte isolations. *Significantly different from respective treatment with 0 U/ml PMN-E. # Significantly different from the respective treatment incubated in 20% O_2 ($p<0.05$).



These data were generated by Patrick Shaw

(Figure 5.5B). Contrasting its ability to kill hepatocytes at 8 h, PMN-E did not cause significant ALT release at any concentration used when hepatocytes were cultured in 20% O_2 for 2 h (Figure 5.5A). However, PMN-E at 6.6 and 8.8 U/ml caused significant ALT release from hepatocytes cultured in 5% O_2 at this time (Figure 5.5A).

5.5 Discussion

Previous experiments demonstrated that LPS/RAN-cotreatment caused hepatotoxicity in rats beginning near 3 h (Luyendyk et al., 2003b). Liver injury in this model is dependent upon an activated coagulation system and the formation of fibrin clots (Luyendyk, 2004b). In addition, liver hypoxia, one possible consequence of hepatic fibrin deposition, occurred in livers of LPS/RAN-cotreated rats. The studies presented here tested the hypothesis that an activated coagulation system causes liver hypoxia in LPS/RAN-treated rats. Heparin administration prevented the slight increase in serum ALT activity and hepatic fibrin deposition observed at 3 h, and confirming previous results (Luyendyk, 2004b), it also attenuated this increase at 6 h (Figure 5.1A). Heparin administration significantly decreased liver PIM-adduct staining by nearly 70% (Figure 5.2), suggesting that coagulation system activation is responsible for the majority of liver hypoxia in LPS/RAN-treated rats. Overall, these results support the hypothesis that hepatic fibrin deposition causes liver hypoxia and hepatotoxicity in LPS/RAN-treated rats. However, whether or not the protective effect of heparin in this model is mediated entirely through its ability to reduce liver hypoxia is not known.

If severe enough, hypoxia alone is sufficient to cause hepatocellular injury in rats (Fassoulaki et al., 1984) and in isolated perfused livers (Lemasters et al., 1981), and it is

capable of inciting cell death in hepatocytes (Khan and O'Brien, 1997). Furthermore, exposure to hypoxia can render the liver and/or isolated hepatocytes sensitive to secondary insult from hepatotoxicants and some drugs (Bacon et al., 1996; Khan and O'Brien, 1995; Silva et al., 1992; McGirr et al., 1990; Khan and O'Brien, 1997; Shen et al., 1982) Overall, these results suggest that otherwise noninjurious degrees of hypoxia can lower the threshold for hepatotoxicity. Lesions in livers of rats coexposed to hypoxia and LPS were LPS-like (Shibayama, 1987), suggesting that hypoxia increases either the degree of LPS-induced inflammation or the sensitivity of the liver to the cytotoxic effects of inflammatory mediators. Indeed, interplay between hypoxia and some inflammatory factors, including macrophages, COX-2, and PMNs, has been reported (Hannah et al., 1995; Zhong et al., 2004; Lahat et al., 2003; Tamura et al., 2002).

PMN accumulation was noted in lesions within livers taken from LPS/RAN-treated rats (Luyendyk et al., 2003b), but their role in this model remains unclear. Treatment with LPS alone caused quantitatively similar increases in serum CINC-1 and hepatic accumulation of PMNs as compared to LPS/RAN at a time near the onset of liver injury in LPS/RAN-treated rats (Figure 5.3). Inasmuch as liver injury does not occur in rats treated with this dose of LPS given alone, this suggests that PMN accumulation is not sufficient to cause hepatotoxicity. A similar observation has been made in livers of rats cotreated with aflatoxin B_1 (AFB_1)/LPS, where PMN accumulation is nearly identical in rats treated with LPS alone; however, PMNs are required for hepatotoxicity in AFB_1/LPS-cotreated rats. Indeed, PMN extravasation rather than PMN accumulation is likely a required event for PMN-mediated hepatocellular damage in models of endotoxemia (Chosay et al., 1997; Jaeschke and Smith, 1997).

Accumulation of PMNs in hepatic sinusoids is independent of adhesion molecules (i.e., VCAM-1, ICAM-1, CD11a/b/CD18) in models of endotoxemia; however, they are required for extravasation of PMNs in the liver and hepatocellular injury (Jaeschke et al., 1996; Jaeschke et al., 1991; Essani et al., 1997; Essani et al., 1995). In addition, chemokine production by hepatocytes can trigger PMN extravastation and tissue injury (Maher et al., 1997; Li et al., 2004). Overall, in light of these results, the possibility that PMNs are important for injury in the LPS/RAN model should not be diminished by the observation that PMN accumulation occurs to a similar degree in both LPS-treated groups. Experiments are underway to evaluate activation of PMNs and the relationship of PMN activation to hepatocellular injury in LPS/RAN-treated rats.

PMN accumulation or activation might occur as a consequence of thrombin generation in LPS/RAN-treated rats. Thrombin and PMNs are sufficient extrahepatic factors to cause hepatocellular injury in isolated, perfused livers from rats treated with large doses of LPS (Moulin et al., 2001), and inhibition of thrombin attenuates PMN activation in rats treated with a large dose of LPS (Copple et al., 2003). Heparin administration did not significantly affect serum CINC-1 or accumulation of PMNs in livers of LPS/RAN-treated rats at any timepoint examined (Figure 5.4). This result suggests that PMN accumulation and increased serum CINC-1 after LPS/RAN-cotreatment occur by a thrombin-independent mechanism. Experiments are underway to test the hypothesis that thrombin activation precedes and is required for PMN activation in LPS/RAN-treated rats. Thrombin can indirectly cause activation of PMNs through a mechanism requiring activation of its receptor, protease activated receptor-1, which is expressed by KCs, SECs, and hepatic stellate cells, but not on PMNs (Copple et al., 2003;

Marra et al., 1998). Another possibility is that thrombin-mediated liver hypoxia modifies PMN accumulation or activation. However, the decrease in liver hypoxia accompanying heparin administration was not associated with a decrease in PMN accumulation in LPS/RAN-treated rats. Nevertheless, hypoxia might promote PMN activation directly, or indirectly through other mechanisms, including altered expression of adhesion molecules on SECs (Tamura et al., 2002; Arnould et al., 1995; Arnould et al., 1994).

PMNs release several cytotoxic factors including the proteases, cathepsin G and elastase, which are sufficient to kill rat hepatocytes *in vitro* (Ho et al., 1996). In addition to possibly enhancing activation of PMNs and release of these proteases, hypoxia might also increase the sensitivity of hepatic parenchymal cells to their cytotoxic effects. Few studies have examined this relationship. In one cell-based model of hypoxia/reoxygenation, cardiac myocytes were rendered more sensitive to elastase cytototoxicity during the reoxygenation phase (Buerke et al., 1994). However, the relative role of hypoxia and reoxygenation phases in increasing sensitivity to elastase cannot be separated. We show here that hepatocytes cultured under hypoxic (5% O_2) conditions are rendered sensitive to killing by normally nontoxic concentrations of PMN-E (Figure 5.5). Interestingly, the timecourse over which hepatocyte killing occurs resembles the timecourse of injury (i.e., beginning near 2-3 h) in the LPS/RAN model (Luyendyk et al., 2003b), whereas killing of hepatocytes by PMN-E in an oxygen-replete atmosphere (20% O_2) requires 8-16 h to reach statistical significance (Ganey et al., 1994). This result suggests that hypoxia can increase the sensitivity to elastase cytotoxicity, as well as accelerate signaling required for PMN-E-induced cell death.

In summary, rats cotreated with LPS/RAN develop liver hypoxia with a timecourse similar to that of hepatocellular injury (Luyendyk et al., 2003b). Anticoagulation with heparin significantly attenuated hepatic fibrin deposition, hypoxia, and hepatocellular injury, suggesting a connection between these 3 events. PMN accumulation and the serum concentration of CINC-1 in LPS/RAN-treated rats were not different from rats treated with LPS alone, and neither of these was affected by heparin administration. Overall, the results are consistent with reduction of LPS/RAN-induced liver injury by heparin through a mechanism related to prevention of fibrin deposition and liver hypoxia, but independent of PMN accumulation. Inasmuch as hypoxia rendered hepatocytes sensitive to killing by PMN-E, PMN activation might be important for injury in livers of LPS/RAN-treated rats. Further studies are required to evaluate the modulating effects of anticoagulation and hypoxia on PMN activation and their connection to injury in this model.

CHAPTER 6

Summary and Conclusions

6.1 Summary of conducted research

Initially, the hypothesis was tested that modest underlying inflammation renders ranitidine hepatotoxic in rats. Dose-response experiments were conducted to identify doses of LPS and RAN that when given alone to rats did not cause hepatotoxicity. In the case of RAN, a dose that would cause hepatotoxicity without significant mortality was not identified. Preliminary dose-ranging studies were performed to identify doses of LPS and RAN that when coadministered produced maximal hepatotoxicity by 24 h (Figure 2.1). Rats treated with a nonhepatotoxic dose of LPS 2 h before a noninjurious dose of RAN developed hepatic parenchymal cell injury (i.e., increased serum ALT and AST activities, Figure 2.2) by 6 h after drug treatment. The primary histopathologic finding in livers of LPS/RAN-treated rats was midzonal oncotic necrosis with neutrophilic infiltrate (Figure 2.3). This lesion increased in frequency and severity between 3 and 6 h in LPS/RAN-treated rats (Table 2.1). As noted earlier, some similarities can be identified between injury in the LPS/RAN model and case reports of RAN hepatotoxicity in people (Table 1.1). For example, increased serum activities of markers of hepatic parenchymal cell injury occured more frequently and were often more dramatic than changes in biomarkers of cholestatic injury. Moreover, histopathologic changes in livers of LPS/RAN-treated rats resemble some changes observed in severe cases of RAN hepatotoxicity in people, including hepatocellular necrosis with inflammatory infiltrate. Overall, these results suggest that RAN is rendered hepatotoxic in rats undergoing a modest inflammatory response.

In a subsequent study, LPS/RAN treatment caused significant hepatotoxicity, whereas cotreatment of rats with the same dose of LPS and FAM at either an equi-

efficacious (EE) or equimolar dose as RAN did not result in significant injury (Figure 2.4). Accordingly, the propensity of each H2-antagonist to cause idiosyncratic hepatotoxicity was predicted by the potential of each to be rendered hepatotoxic by inflammation. Furthermore, this result suggests that H2-antagonism is not sufficient to cause hepatotoxicity in this model. Similar results were observed *in vitro* when rat hepatocytes were exposed to PMN-conditioned medium. RAN treatment did not cause significant cytotoxicity at any concentration tested, but it did render hepatocytes sensitive to the cytotoxic effects of PMN-CM (Figure 2.5). Matching the *in vivo* result, FAM treatment did not cause this effect. This suggests that, at least in part, RAN might alter hepatocellular sensitivity to cytotoxic factors released by activated PMNs. However, the millimolar concentrations required to cause this effect are likely not reached in RAN-treated rats, so that the relevance of this observation to the mechanism of LPS/RAN-hepatotoxicity is therefore unclear.

Inasmuch as changes in gene expression are important for manifestation of inflammatory responses, hepatic gene expression was evaluated by Affymetrix microarray analysis in rats treated with LPS and/or RAN at a time just before the development of significant hepatic parenchymal cell injury (i.e., 3 h, Figure 3.1). At this time, hierarchical clustering of active genes was sufficient to segregate rats to their respective treatments (Figure 3.2). Several probesets were changed only after treatment with LPS, RAN, or LPS/RAN, and others were changed after more than one treatment (Figure 3.3). Within the population of active genes, 4 patterns of potential mechanistic interest were identified. Genes changing similarly after treatment with LPS alone or RAN alone and in LPS/RAN-cotreated rats (LPS $\cong$ LPS/RAN, RAN $\cong$ LPS/RAN) were

involved in inflammation and/or associated with cell death signaling (Table 3.1). However, treatment with RAN or LPS alone did not cause hepatotoxicity, suggesting that the products of genes with this expression pattern are likely not sufficient to cause injury. They might, however, interact with other changes to cause toxicity.

RAN cotreatment attenuated the LPS-induced change in several genes (LPS/RAN < LPS, Table 3.1). This group of genes might point to an ability of RAN to prevent upregulation of cytoprotective or anti-inflammatory genes. Other genes were increased to a greater degree in LPS/RAN-treated rats as compared to any other treatment group (LPS/RAN > LPS and > RAN, Table 3.1). Gene products in this group were related to inflammation and many were hypoxia-inducible, including PAI-1, Egr-1, Btg-2, and others (Table 3.2). The change in expression for three of these (PAI-1, Egr-1, Btg-2) was confirmed by real-time PCR (Figure 3.4). Inasmuch as injury only occurs in the LPS/RAN group, augmented expression of one or more of these gene products might be involved in the injury. Further investigation and data-mining may reveal interactions and relationships between these genes as they relate to LPS/RAN-induced liver injury.

Alterations in hepatic gene expression elucidated by microarray analysis suggested altered liver homeostasis (i.e., hypoxia) and involvement of the hemostatic system (i.e., increased PAI-1 mRNA) in LPS/RAN-induced liver injury. Indeed, the change in PAI-1 mRNA in liver was reflected by a change in serum PAI-1 concentration at 3 h (Figure 3.5). Further examination revealed a persistently augmented serum PAI-1 concentration in LPS/RAN-treated rats, and a less robust increase in rats treated with LPS that faded by 6 h (Figure 4.5). Consistent with the change in serum PAI-1 concentration, hepatic fibrin deposition was observed in livers of LPS/RAN-treated rats both before

(Figure 3.7 and 4.3) and after (Figure 4.4) the development of liver injury. SK treatment significantly reduced hepatic fibrin deposition and parenchymal cell injury in LPS/RAN-treated rats (Figure 4.6). The results suggest that hepatic fibrin deposition is a mediator of liver injury in LPS/RAN-treated rats and are consistent with impaired fibrinolysis as a consequence of augmented PAI-1 expression.

Hepatic fibrin deposition might result from activation of the coagulation system, impaired fibrinolysis, or both. Serum HA was increased in LPS/RAN-treated rats in the absence of a change in RECA-1 staining in liver, suggesting perturbation in the function of SECs in the absence of overt cell destruction (Figure 3.6 and Table 4.1). In addition to causing increased PAI-1 production, altered SEC function can result in activation of the coagulation system. Indeed, LPS/RAN-treatment caused coagulation system activation prior to hepatocellular injury. Inhibition of coagulation with heparin significantly reduced LPS/RAN-induced fibrin deposition and hepatic parenchymal cell injury (Figure 4.7 and 5.1). Overall, the results suggest that RAN augments LPS-mediated activation of the hemostatic system (Figure 4.20). In light of the protection provided by SK in this model, the results suggest that the protection provided by heparin is likely mediated by its prevention of hepatic fibrin deposition in LPS/RAN-treated rats.

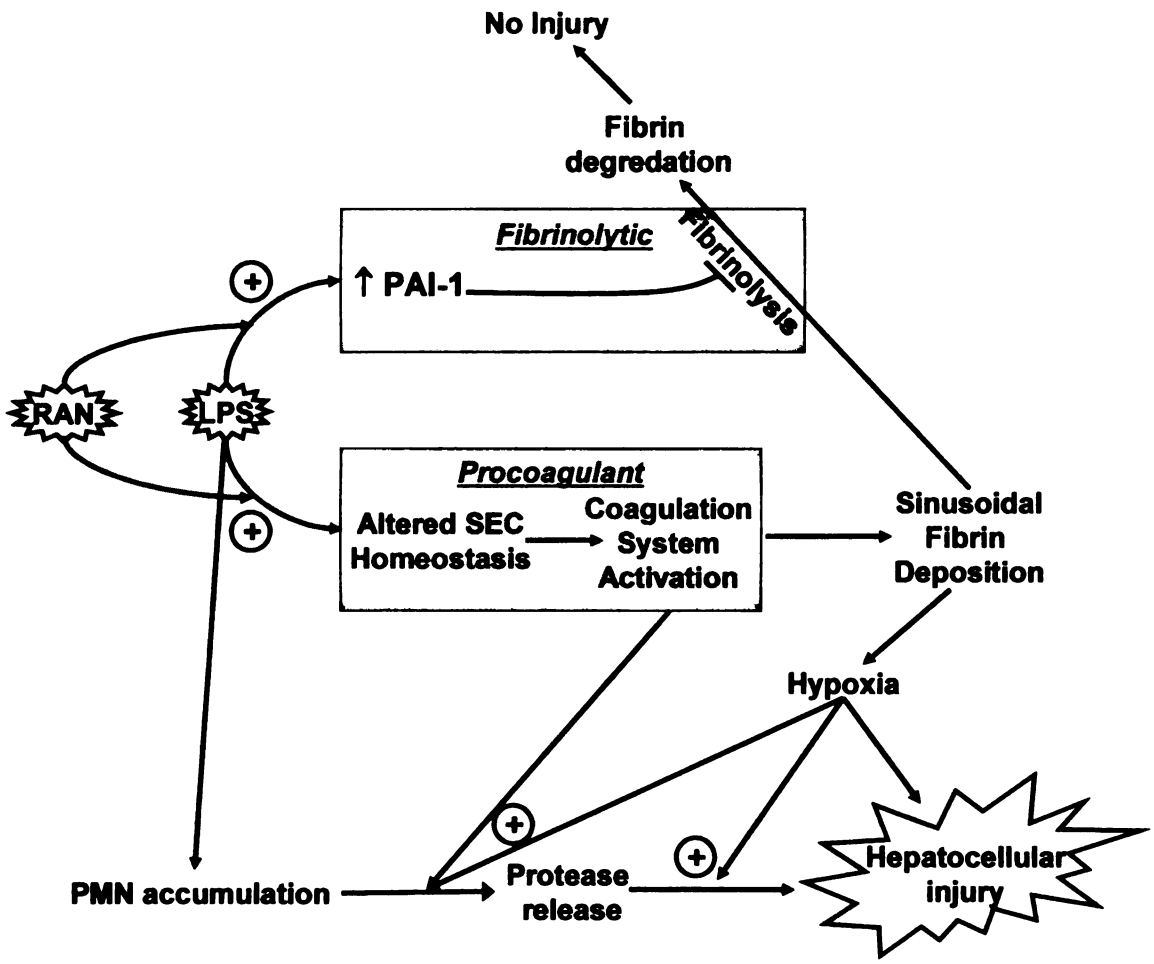
Possible consequences of hepatic fibrin deposition include disruption of liver blood flow and hypoxia. Indeed, LPS/RAN-treatment caused liver hypoxia at a time near the onset of liver injury (Figure 4.8 and 4.8), a response that was attenuated by heparin administration. Although hypoxia alone might be sufficient to cause injury in LPS/RAN-treated rats, it is likely that it influences the action of other inflammatory cells/mediators. Since PMNs accumulate in lesions after LPS/RAN-treated rats, preliminary studies were

conducted to evaluate a possible effect of hypoxia on their contribution to liver injury. Interestingly, serum CINC-1 and hepatic PMN accumulation were similar in LPS/Veh and LPS/RAN-treated rats, suggesting that if PMNs are involved in the injury, their accumulation in liver is not sufficient and that secondary activation signals are involved. Heparin affected neither CINC-1 nor PMN accumulation in LPS/RAN-treated rats. This result suggests that activation of the coagulation system is not required for PMN accumulation and that a reduction in liver hypoxia does not influence PMN accumulation. Nevertheless, hypoxia rendered hepatocytes sensitive to killing by PMN-E, suggesting that if PMN activation occurs in LPS/RAN-treated rats, the liver might be a sensitive target. Furthermore, the timecourse of hypoxia-enhanced HPC killing by PMN-E was also consistent with the development of liver injury in LPS/RAN-treated rats.

6.2 Proposed mechanism

Figure 6.1 illustrates a proposed mechanism for LPS/RAN-induced liver injury. This mechanism is based on the results presented in this dissertation but will most certainly evolve as we learn more about this model. RAN can augment the effects of LPS on the hemostatic system, including activation of thrombin (Figure 4.2) and PAI-1 expression (Figure 4.4). Inasmuch as fibrin clots are required for injury in this model (Figure 4.6), impaired fibrinolysis by PAI-1 likely contributes to injury. One contribution

Figure 6.1: Proposed mechanism of hepatotoxicity caused by LPS/RAN-cotreatment in rats. *See section 6.2 for details.*


No Injury
Fibrin degredation
Fibrinolytic
↑ PAI-1
Fibrinolysis
RAN
LPS
Procoagulant
Altered SEC Homeostasis
Coagulation System Activation
Sinusoidal Fibrin Deposition
Hypoxia
PMN accumulation
Protease release
Hepatocellular injury

of fibrin clots to LPS/RAN-induced liver injury might be mediated through ischemia and hypoxia. Indeed, LPS/RAN-treatment caused liver hypoxia (Figure 4.8 and 4.9), and the protective effect of heparin on HPC injury was associated with a reduction in liver hypoxia (Figure 5.1 and 5.2). Hypoxia might cause hepatocellular injury directly or indirectly by affecting other inflammatory factors or hepatocellular homeostasis. One possibility is that activation of the coagulation system and dramatic liver hypoxia contribute to hepatocellular injury in LPS/RAN-treated rats by causing PMN activation and release of toxic proteases (e.g., elastase). Furthermore, hypoxia might increase sensitivity of hepatic parenchymal cells to killing by PMN-derived proteases (Figure 5.5). Not illustrated in Figure 6.1, but of potential importance are direct effects of RAN (Figure 2.5) on hepatocellular sensitivity to these mediators.

6.3 Significance of research, knowledge gaps, and future studies

Several important findings were identified by these studies. First, the observation that idiosyncrasy-like hepatotoxicity develops in rats cotreated with LPS and RAN, but not FAM, suggests an ability of this model to predict the propensity of these two H2-antagonists to cause idiosyncratic liver injury. Additional studies evaluating other drugs associated with idiosyncratic hepatotoxicity within this pharmacologic class (e.g., cimetidine and nizatidine) and others are needed to validate further its use as preclinical predictor of some idiosyncratic drug reactions. Given the potential impact of losing

a drug during development, the inclusion of "negative comparators" in validation of the LPS/drug-cotreatment model would assist in identification of the apparent "false-positive" rate. Overall, these studies add another layer of promise to a growing body of evidence suggesting the predictive potential of this model (Buchweitz et al., 2002).

In addition to its use as a "negative comparator" in these studies, treatment of rats with FAM and LPS provided useful insight into the mechanism of injury in the LPS/RAN model. LPS-treated rats given a pharmacologically equiefficacious dose of FAM (relative to RAN) did not develop liver injury, suggesting that the pharmacologic effect of the drug is not sufficient to cause toxicity. Consistent with this observation is the differing propensity of marketed H2-antagonists to cause liver injury (see Chapter 1). Nevertheless, H2-receptor blockade might be a required factor for LPS/RAN-induced liver injury. For example, histamine attenuates LPS-induced TNF synthesis by an H2-receptor-dependent mechanism in monocytes (Vannier et al., 1991). Furthermore, histamine affords protection against *P. acnes*/LPS- and ethanol-induced liver injury by a H2-receptor dependent mechanism (Hornyak et al., 2003; Yokoyama et al., 2004). Accordingly, a hypothesis to be tested is that H2-antagonism is a permissive event for liver injury in LPS/RAN-treated rats, but alone it is insufficient to cause injury. Administration of histamine dihydrochloride would be expected to compete with RAN for H2-receptor binding and to attenuate LPS/RAN-induced liver injury. Although the role of endogenous histamine antagonism in LPS/RAN-induced liver injury remains unknown, it is clear that H2-independent properties of these drugs determine their ability to interact with LPS to cause hepatotoxicity.

Evaluation of liver gene expression using microarray technology provided a wealth of information about effects of RAN alone and in combination with LPS. Selection of specific expression patterns revealed useful information regarding the function/association of gene products changing in each treatment group. However, important changes in expression likely remain undiscovered within the dataset presented herein. Future data mining efforts should build on statistical and filtering strategies outlined here to include the use of updated rat gene nomenclature and ontologies, as well as tools designed to investigate interactions among gene products (e.g., in cell death signaling). In addition, analytical strategies designed for identification of synergistic changes in gene expression could be applied to this experimental design. As noted in Chapter 3, the addition of timecourse studies evaluating gene expression would most certainly be useful in understanding the relationship of gene expression to liver toxicity in LPS/RAN-treated rats. One advantage of the time selected for the studies presented here is that it provided a snapshot of gene expression before the onset of liver injury, thereby allowing for potential mechanistic association of a change in expression with the injury. However, as interesting hypotheses emerge, it is important to recognize that a change in gene expression is not always causal to injury, making post-hoc mechanistic analysis an absolute requirement for elucidating mechanisms.

In the case of PAI-1, real-time PCR and ELISA were used to confirm augmented mRNA and protein in LPS/RAN-treated rats. However, it is not known whether PAI-1 is a mediator of liver injury in this model. Studies to test this hypothesis using an inhibitor of PAI-1 activity are in the planning stage. However, the potential for impaired fibrinolysis as a result of increased PAI-1 expression prompted us to test, and ultimately

prove true, the hypothesis that fibrin clots are important for liver injury in this model. Analysis of liver gene expression in LPS/RAN-treated rats revealed a large set of genes that were expressed under hypoxic conditions (Table 3.1 and 3.2). Follow-up investigation of this observation using two other markers (PIM-adducts and HIF-1alpha) demonstrated that LPS/RAN-treatment does cause liver hypoxia. By and large, analysis of gene expression in LPS/RAN-treated yielded several hypotheses that proved useful in identification of potential mediators of liver injury.

A unique challenge presented by this model is the abrupt and somewhat variable time to onset and severity of liver injury in LPS/RAN-treated rats. For example, whereas some LPS/RAN-treated rats never develop liver injury, the maximal increase in ALT observed in LPS/RAN-treated rats is variable and can reach values as high as 1000 U/L (unpublished observation, JPL). In addition, the time to onset of hepatotoxicity is variable, occurring sometimes as early as 2 h. Therefore, it is somewhat challenging to use the temporal relationship between exposure and injury onset to support a causal association between a particular mediator and liver injury. For example, the experiments in Chapter 4 showed that liver hypoxia in LPS/RAN-treated rats accompanied hepatocellular injury but they did not distinguish the temporal relationship between these two outcomes (i.e., Figure 2.2 and 3.1). Accordingly, identification of liver hypoxia in LPS/RAN-treated rats before the onset of injury should be a focus of future experiments. The hypothesis that liver hypoxia causes LPS/RAN-induced liver injury has not yet been tested. However, the result that heparin decreases both liver hypoxia and injury is consistent with a protective effect mediated through reduction of hypoxia, especially considering the importance of fibrin clots in this model. Further experiments are required

to identify hypoxia as a cause rather than a consequence of liver injury in this model. For example, one hypothesis to test is that exposure to hypoxia renders rats susceptible to RAN hepatotoxicity (i.e., hypoxia can replace LPS). Furthermore, evaluation of gene expression data for hypoxia-inducible, cell death-related factors in LPS/RAN-treated rats might provide further insight.

As eluded to in Chapter 5, hypoxia might increase PMN activation or increase the sensitivity to killing by PMN-derived products in livers of LPS/RAN-treated rats. Indeed, several clues suggesting a role for PMNs in LPS/RAN hepatotoxicity have been identified. PMN accumulation occurs in livers of LPS/RAN-treated rats (Figure 2.3 and 5.3), RAN renders hepatocytes sensitive to PMN-CM (Figure 2.5), and hypoxia renders hepatocytes sensitive to PMN-E (Figure 5.5). An important hypothesis to test before proceeding with further investigation of this hypothesis is that PMNs are critical mediators in LPS/RAN-induced liver injury. If a hypoxia/PMN interaction is required for LPS/RAN-induced liver injury, prevention of either PMN accumulation or hypoxia should result in a reduction in liver injury. Current efforts are focused on development of assays for assessment of PMN activation in LPS/RAN-treated rats. In addition, plans for studies evaluating the role of PMNs (antibody-mediated PMN depletion) and PMN elastase (elastase inhibitor GW 311616) in LPS/RAN-induced liver injury are currently underway.

The results presented in this dissertation demonstrate that a modest inflammatory response renders RAN hepatotoxic in rats. The results provide a rationale for further evaluation of the hypothesis that inflammation is involved in some idiosyncratic drug reactions. Further validation of the LPS/drug cotreatment model may lead to a predictive

preclinical tool. Studies comparing RAN and FAM support this, but clearly testing more drugs and their "negative comparators" is required for complete validation. In addition, the results of these studies point to the hemostatic system as a critical mediator of LPS/RAN-induced liver injury and contribute to our understanding of the consequences of drug/inflammation interaction associated with hemostatic system activation in hepatotoxicity (i.e, fibrin deposition, hypoxia). Finally, results of *in vitro* studies suggest that hypoxia can increase sensitivity to cytotoxic effects of PMN-E. This relationship has implications for our understanding of mechanisms in other models of PMN-dependent hepatotoxicity.

Bibliography

Abrahamsson T, Nerme V, Stromqvist M, Akerblom B, Legnehed A, Pettersson K and Westin EA (1996) Anti-thrombotic effect of a PAI-1 inhibitor in rats given endotoxin. *Thromb Haemost* **75**:118-126.

Aithal GP, Day CP, Leathart JB and Daly AK (2000) Relationship of polymorphism in CYP2C9 to genetic susceptibility to diclofenac-induced hepatitis. *Pharmacogenetics* **10**:511-518.

Aithal GP, Ramsay L, Daly AK, Sonchit N, Leathart JB, Alexander G, Kenna JG, Caldwell J and Day CP (2004) Hepatic adducts, circulating antibodies, and cytokine polymorphisms in patients with diclofenac hepatotoxicity. *Hepatology* **39**:1430-1440.

Akahane K, Okamoto K, Kikuchi M, Todoroki H, Higure A, Ohuchida T, Kitahara K, Takeda S, Itoh H and Ohsato K (2001) Inhibition of factor Xa suppresses the expression of tissue factor in human monocytes and lipopolysaccharide-induced endotoxemia in rats. *Surgery* **130**:809-818.

Akita S, Kawahara M, Takeshita T, Morio M and Fujii K (1989) Halothane-induced hepatic microsomal lipid peroxidation in guinea pigs and rats. *J Appl Toxicol* **9**:9-14.

Akita S, Morio M, Kawahara M, Takeshita T, Fujii K and Yamamoto M (1988) Halothane-induced liver injury as a consequence of enhanced microsomal lipid peroxidation in guinea pigs. *Res Commun Chem Pathol Pharmacol* **61**:227-243.

Alden C, Lin J, and Smith P (2003) Predictive Toxicology Technology for Avoiding Idiosyncratic Liver Injury. *Preclinica* **May/June**: 27-35.

Ament PW, Roth JD and Fox CJ (1994) Famotidine-induced mixed hepatocellular jaundice. *Ann Pharmacother* **28**:40-42.

Amouzadeh HR and Pohl LR (1995) Processing of endoplasmic reticulum luminal antigens associated with halothane hepatitis in rat hepatocytes. *Hepatology* **22**:936-943.

Arnould T, Michiels C, Janssens D, Delaive E and Remacle J (1995) Hypoxia induces PMN adherence to umbilical vein endothelium. *Cardiovasc Res* **30**:1009-1016.

Arnould T, Michiels C and Remacle J (1994) Hypoxic human umbilical vein endothelial cells induce activation of adherent polymorphonuclear leukocytes. *Blood* **83**:3705-3716.

Arteel GE, Thurman RG and Raleigh JA (1998) Reductive metabolism of the hypoxia marker pimonidazole is regulated by oxygen tension independent of the pyridine nucleotide redox state. *Eur J Biochem* **253**:743-750.

Arteel GE, Thurman RG, Yates JM and Raleigh JA (1995) Evidence that hypoxia markers detect oxygen gradients in liver: pimonidazole and retrograde perfusion of rat liver. *Br J Cancer* **72**:889-895.

Ashton T, Young IS, Davison GW, Rowlands CC, McEneny J, Van BC, Jones E, Peters JR and Jackson SK (2003) Exercise-induced endotoxemia: the effect of ascorbic acid supplementation. *Free Radic Biol Med* **35**:284-291.

Awad JA, Horn JL, Roberts LJ and Franks JJ (1996) Demonstration of halothane-induced hepatic lipid peroxidation in rats by quantification of F2-isoprostanes. *Anesthesiology* **84**:910-916.

Bacon JA, Cramer CT, Petrella DK, Sun EL and Ulrich RG (1996) Potentiation of hypoxic injury in cultured rabbit hepatocytes by the quinoxalinone anxiolytic, panadiplon. *Toxicology* **108**:9-16.

Bagheri H, Michel F, Lapeyre-Mestre M, Lagier E, Cambus JP, Valdiguie P and Montastruc JL (2000) Detection and incidence of drug-induced liver injuries in hospital: a prospective analysis from laboratory signals. *Br J Clin Pharmacol* **50**:479-484.

Bahrami S, Redl H, Leichtfried G, Yu Y and Schlag G (1994) Similar cytokine but different coagulation responses to lipopolysaccharide injection in D-galactosamine-sensitized versus nonsensitized rats. *Infect Immun* **62**:99-105.

Banks AT, Zimmerman HJ, Ishak KG and Harter JG (1995) Diclofenac-associated hepatotoxicity: analysis of 180 cases reported to the Food and Drug Administration as adverse reactions. *Hepatology* **22**:820-827.

Barcia C, Fernandez BA, Poza M and Herrero MT (2003) Parkinson's disease and inflammatory changes. *Neurotox Res* **5**:411-418.

Barr GD and Piper DW (1981) Possible ranitidine hepatitis. *Med J Aust* **2**:421.

Barton CC, Barton EX, Ganey PE, Kunkel SL and Roth RA (2001) Bacterial lipopolysaccharide enhances aflatoxin B1 hepatotoxicity in rats by a mechanism that depends on tumor necrosis factor alpha. *Hepatology* **33**:66-73.

Barton CC, Ganey PE and Roth RA (2000a) Lipopolysaccharide augments aflatoxin B(1)-induced liver injury through neutrophil-dependent and -independent mechanisms. *Toxicol Sci* **58**:208-215.

Barton CC, Hill DA, Yee SB, Barton EX, Ganey PE and Roth RA (2000b) Bacterial lipopolysaccharide exposure augments aflatoxin B(1)-induced liver injury. *Toxicol Sci* **55**:444-452.

Benjamini Y and Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc* **57**: 289-300.

Beutler B (2002) TLR4 as the mammalian endotoxin sensor. *Curr Top Microbiol Immunol* **270**:109-120.

Binder BR, Christ G, Gruber F, Grubic N, Hufnagl P, Krebs M, Mihaly J and Prager GW (2002) Plasminogen activator inhibitor 1: physiological and pathophysiological roles. *News Physiol Sci* **17**:56-61.

Bini A, Itoh Y, Kudryk BJ and Nagase H (1996) Degradation of cross-linked fibrin by matrix metalloproteinase 3 (stromelysin 1): hydrolysis of the gamma Gly 404-Ala 405 peptide bond. *Biochemistry* **35**:13056-13063.

Bird GL and Williams R (1989) Detection of antibodies to a halothane metabolite hapten in sera from patients with halothane-associated hepatitis. *J Hepatol* **9**:366-373.

Black M, Scott WE, Jr. and Kanter R (1984) Possible ranitidine hepatotoxicity. *Ann Intern Med* **101**:208-210.

Boelsterli UA Disease-related determinants of susceptibility to drug-induced idiosyncratic hepatotoxicity. (2003a) *Curr Opin Drug Discov Devel* **6**:81-91.

Boelsterli UA (2003b) Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Toxicol Appl Pharmacol* **192**:307-322.

Boelsterli UA, Zimmerman HJ and Kretz-Rommel A (1995) Idiosyncratic liver toxicity of nonsteroidal antiinflammatory drugs: molecular mechanisms and pathology. *Crit Rev Toxicol* **25**:207-235.

Bort R, Mace K, Boobis A, Gomez-Lechon MJ, Pfeifer A and Castell J (1999) Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. *Biochem Pharmacol* **58**:787-796.

Bougie D, Johnson ST, Weitekamp LA and Aster RH (1997) Sensitivity to a metabolite of diclofenac as a cause of acute immune hemolytic anemia. *Blood* **90**:407-413.

Bourdi M, Amouzadeh HR, Rushmore TH, Martin JL and Pohl LR (2001) Halothane-induced liver injury in outbred guinea pigs: role of trifluoroacetylated protein adducts in animal susceptibility. *Chem Res Toxicol* **14**:362-370.

Bredfeldt JE and von HC (1984) Ranitidine, acetaminophen, and hepatotoxicity. *Ann Intern Med* **101**:719.

Brown AP, Schultze AE, Holdan WL, Buchweitz JP, Roth RA and Ganey PE (1996) Lipopolysaccharide-induced hepatic injury is enhanced by polychlorinated biphenyls. *Environ Health Perspect* **104**:634-640.

Buchweitz JP, Ganey PE, Bursian SJ and Roth RA (2002) Underlying endotoxemia augments toxic responses to chlorpromazine: is there a relationship to drug idiosyncrasy? *J Pharmacol Exp Ther* **300**:460-467.

Buerke M, Weyrich AS and Lefer AM (1994) Isolated cardiac myocytes are sensitized by hypoxia-reoxygenation to neutrophil-released mediators. *Am J Physiol* **266**:H128-H136.

Calkins CM, Bensard DD, Shames BD, Pulido EJ, Abraham E, Fernandez N, Meng X, Dinarello CA and McIntyre RC, Jr. (2002) IL-1 regulates in vivo C-X-C chemokine induction and neutrophil sequestration following endotoxemia. *J Endotoxin Res* **8**:59-67.

Camus G, Nys M, Poortmans JR, Venneman I, Monfils T, by-Dupont G, Juchmes-Ferir A, Deby C, Lamy M and Duchateau J (1998) Endotoxaemia, production of tumour necrosis factor alpha and polymorphonuclear neutrophil activation following strenuous exercise in humans. *Eur J Appl Physiol Occup Physiol* **79**:62-68.

Camus G, Poortmans J, Nys M, by-Dupont G, Duchateau J, Deby C and Lamy M (1997) Mild endotoxaemia and the inflammatory response induced by a marathon race. *Clin Sci (Lond)* **92**:415-422.

Chamulitrat W, Blazka ME, Jordan SJ, Luster MI and Mason RP (1995) Tumor necrosis factor-alpha and nitric oxide production in endotoxin-primed rats administered carbon tetrachloride. *Life Sci* **57**:2273-2280.

Chen D, Giannopoulos K, Shiels PG, Webster Z, McVey JH, Kemball-Cook G, Tuddenham E, Moore M, Lechler R and Dorling A (2004) Inhibition of intravascular thrombosis in murine endotoxemia by targeted expression of hirudin and tissue factor pathway inhibitor analogs to activated endothelium. *Blood* **104**:1344-1349.

Choi JW, Park SC, Kang GH, Liu JO and Youn HD (2004) Nur77 activated by hypoxia-inducible factor-1alpha overproduces proopiomelanocortin in von Hippel-Lindau-mutated renal cell carcinoma. *Cancer Res* **64**:35-39.

Chosay JG, Essani NA, Dunn CJ and Jaeschke H (1997) Neutrophil margination and extravasation in sinusoids and venules of liver during endotoxin-induced injury. *Am J Physiol* **272**:G1195-G1200.

Christen U, Burgin M and Gut J (1991) Halothane metabolism: Kupffer cells carry and partially process trifluoroacetylated protein adducts. *Biochem Biophys Res Commun* **175**:256-262.

Cohen A and Fabre L (1983) Tolerance to repeated intravenous doses of ranitidine HCl and cimetidine HCl in normal volunteers. Cur. Ther. Res. **34**: 475-482.

Colin-Jones DG, Ireland A, Gear P, Golding PL, Ramage JK, Williams JG, Leicester RJ, Smith CL, Ross G, Bamforth J and . (1984) Reducing overnight secretion of acid to heal duodenal ulcers. Comparison of standard divided dose of ranitidine with a single dose administered at night. *Am J Med* **77**:116-122.

Colman RW, MarderVJ, Salzman EW, and Hirsh J (1994) Overview of hemostasis., in *Hemostasis and Thrombosis* (Colman RW, Hirsh J, Marder J, Salzmaned, eds) p 3, Lippincott, Philadelphia.

Cook JA, Marconi EA and Di Luzio NR (1974) Lead, cadmium, endotoxin interaction: effect on mortality and hepatic function. *Toxicol Appl Pharmacol* **28**:292-302.

Copple BL, Banes A, Ganey PE and Roth RA (2002a) Endothelial cell injury and fibrin deposition in rat liver after monocrotaline exposure. *Toxicol Sci* **65**:309-318.

Copple BL, Ganey PE and Roth RA (2004a) Hypoxia in rat liver after monocrotaline exposure. *Toxicol Sci* **78 (S-1)**, 491.

Copple BL, Moulin F, Hanumegowda UM, Ganey PE and Roth RA (2003) Thrombin and protease-activated receptor-1 agonists promote lipopolysaccharide-induced hepatocellular injury in perfused livers. *J Pharmacol Exp Ther* **305**:417-425.

Copple BL, Rondelli CM, Maddox JF, Hoglen NC, Ganey PE and Roth RA (2004b) Modes of cell death in rat liver after monocrotaline exposure. *Toxicol Sci* **77**:172-182.

Copple BL, Woolley B, Banes A, Ganey PE and Roth RA (2002b) Anticoagulants prevent monocrotaline-induced hepatic parenchymal cell injury but not endothelial cell injury in the rat. *Toxicol Appl Pharmacol* **180**:186-196.

Cottam DR, Mattar SG, Barinas-Mitchell E, Eid G, Kuller L, Kelley DE and Schauer PR (2004) The chronic inflammatory hypothesis for the morbidity associated with morbid obesity: implications and effects of weight loss. *Obes Surg* **14**:589-600.

Coutellier A, Pelletier S, Ekert P, Hauw JJ, Soubrie C and Herson S (1993) Inflammatory myopathy during ranitidine therapy. *J Rheumatol* **20**:1453-1454.

Dalgleish AG and O'Byrne KJ (2002) Chronic immune activation and inflammation in the pathogenesis of AIDS and cancer. *Adv Cancer Res* **84**:231-276.

de GH and Noll T (1984) The crucial role of hypoxia in halothane-induced lipid peroxidation. *Biochem Biophys Res Commun* **119**:139-143.

Deaciuc IV, McDonough KH, Bagby GJ and Spitzer JJ (1993) Alcohol consumption in rats potentiates the deleterious effect of gram-negative sepsis on hepatic hyaluronan uptake. *Alcohol Clin Exp Res* **17**:1002-1008.

Derevianko A, D'Amico R and Simms H (1996) Polymorphonuclear leucocyte (PMN)-derived inflammatory cytokines--regulation by oxygen tension and extracellular matrix. *Clin Exp Immunol* **106**:560-567.

Desaint B, Alperine M, Legendre C, Florent C and Levy VG (1985) [Hepatitis caused by ranitidine]. *Gastroenterol Clin Biol* **9**:746-747.

Devuyst O, Lefebvre C, Geubel A and Coche E (1993) Acute cholestatic hepatitis with rash and hypereosinophilia associated with ranitidine treatment. *Acta Clin Belg* **48**:109-114.

Dieckhaus CM, Thompson CD, Roller SG and Macdonald TL (2002) Mechanisms of idiosyncratic drug reactions: the case of felbamate. *Chem Biol Interact* **142**:99-117.

Duijvestijn AM, van GH, Klatter F, Majoor GD, van BE and van B, V (1992) Antibodies defining rat endothelial cells: RECA-1, a pan-endothelial cell-specific monoclonal antibody. *Lab Invest* **66**:459-466.

El-Bassiouni EA, bo-Ollo MM, Helmy MH, Ismail S and Ramadan MI (1998) Changes in the defense against free radicals in the liver and plasma of the dog during hypoxia and/or halothane anaesthesia. *Toxicology* **128**:25-34.

Engelmann B, Luther T and Muller I (2003) Intravascular tissue factor pathway--a model for rapid initiation of coagulation within the blood vessel. *Thromb Haemost* **89**:3-8.

Essani NA, Bajt ML, Farhood A, Vonderfecht SL and Jaeschke H (1997) Transcriptional activation of vascular cell adhesion molecule-1 gene in vivo and its role in the pathophysiology of neutrophil-induced liver injury in murine endotoxin shock. *J Immunol* **158**:5941-5948.

Essani NA, Fisher MA, Farhood A, Manning AM, Smith CW and Jaeschke H (1995) Cytokine-induced upregulation of hepatic intercellular adhesion molecule-1 messenger RNA expression and its role in the pathophysiology of murine endotoxin shock and acute liver failure. *Hepatology* **21**:1632-1639.

Essayan DM (1999) Cyclic nucleotide phosphodiesterase (PDE) inhibitors and immunomodulation. *Biochem Pharmacol* **57**:965-973.

Faich GA and Moseley RH (2001) Troglitazone (Rezulin) and hepatic injury. *Pharmacoepidemiol Drug Saf* **10**:537-547.

Fassoulaki A, Eger EI, Johnson BH, Ferrell LD, Smuckler EA, Harper MH, Eger RR and Cahalan MK (1984) Brief periods of hypoxia can produce hepatic injury in rats. *Anesth Analg* **63**:885-887.

Francavilla A, Panella C, Polimeno L, Di Leo A, Makowka L, Barone M, Amoruso A, Ingrosso M and Starzl TE (1989) Effect of cimetidine, ranitidine, famotidine and omeprazole on hepatocyte proliferation in vitro. *J Hepatol* **8**:32-41.

Fujii K, Morio M, Kikuchi H, Ishihara S, Okida M and Ficor F (1984) In vivo spin-trap study on anaerobic dehalogenation of halothane. *Life Sci* **35**:463-468.

Fujiwara K, Ogata I, Ohta Y, Hirata K, Oka Y, Yamada S, Sato Y, Masaki N and Oka H (1988) Intravascular coagulation in acute liver failure in rats and its treatment with antithrombin III. *Gut* **29**:1103-1108.

Funk C, Ponelle C, Scheuermann G and Pantze M (2001) Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in

vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat. *Mol Pharmacol* **59**:627-635.

Furst SM and Gandolfi AJ (1997) Interaction of lymphocytes with Kupffer cells from halothane-exposed guinea pigs. *Int Arch Allergy Immunol* **114**:46-53.

Furst SM, Luedke D and Gandolfi AJ (1997) Kupffer cells from halothane-exposed guinea pigs carry trifluoroacetylated protein adducts. *Toxicology* **120**:119-132.

Ganey PE, Luyendyk JP, Maddox JF, and Roth RA. (2004). Adverse hepatic drug reactions: inflammatory episodes as consequence and contributor. *Chem Biol Interact.* **150**:35-51.

Ganey PE, Bailie MB, VanCise S, Colligan ME, Madhukar BV, Robinson JP and Roth RA (1994) Activated neutrophils from rat injured isolated hepatocytes. *Lab Invest* **70**:53-60.

Ganey PE, Barton YW, Kinser S, Sneed RA, Barton CC and Roth RA (2001) Involvement of cyclooxygenase-2 in the potentiation of allyl alcohol-induced liver injury by bacterial lipopolysaccharide. *Toxicol Appl Pharmacol* **174**:113-121.

Ganey PE and Roth RA (2001) Concurrent inflammation as a determinant of susceptibility to toxicity from xenobiotic agents. *Toxicology* **169**:195-208.

Gao JJ, Diesl V, Wittmann T, Morrison DC, Ryan JL, Vogel SN and Follettie MT (2002) Regulation of gene expression in mouse macrophages stimulated with bacterial CpG-DNA and lipopolysaccharide. *J Leukoc Biol* **72**:1234-1245.

Garcia RL, Wallander MA and Stricker BH (1997) The risk of acute liver injury associated with cimetidine and other acid-suppressing anti-ulcer drugs. *Br J Clin Pharmacol* **43**:183-188.

Gardiner KR, Halliday MI, Barclay GR, Milne L, Brown D, Stephens S, Maxwell RJ and Rowlands BJ (1995) Significance of systemic endotoxaemia in inflammatory bowel disease. *Gut* **36**:897-901.

Gereda JE, Klinnert MD, Price MR, Leung DY and Liu AH (2001) Metropolitan home living conditions associated with indoor endotoxin levels. *J Allergy Clin Immunol* **107**:790-796.

Gill RQ and Sterling RK (2001) Acute liver failure. *J Clin Gastroenterol* **33**:191-198.

Gomez-Lechon MJ, Ponsoda X, O'Connor E, Donato T, Castell JV and Jover R (2003a) Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. *Biochem Pharmacol* **66**:2155-2167.

Gomez-Lechon MJ, Ponsoda X, O'Connor E, Donato T, Jover R and Castell JV (2003b) Diclofenac induces apoptosis in hepatocytes. *Toxicol In Vitro* **17**:675-680.

Graham DY, Opekun AR, Smith JL and Schwartz JT (1985) Ranitidine and hepatotoxicity. *Ann Intern Med* **102**:416.

Gray MO, Karliner JS and Mochly-Rosen D (1997) A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem* **272**:30945-30951.

Gruber F, Hufnagl P, Hofer-Warbinek R, Schmid JA, Breuss JM, Huber-Beckmann R, Lucerna M, Papac N, Harant H, Lindley I, de MR and Binder BR (2003) Direct binding of Nur77/NAK-1 to the plasminogen activator inhibitor 1 (PAI-1) promoter regulates TNF alpha -induced PAI-1 expression. *Blood* **101**:3042-3048.

Gujral JS, Hinson JA, Farhood A and Jaeschke H (2004) NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia. *Am J Physiol Gastrointest Liver Physiol* **287**:G243-G252.

Gunawan B and Kaplowitz N (2004) Clinical perspectives on xenobiotic-induced hepatotoxicity. *Drug Metab Rev* **36**:301-312.

Gut J, Christen U and Huwyler J (1993) Mechanisms of halothane toxicity: novel insights. *Pharmacol Ther* **58**:133-155.

Halparin LS (1984) Adverse effects of ranitidine therapy. *Can Med Assoc J* **130**:668, 672.

Hamaguchi E, Takamura T, Shimizu A and Nagai Y (2003) Tumor necrosis factor-alpha and troglitazone regulate plasminogen activator inhibitor type 1 production through extracellular signal-regulated kinase- and nuclear factor-kappaB-dependent pathways in cultured human umbilical vein endothelial cells. *J Pharmacol Exp Ther* **307**:987-994.

Hannah S, Mecklenburgh K, Rahman I, Bellingan GJ, Greening A, Haslett C and Chilvers ER (1995) Hypoxia prolongs neutrophil survival in vitro. *FEBS Lett* **372**:233-237.

Hansen J, Cherwitz DL and Allen JI (1994) The role of tumor necrosis factor-alpha in acute endotoxin-induced hepatotoxicity in ethanol-fed rats. *Hepatology* **20**:461-474.

Hashimoto F, Davis RL and Egli D (1994) Hepatitis following treatments with famotidine and then cimetidine. *Ann Pharmacother* **28**:37-39.

Hastings KL, Thomas C, Brown AP and Gandolfi AJ (1995) Trifluoroacetylation potentiates the humoral immune response to halothane in the guinea pig. *Immunopharmacol Immunotoxicol* **17**:201-213.

Hayashi T and Faustman D (2000) Essential role of human leukocyte antigen-encoded proteasome subunits in NF-kappaB activation and prevention of tumor necrosis factor-alpha-induced apoptosis. *J Biol Chem* **275**:5238-5247.

Heinrich J, Gehring U, Douwes J, Koch A, Fahlbusch B, Bischof W and Wichmann HE (2001) Pets and vermin are associated with high endotoxin levels in house dust. *Clin Exp Allergy* **31**:1839-1845.

Helfgott SM, Sandberg-Cook J, Zakim D and Nestler J (1990) Diclofenac-associated hepatotoxicity. *JAMA* **264**:2660-2662.

Hewett JA, Jean PA, Kunkel SL and Roth RA (1993) Relationship between tumor necrosis factor-alpha and neutrophils in endotoxin-induced liver injury. *Am J Physiol* **265**:G1011-G1015.

Hewett JA and Roth RA (1993) Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. *Pharmacol Rev* **45**:382-411.

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

Hewett JA, Schultze AE, VanCise S and Roth RA (1992) Neutrophil depletion protects against liver injury from bacterial endotoxin. *Lab Invest* **66**:347-361.

Hiesse C, Cantarovich M, Santelli C, Francais P, Charpentier B, Fries D and Buffet C (1985) Ranitidine hepatotoxicity in renal transplant patient. *Lancet* **1**:1280.

Hirschowitz BI, Berenson MM, Berkowitz JM, Bright-Asare P, DeLuca VA, Jr., Eshelman FN, Font RG, Griffin JW, Jr., Kozarek RA, McCray RS et al. (1986) A multicenter study of ranitidine treatment of duodenal ulcers in the United States. *J Clin Gastroenterol* **8**:359-366.

Ho JS, Buchweitz JP, Roth RA and Ganey PE (1996) Identification of factors from rat neutrophils responsible for cytotoxicity to isolated hepatocytes. *J Leukoc Biol* **59**:716-724.

Honchel R, Marsano L, Cohen D, Shedlofsky S and McClain CJ (1991) Lead enhances lipopolysaccharide and tumor necrosis factor liver injury. *J Lab Clin Med* **117**:202-208.

Hornyak SC, Gehlsen KR and Haaparanta T (2003) Histamine dihydrochloride protects against early alcohol-induced liver injury in a rat model. *Inflammation* **27**:317-327.

Horton MR, Olman MA, Bao C, White KE, Choi AM, Chin BY, Noble PW and Lowenstein CJ (2000) Regulation of plasminogen activator inhibitor-1 and urokinase by hyaluronan fragments in mouse macrophages. *Am J Physiol Lung Cell Mol Physiol* **279**:L707-L715.

Ilyin GP, Rissel M, Malledant Y, Tanguy M and Guillouzo A (1994) Human hepatocytes express trifluoroacetylated neoantigens after in vitro exposure to halothane. *Biochem Pharmacol* **48**:561-567.

Ishii K, Ito Y, Katagiri H, Matsumoto Y, Kakita A and Majima M (2002) Neutrophil elastase inhibitor attenuates lipopolysaccharide-induced hepatic microvascular dysfunction in mice. *Shock* **18**:163-168.

Jacob AI, Goldberg PK, Bloom N, Degenshein GA and Kozinn PJ (1977) Endotoxin and bacteria in portal blood. *Gastroenterology* **72**:1268-1270.

Jaeschke H, Farhood A, Fisher MA and Smith CW (1996) Sequestration of neutrophils in the hepatic vasculature during endotoxemia is independent of beta 2 integrins and intercellular adhesion molecule-1. *Shock* **6**:351-356.

Jaeschke H, Farhood A and Smith CW (1991) Neutrophil-induced liver cell injury in endotoxin shock is a CD11b/CD18-dependent mechanism. *Am J Physiol* **261**:G1051-G1056.

Jaeschke H and Smith CW (1997) Mechanisms of neutrophil-induced parenchymal cell injury. *J Leukoc Biol* **61**:647-653.

Jeukendrup AE, Vet-Joop K, Sturk A, Stegen JH, Senden J, Saris WH and Wagenmakers AJ (2000) Relationship between gastro-intestinal complaints and endotoxaemia, cytokine release and the acute-phase reaction during and after a long-distance triathlon in highly trained men. *Clin Sci (Lond)* **98**:47-55.

Jimenez-Saenz M, rguelles-Arias F, Herrerias-Gutierrez JM and Duran-Quintana JA (2000) Acute cholestatic hepatitis in a child treated with famotidine. *Am J Gastroenterol* **95**:3665-3666.

Jin SL and Conti M (2002) Induction of the cyclic nucleotide phosphodiesterase PDE4B is essential for LPS-activated TNF-alpha responses. *Proc Natl Acad Sci U S A* **99**:7628-7633.

Jones BE, Lo CR, Liu H, Srinivasan A, Streetz K, Valentino KL and Czaja MJ (2000) Hepatocytes sensitized to tumor necrosis factor-alpha cytotoxicity undergo apoptosis through caspase-dependent and caspase-independent pathways. *J Biol Chem* **275**:705-712.

Jones DB, Hanson RG, Mihaly GW, Johnson RD, Louis WJ, Yeomans ND and Smallwood RA (1982) Double-blind controlled trial of ranitidine versus cimetidine in the treatment of duodenal ulceration. *Aust N Z J Med* **12**:547-549.

Ju C and Uetrecht JP (2002) Mechanism of idiosyncratic drug reactions: reactive metabolite formation, protein binding and the regulation of the immune system. *Curr Drug Metab* **3**:367-377.

Kaplowitz N (2004) Drug-induced liver injury. *Clin Infect Dis* **38 (S-2)**:S44-8.

Kaplowitz N (2004b) Acetaminophen hepatoxicity: what do we know, what don't we know, and what do we do next? *Hepatology* **40**:23-26.

Karachalios GN (1985) Ranitidine and hepatitis. *Ann Intern Med* **103**:634-635.

Kenna JG, Martin JL, Satoh H and Pohl LR (1990) Factors affecting the expression of trifluoroacetylated liver microsomal protein neoantigens in rats treated with halothane. *Drug Metab Dispos* **18**:788-793.

Kenna JG, Neuberger J and Williams R (1987) Specific antibodies to halothane-induced liver antigens in halothane-associated hepatitis. *Br J Anaesth* **59**:1286-1290.

Kerb R, Hoffmeyer S and Brinkmann U (2001) ABC drug transporters: hereditary polymorphisms and pharmacological impact in MDR1, MRP1 and MRP2. *Pharmacogenomics* **2**:51-64.

Khan S and O'Brien PJ (1997) Rapid and specific efflux of glutathione before hepatocyte injury induced by hypoxia. *Biochem Biophys Res Commun* **238**:320-322.

Khan S and O'Brien PJ (1995) Molecular mechanisms of tirapazamine (SR 4233, Win 59075)-induced hepatocyte toxicity under low oxygen concentrations. *Br J Cancer* **71**:780-785.

Kharasch ED, Hankins D, Mautz D and Thummel KE (1996) Identification of the enzyme responsible for oxidative halothane metabolism: implications for prevention of halothane hepatitis. *Lancet* **347**:1367-1371.

Kietzmann T, Jungermann K and Gorlach A (2003) Regulation of the hypoxia-dependent plasminogen activator inhibitor 1 expression by MAP kinases. *Thromb Haemost* **89**:666-673.

Kietzmann T, Roth U and Jungermann K (1999) Induction of the plasminogen activator inhibitor-1 gene expression by mild hypoxia via a hypoxia response element binding the hypoxia-inducible factor-1 in rat hepatocytes. *Blood* **94**:4177-4185.

Kinser S, Copple BL, Roth RA and Ganey PE (2002) Enhancement of allyl alcohol hepatotoxicity by endotoxin requires extrahepatic factors. *Toxicol Sci* **69**:470-481.

Kinser S, Sneed R, Roth R and Ganey P (2004) Neutrophils contribute to endotoxin enhancement of allyl alcohol hepatotoxicity. *J Toxicol Environ Health A* **67**:911-928.

Kitteringham NR, Kenna JG and Park BK (1995) Detection of autoantibodies directed against human hepatic endoplasmic reticulum in sera from patients with halothane-associated hepatitis. *Br J Clin Pharmacol* **40**:379-386.

Knolle PA and Gerken G (2000) Local control of the immune response in the liver. *Immunol Rev* **174**:21-34.

Kobayashi H, Horikoshi K, Yamataka A, Yamataka T, Okazaki T, Lane GJ and Miyano T (1999) Hyaluronic acid: a specific prognostic indicator of hepatic damage in biliary atresia. *J Pediatr Surg* **34**:1791-1794.

Kohlroser J, Mathai J, Reichheld J, Banner BF and Bonkovsky HL (2000) Hepatotoxicity due to troglitazone: report of two cases and review of adverse events reported to the United States Food and Drug Administration. *Am J Gastroenterol* **95**:272-276.

Kretz-Rommel A and Boelsterli UA (1995) Cytotoxic activity of T cells and non-T cells from diclofenac-immunized mice against cultured syngeneic hepatocytes exposed to diclofenac. *Hepatology* **22**:213-222.

Kretz-Rommel A and Boelsterli UA (1993) Diclofenac covalent protein binding is dependent on acyl glucuronide formation and is inversely related to P450-mediated acute cell injury in cultured rat hepatocytes. *Toxicol Appl Pharmacol* **120**:155-161.

Kretz-Rommel A and Boelsterli UA (1994) Selective protein adducts to membrane proteins in cultured rat hepatocytes exposed to diclofenac: radiochemical and immunochemical analysis. *Mol Pharmacol* **45**:237-244.

Labib R, Turkall R and bdel-Rahman MS (2002) Endotoxin potentiates the hepatotoxicity of cocaine in male mice. *J Toxicol Environ Health A* **65**:977-993.

Lahat N, Rahat MA, Ballan M, Weiss-Cerem L, Engelmayer M and Bitterman H (2003) Hypoxia reduces CD80 expression on monocytes but enhances their LPS-stimulated TNF-alpha secretion. *J Leukoc Biol* **74**:197-205.

Lane DA and Grant PJ (2000) Role of hemostatic gene polymorphisms in venous and arterial thrombotic disease. *Blood* **95**:1517-1532.

Lane SR, Nicholls PJ and Sewell RD (2004) The measurement and health impact of endotoxin contamination in organic dusts from multiple sources: focus on the cotton industry. *Inhal Toxicol* **16**:217-229.

Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM and Bor DH (2002) Timing of new black box warnings and withdrawals for prescription medications. *JAMA* **287**:2215-2220.

Lauritsen K, Havelund T and Rask-Madsen J (1984) Ranitidine and hepatotoxicity. *Lancet* **2**:1471.

Lazarou J, Pomeranz BH and Corey PN (1998) Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies. *JAMA* **279**:1200-1205.

Lee FI, Reed PI, Crowe JP, McIsaac RL and Wood JR (1986) Acute treatment of duodenal ulcer: a multicentre study to compare ranitidine 150 mg twice daily with ranitidine 300 mg once at night. *Gut* **27**:1091-1095.

Lemasters JJ, Ji S and Thurman RG (1981) Centrilobular injury following hypoxia in isolated, perfused rat liver. *Science* **213**:661-663.

Lepper PM, Held TK, Schneider EM, Bolke E, Gerlach H and Trautmann M (2002) Clinical implications of antibiotic-induced endotoxin release in septic shock. *Intensive Care Med* **28**:824-833.

Lewis J.H. (2003) Nonsteroidal anti-inflammatory drugs: pathology and clinical presentation of hepatoxicity, in *Drug-Induced Liver Disease* (Kaplowitz N and DeLeve LD ed) pp 377-404, Marcel Dekker, New York.

Li AP (2002) A review of the common properties of drugs with idiosyncratic hepatotoxicity and the "multiple determinant hypothesis" for the manifestation of idiosyncratic drug toxicity. *Chem Biol Interact* **142**:7-23.

Li C, Liu J, Waalkes MP and Jaeschke H (2003) Gene array analysis of the hepatic response to endotoxin in glutathione peroxidase-deficient mice. *Toxicol Lett* **144**:397-406.

Li X, Klintman D, Liu Q, Sato T, Jeppsson B and Thorlacius H (2004) Critical role of CXC chemokines in endotoxemic liver injury in mice. *J Leukoc Biol* **75**:443-452.

Liberopoulos EN, Nonni AB, Tsianos EV and Elisaf MS (2002) Possible ranitidine-induced cholestatic jaundice. *Ann Pharmacother* **36**:172.

Lin JH (1991) Pharmacokinetic and pharmacodynamic properties of histamine H2-receptor antagonists. Relationship between intrinsic potency and effective plasma concentrations. *Clin Pharmacokinet* **20**:218-236.

Lind L (2003) Circulating markers of inflammation and atherosclerosis. *Atherosclerosis* **169**:203-214.

Lind RC and Gandolfi AJ (1997) Late dimethyl sulfoxide administration provides a protective action against chemically induced injury in both the liver and the kidney. *Toxicol Appl Pharmacol* **142**:201-207.

Lind RC, Gandolfi AJ and Hall PM (1992) Glutathione depletion enhances subanesthetic halothane hepatotoxicity in guinea pigs. *Anesthesiology* **77**:721-727.

Lind RC, Gandolfi AJ and Hall PM (1994) A model for fatal halothane hepatitis in the guinea pig. *Anesthesiology* **81**:478-487.

Lind RC, Gandolfi AJ, Sipes IG and Brown BRJ (1984) The involvement of endotoxin in halothane-associated liver injury. *Anesthesiology* **61**:544-550.

Liu H, Lo CR and Czaja MJ (2002) NF-kappaB inhibition sensitizes hepatocytes to TNF-induced apoptosis through a sustained activation of JNK and c-Jun. *Hepatology* **35**:772-778.

Luparini RL, Rotundo A, Mattace R and Marigliano V (2000) [Possibly ranitidine-induced autoimmune hepatitis]. *Ann Ital Med Int* **15**:214-217.

Luyendyk JP, Copple BL, Barton CC, Ganey PE and Roth RA (2003a) Augmentation of aflatoxin B1 hepatotoxicity by endotoxin: involvement of endothelium and the coagulation system. *Toxicol Sci* **72**:171-181.

Luyendyk JP, Maddox JF, Cosma GN, Ganey PE, Cockerell GL and Roth RA (2003b) Ranitidine treatment during a modest inflammatory response precipitates idiosyncrasy-like liver injury in rats. *J Pharmacol Exp Ther* **307**:9-16.

Luyendyk JP, Mattes WB, Burgoon LD, Zacharewski TR, Maddox JF, Cosma GN, Ganey PE and Roth RA (2004c) Gene expression analysis points to hemostasis in livers of rats cotreated with lipopolysaccharide and ranitidine. *Toxicol Sci* **80**:203-213.

Luyendyk JP, Shores KC, Ganey PE and Roth RA (2002) Bacterial lipopolysaccharide exposure alters aflatoxin B(1) hepatotoxicity: benchmark dose analysis for markers of liver injury. *Toxicol Sci* **68**:220-225.

Luyendyk JP, Maddox JF, Ganey PE, and Roth RA. (2004a). Coagulation system activation and fibrin deposition are critical factors in idiosyncrasy-like liver injury from lipopolysaccharide and ranitidine coexposure. FASEB J in press. Abstract

Luyendyk JP, Maddox JF, Green, CD Ganey PE, and Roth RA. (2004b) Role of hepatic fibrin in idiosyncrasy-like liver injury from lipopolysaccharide-ranitidine coexposure in rats. Hepatology **in press.**

Mackman N (1996) Regulation of tissue factor gene expression in human monocytic and endothelial cells. *Haemostasis* **26 Suppl 1**:17-19.

Maddox JF, Domzalski AC, Roth RA and Ganey PE (2004) 15-deoxy prostaglandin J2 enhances allyl alcohol-induced toxicity in rat hepatocytes. *Toxicol Sci* **77**:290-298.

Maher JJ, Scott MK, Saito JM and Burton MC (1997) Adenovirus-mediated expression of cytokine-induced neutrophil chemoattractant in rat liver induces a neutrophilic hepatitis. *Hepatology* **25**:624-630.

Marotto ME, Thurman RG and Lemasters JJ (1988) Early midzonal cell death during low-flow hypoxia in the isolated, perfused rat liver: protection by allopurinol. *Hepatology* **8**:585-590.

Marra F, DeFranco R, Grappone C, Milani S, Pinzani M, Pellegrini G, Laffi G and Gentilini P (1998) Expression of the thrombin receptor in human liver: up-regulation during acute and chronic injury. *Hepatology* **27**:462-471.

Marshall LJ, Ramdin LS, Brooks T, DPhil PC and Shute JK (2003) Plasminogen activator inhibitor-1 supports IL-8-mediated neutrophil transendothelial migration by inhibition of the constitutive shedding of endothelial IL-8/heparan sulfate/syndecan-1 complexes. *J Immunol* **171**:2057-2065.

Martin JL, Kenna JG, Martin BM, Thomassen D, Reed GF and Pohl LR (1993a) Halothane hepatitis patients have serum antibodies that react with protein disulfide isomerase. *Hepatology* **18**:858-863.

Martin JL, Reed GF and Pohl LR (1993b) Association of anti-58 kDa endoplasmic reticulum antibodies with halothane hepatitis. *Biochem Pharmacol* **46**:1247-1250.

Martino G, Adorini L, Rieckmann P, Hillert J, Kallmann B, Comi G and Filippi M (2002) Inflammation in multiple sclerosis: the good, the bad, and the complex. *Lancet Neurol* **1**:499-509.

Masubuchi Y, Nakayama S and Horie T (2002) Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. *Hepatology* **35**:544-551.

Matsumaru K, Ji C and Kaplowitz N (2003) Mechanisms for sensitization to TNF-induced apoptosis by acute glutathione depletion in murine hepatocytes. *Hepatology* **37**:1425-1434.

Mattes WB (2004) Annotation and cross-indexing of array elements on multiple platforms. *Environ Health Perspect* **112**:506-510.

Mavier P, Preaux AM, Guigui B, Lescs MC, Zafrani ES and Dhumeaux D (1988) In vitro toxicity of polymorphonuclear neutrophils to rat hepatocytes: evidence for a proteinase-mediated mechanism. *Hepatology* **8**:254-258.

McGirr LG, Khan S, Lauriault V and O'Brien PJ (1990) Molecular mechanisms for bromotrichloromethane cytotoxicity in isolated rat hepatocytes. *Xenobiotica* **20**:933-943.

McLain GE, Sipes IG and Brown BR, Jr. (1979) An animal model of halothane hepatotoxicity: roles of enzyme induction and hypoxia. *Anesthesiology* **51**:321-326.

Meyer O, Hoffmann T, Aslan T, Ahrens N, Kiesewetter H and Salama A (2003) Diclofenac-induced antibodies against RBCs and platelets: two case reports and a concise review. *Transfusion* **43**:345-349.

Mills JG, Koch KM, Webster C, Sirgo MA, Fitzgerald K and Wood JR (1997) The safety of ranitidine in over a decade of use. *Aliment Pharmacol Ther* **11**:129-137.

Minoda Y and Kharasch ED (2001) Halothane-dependent lipid peroxidation in human liver microsomes is catalyzed by cytochrome P4502A6 (CYP2A6). *Anesthesiology* **95**:509-514.

Mizoguchi Y, Shin T, Mizuno M, Seki S, Kobayashi K and Morisawa S (1990) The enhancement of liver injury by lipopolysaccharide in an experimental drug-induced allergic hepatitis model. *Gastroenterol Jpn* **25**:458-462.

Montes R, Declerck PJ, Calvo A, Montes M, Hermida J, Munoz MC and Rocha E (2000) Prevention of renal fibrin deposition in endotoxin-induced DIC through inhibition of PAI-1. *Thromb Haemost* **84**:65-70.

Moulin F, Copple BL, Ganey PE and Roth RA (2001) Hepatic and extrahepatic factors critical for liver injury during lipopolysaccharide exposure. *Am J Physiol Gastrointest Liver Physiol* **281**:G1423-G1431.

Moulin F, Pearson JM, Schultze AE, Scott MA, Schwartz KA, Davis JM, Ganey PE and Roth RA (1996) Thrombin is a distal mediator of lipopolysaccharide-induced liver injury in the rat. *J Surg Res* **65**:149-158.

Murphy EJ, Davern TJ, Shakil AO, Shick L, Masharani U, Chow H, Freise C, Lee WM and Bass NM (2000) Troglitazone-induced fulminant hepatic failure. Acute Liver Failure Study Group. *Dig Dis Sci 2000* **45**:549-553.

Nagai H, Matsumaru K, Feng G and Kaplowitz N (2002) Reduced glutathione depletion causes necrosis and sensitization to tumor necrosis factor-alpha-induced apoptosis in cultured mouse hepatocytes. *Hepatology* **36**:55-64.

Nakamoto Y and Kaneko S (2003) Mechanisms of viral hepatitis induced liver injury. *Curr Mol Med* **3**:537-544.

Neville LF, Mathiak G and Bagasra O (1997) The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev* **8**:207-219.

Njoku DB, Greenberg RS, Bourdi M, Borkowf CB, Dake EM, Martin JL and Pohl LR (2002) Autoantibodies associated with volatile anesthetic hepatitis found in the sera of a large cohort of pediatric anesthesiologists. *Anesth Analg* **94**:243-9, table.

Nolan JP (1989) Intestinal endotoxins as mediators of hepatic injury--an idea whose time has come again. *Hepatology* **10**:887-891.

Offit KO (1984) Ranitidine: Correspondence. N. Engl. J. Med. **310**:1603-1604.

Okajima K, Harada N and Uchiba M (2002) Ranitidine reduces ischemia/reperfusion-induced liver injury in rats by inhibiting neutrophil activation. *J Pharmacol Exp Ther* **301**:1157-1165.

Oldham HG, Norman SJ and Chenery RJ (1985) Primary cultures of adult rat hepatocytes--a model for the toxicity of histamine H2-receptor antagonists. *Toxicology* **36**:215-229.

Ozawa K, Kuwabara K, Tamatani M, Takatsuji K, Tsukamoto Y, Kaneda S, Yanagi H, Stern DM, Eguchi Y, Tsujimoto Y, Ogawa S and Tohyama M (1999) 150-kDa oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. *J Biol Chem* **274**:6397-6404.

Park JH, Spiegelman DL, Burge HA, Gold DR, Chew GL and Milton DK (2000) Longitudinal study of dust and airborne endotoxin in the home. *Environ Health Perspect* **108**:1023-1028.

Patterson RM, Stachlewitz R and Germolec D (2003) Induction of apoptosis by 2,3,7,8-tetrachlorodibenzo-p-dioxin following endotoxin exposure. *Toxicol Appl Pharmacol* **190**:120-134.

Pawlinski R, Pedersen B, Kehrle B, Aird WC, Frank RD, Guha M and Mackman N (2003) Regulation of tissue factor and inflammatory mediators by Egr-1 in a mouse endotoxemia model. *Blood* **101**:3940-3947.

Pawlinski R, Pedersen B, Schabbauer G, Tencati M, Holscher T, Boisvert W, ndrade-Gordon P, Frank RD and Mackman N (2004) Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. *Blood* **103**:1342-1347.

Pearson JM, Brown AP, Schultze AE, Ganey PE and Roth RA (1996a) Gadolinium chloride treatment attenuates hepatic platelet accumulation after lipopolysaccharide administration. *Shock* **5**:408-415.

Pearson JM, Schultze AE, Jean PA and Roth RA (1995) Platelet participation in liver injury from gram-negative bacterial lipopolysaccharide in the rat. *Shock* **4**:178-186.

Pearson JM, Schultze AE, Schwartz KA, Scott MA, Davis JM and Roth RA (1996b) The thrombin inhibitor, hirudin, attenuates lipopolysaccharide-induced liver injury in the rat. *J Pharmacol Exp Ther* **278**:378-383.

Pellock JM (1999) Felbamate. *Epilepsia* **40 Suppl 5**:S57-S62.

Pfeilschifter J, Koditz R, Pfohl M and Schatz H (2002) Changes in proinflammatory cytokine activity after menopause. *Endocr Rev* **23**:90-119.

Pirmohamed M, Breckenridge AM, Kitteringham NR and Park BK (1998) Adverse drug reactions. *BMJ* **316**:1295-1298.

Pirmohamed M, Madden S and Park BK (1996) Idiosyncratic drug reactions. Metabolic bioactivation as a pathogenic mechanism. *Clin Pharmacokinet* **31**:215-230.

Pirmohamed M, Naisbitt DJ, Gordon F and Park BK (2002) The danger hypothesis--potential role in idiosyncratic drug reactions. *Toxicology* **181-182**:55-63.

Platz EA and De Marzo AM (2004) Epidemiology of inflammation and prostate cancer. *J Urol* **171**:S36-S40.

Price CJ, Warburton EA and Menon DK (2003) Human cellular inflammation in the pathology of acute cerebral ischaemia. *J Neurol Neurosurg Psychiatry* **74**:1476-1484.

Proctor JD (1984) Hepatitis associated with ranitidine. *JAMA* **251**:1554.

Pumford NR, Martin BM, Thomassen D, Burris JA, Kenna JG, Martin JL and Pohl LR (1993a) Serum antibodies from halothane hepatitis patients react with the rat endoplasmic reticulum protein ERp72. *Chem Res Toxicol* **6**:609-615.

Pumford NR, Myers TG, Davila JC, Highet RJ and Pohl LR (1993b) Immunochemical detection of liver protein adducts of the nonsteroidal antiinflammatory drug diclofenac. *Chem Res Toxicol* **6**:147-150.

Purcell P, Henry D and Melville G (1991) Diclofenac hepatitis. *Gut* **32**:1381-1385.

Ramrakhiani S, Brunt EM and Bacon BR (1998) Possible cholestatic injury from ranitidine with a review of the literature. *Am J Gastroenterol* **93**:822-826.

Ribeiro JM, Lucas M, Baptista A and Victorino RM (2000) Fatal hepatitis associated with ranitidine. *Am J Gastroenterol* **95**:559-560.

Rice SA, Maze M, Smith CM, Kosek JC and Mazze RI (1987) Halothane hepatotoxicity in Fischer 344 rats pretreated with isoniazid. *Toxicol Appl Pharmacol* **87**:411-419.

Roth RA, Harkema JR, Pestka JP and Ganey PE (1997) Is exposure to bacterial endotoxin a determinant of susceptibility to intoxication from xenobiotic agents? *Toxicol Appl Pharmacol* **147**:300-311.

Roth RA, Luyendyk JP, Maddox JF and Ganey PE (2003) Inflammation and drug idiosyncrasy--is there a connection? *J Pharmacol Exp Ther* **307**:1-8.

Rumbeiha WK, Fitzgerald SD, Braselton WE, Roth RA and Kaneene JB (2000) Potentiation of mercury-induced nephrotoxicity by endotoxin in the Sprague-Dawley rat. *Toxicology* **149**:75-87.

Rush GF, Ripple M and Chenery R (1985) Mechanism of oxmetidine (SK&F 92994) cytotoxicity in isolated rat hepatocytes. *J Pharmacol Exp Ther* **233**:741-746.

Sachs UJ, Santoso S, Roder L, Smart E, Bein G and Kroll H (2004) Diclofenac-induced antibodies against red blood cells are heterogeneous and recognize different epitopes. *Transfusion* **44**:1226-1230.

Sallustio BC and Holbrook FL (2001) In vivo perturbation of rat hepatocyte canalicular membrane function by diclofenac. *Drug Metab Dispos* **29**:1535-1538.

Sato N, Fujii K, Yuge O and Morio M (1990) The association of halothane-induced lipid peroxidation with the anaerobic metabolism of halothane: an in vitro study in guinea pig liver microsomes. *Hiroshima J Med Sci* **39**:1-6.

Satoh H, Fukuda Y, Anderson DK, Ferrans VJ, Gillette JR and Pohl LR (1985) Immunological studies on the mechanism of halothane-induced hepatotoxicity: immunohistochemical evidence of trifluoroacetylated hepatocytes. *J Pharmacol Exp Ther* **233**:857-862.

Savov JD, Brass DM, Berman KG, McElvania E and Schwartz DA (2003) Fibrinolysis in LPS-induced chronic airway disease. *Am J Physiol Lung Cell Mol Physiol* **285**:L940-L948.

Sawdey MS and Loskutoff DJ (1991) Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. *J Clin Invest* **88**:1346-1353.

Scarpignato C, Tramacere R and Zappia L (1987) Antisecretory and antiulcer effect of the H2-receptor antagonist famotidine in the rat: comparison with ranitidine. *Br J Pharmacol* **92**:153-159.

Schnitt SJ, Stillman IE, Owings DV, Kishimoto C, Dvorak HF and Abelmann WH (1993) Myocardial fibrin deposition in experimental viral myocarditis that progresses to dilated cardiomyopathy. *Circ Res* **72**:914-920.

Schultze AE and Roth RA (1998) Chronic pulmonary hypertension--the monocrotaline model and involvement of the hemostatic system. *J Toxicol Environ Health B Crit Rev* **1**:271-346.

Schwabe RF, Uchinami H, Qian T, Bennett BL, Lemasters JJ and Brenner DA (2004) Differential requirement for c-Jun NH2-terminal kinase in TNFalpha- and Fas-mediated apoptosis in hepatocytes. *FASEB J* **18**:720-722.

Seguin B and Uetrecht J. (2003). The danger hypothesis applied to idiosyncratic drug reactions. *Curr Opin Allergy Clin Immunol* **3**:235-242.

Seitz S, Kretz-Rommel A, Oude Elferink RP and Boelsterli UA (1998) Selective protein adduct formation of diclofenac glucuronide is critically dependent on the rat canalicular conjugate export pump (Mrp2). *Chem Res Toxicol* **11**:513-519.

Semenza GL (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* **15**:551-578.

Sgro C, Clinard F, Ouazir K, Chanay H, Allard C, Guilleminet C, Lenoir C, Lemoine A and Hillon P (2002) Incidence of drug-induced hepatic injuries: a French population-based study. *Hepatology* **36**:451-455.

Shayiq RM, Roberts DW, Rothstein K, Snawder JE, Benson W, Ma X and Black M (1999) Repeat exposure to incremental doses of acetaminophen provides protection against acetaminophen-induced lethality in mice: an explanation for high acetaminophen dosage in humans without hepatic injury. *Hepatology* **29**:451-463.

Shen ES, Garry VF and Anders MW (1982) Effect of hypoxia on carbon tetrachloride hepatotoxicity. *Biochem Pharmacol* **31**:3787-3793.

Shi L, Kishore R, McMullen MR and Nagy LE (2002) Lipopolysaccharide stimulation of ERK1/2 increases TNF-alpha production via Egr-1. *Am J Physiol Cell Physiol* **282**:C1205-C1211.

Shibayama Y (1987) Enhanced hepatotoxicity of endotoxin by hypoxia. *Pathol Res Pract* **182**:390-395.

Shishehbor MH and Hazen SL (2004) Inflammatory and oxidative markers in atherosclerosis: relationship to outcome. *Curr Atheroscler Rep* **6**:243-250.

Silva JM, Rao DN and O'Brien PJ (1992) Modulation of trenimon-induced cytotoxicity by DT-diaphorase in isolated rat hepatocytes under aerobic versus hypoxic conditions. *Cancer Res* **52**:3015-3021.

Smith AC, Roberts SM, Berman LM, Harbison RD and James RC (1988) Effects of piperonyl butoxide on halothane hepatotoxicity and metabolism in the hyperthyroid rat. *Toxicology* **50**:95-105.

Sneed RA, Buchweitz JP, Jean PA and Ganey PE (2000) Pentoxifylline attenuates bacterial lipopolysaccharide-induced enhancement of allyl alcohol hepatotoxicity. *Toxicol Sci* **56**:203-210.

Sneed RA, Grimes SD, Schultze AE, Brown AP and Ganey PE (1997) Bacterial endotoxin enhances the hepatotoxicity of allyl alcohol. *Toxicol Appl Pharmacol* **144**:77-87.

Souza Lima MA (1984) Hepatitis associated with ranitidine. *Ann Intern Med* **101**:207-208.

Spracklin DK, Emery ME, Thummel KE and Kharasch ED (2003) Concordance between trifluoroacetic acid and hepatic protein trifluoroacetylation after disulfiram inhibition of halothane metabolism in rats. *Acta Anaesthesiol Scand* **47**:765-770.

Spracklin DK, Hankins DC, Fisher JM, Thummel KE and Kharasch ED (1997) Cytochrome P450 2E1 is the principal catalyst of human oxidative halothane metabolism in vitro. *J Pharmacol Exp Ther* **281**:400-411.

Spracklin DK, Thummel KE and Kharasch ED (1996) Human reductive halothane metabolism in vitro is catalyzed by cytochrome P450 2A6 and 3A4. *Drug Metab Dispos* **24**:976-983.

Stadnicki A (1984) Ranitidine and haemostasis in man. *Hepatogastroenterology* **31**:230-232.

Stier A (1964) TRIFLUOROACETIC ACID AS METABOLITE OF HALOTHANE. *Biochem Pharmacol* **13**:1544.

Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M and Candinas D (2001) HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J* **15**:2445-2453.

Su GL, Klein RD, Aminlari A, Zhang HY, Steinstraesser L, Alarcon WH, Remick DG and Wang SC (2000) Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* **31**:932-936.

Suzuki H and Sugiyama Y (2002) Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. *Adv Drug Deliv Rev* **54**:1311-1331.

Tai JH and Pestka JJ (1988) Synergistic interaction between the trichothecene T-2 toxin and Salmonella typhimurium lipopolysaccharide in C3H/HeN and C3H/HeJ mice. *Toxicol Lett* **44**:191-200.

Tamura DY, Moore EE, Partrick DA, Johnson JL, Offner PJ and Silliman CC (2002) Acute hypoxemia in humans enhances the neutrophil inflammatory response. *Shock* **17**:269-273.

Tang W (2003) The metabolism of diclofenac--enzymology and toxicology perspectives. *Curr Drug Metab* **4**:319-329.

Thiel G and Cibelli G (2002) Regulation of life and death by the zinc finger transcription factor Egr-1. *J Cell Physiol* **193**:287-292.

Tolman KG and Chandramouli J (2003) Hepatotoxicity of the thiazolidinediones. *Clin Liver Dis* **7**:369 -79

Tracy RP (2003) Emerging relationships of inflammation, cardiovascular disease and chronic diseases of aging. *Int J Obes Relat Metab Disord* **27 Suppl 3**:S29-S34.

Tredger JM, Thuluvath P, Williams R and Murray-Lyon IM (1995) Metabolic basis for high paracetamol dosage without hepatic injury: a case study. *Hum Exp Toxicol* **14**:8-12.

Tuder RM and Voelkel NF (1998) Pulmonary hypertension and inflammation. *J Lab Clin Med* **132**:16-24.

Uetrecht J (2001) Prediction of a new drug's potential to cause idiosyncratic reactions. *Curr Opin Drug Discov Devel* **4**:55-59.

Uetrecht J (1991) Metabolism of drugs by activated leukocytes: implications for drug-induced lupus and other drug hypersensitivity reactions. *Adv Exp Med Biol* **283**:121-132.

Ulrich RG, Bacon JA, Brass EP, Cramer CT, Petrella DK and Sun EL (2001) Metabolic, idiosyncratic toxicity of drugs: overview of the hepatic toxicity induced by the anxiolytic, panadiplon. *Chem Biol Interact* **134**:251-270.

Valois M, Cooper MA and Shear NH (2003) Clinical pharmacology consultations: consultation requests may be misleading -- an organized approach to drug-induced hepatitis. *Can J Clin Pharmacol* **10**:59-62.

van Bommel EF and Meyboom RH (1992) [Liver damage caused by ranitidine]. *Ned Tijdschr Geneeskd* **136**:435-437.

Van Dyke TE and Serhan CN (2003) Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. *J Dent Res* **82**:82-90.

Van Pelt FN and Kenna JG (1994) Formation of trifluoroacetylated protein antigens in cultured rat hepatocytes exposed to halothane in vitro. *Biochem Pharmacol* **48**:461-471.

Vannier E, Miller LC and Dinarello CA (1991) Histamine suppresses gene expression and synthesis of tumor necrosis factor alpha via histamine H2 receptors. *J Exp Med* **174**:281-284.

Vergani D, Mieli-Vergani G, Alberti A, Neuberger J, Eddleston AL, Davis M and Williams R (1980) Antibodies to the surface of halothane-altered rabbit hepatocytes in patients with severe halothane-associated hepatitis. *N Engl J Med* **303**:66-71.

Vial T, Goubier C, Bergeret A, Cabrera F, Evreux JC and Descotes J (1991) Side effects of ranitidine. *Drug Saf* **6**:94-117.

Visser M, Bouter LM, McQuillan GM, Wener MH and Harris TB (1999) Elevated C-reactive protein levels in overweight and obese adults. *JAMA* **282**:2131-2135.

Visser M, Bouter LM, McQuillan GM, Wener MH and Harris TB (2001) Low-grade systemic inflammation in overweight children. *Pediatrics* **107**:E13.

Vollmar B, Ruttinger D, Wanner GA, Leiderer R and Menger MD (1996) Modulation of kupffer cell activity by gadolinium chloride in endotoxemic rats. *Shock* **6**:434-441.

Wagner JG, Van Dyken SJ, Hotchkiss JA and Harkema JR (2001) Endotoxin enhancement of ozone-induced mucous cell metaplasia is neutrophil-dependent in rat nasal epithelium. *Toxicol Sci* **60**:338-347.

Walker AM (1997) Quantitative studies of the risk of serious hepatic injury in persons using nonsteroidal antiinflammatory drugs. *Arthritis Rheum* **40**:201-208.

Whitcomb DC (2004) Inflammation and Cancer V. Chronic pancreatitis and pancreatic cancer. *Am J Physiol Gastrointest Liver Physiol* **287**:G315-G319.

Willerson JT and Ridker PM (2004) Inflammation as a cardiovascular risk factor. *Circulation* **109**:II2-10.

Wood CL, Gandolfi AJ and Van Dyke RA (1976) Lipid binding of a halothane metabolite. Relationship to lipid peroxidation in vitro. *Drug Metab Dispos* **4**:305-313.

Yamasawa H, Ishii Y and Kitamura S (1999) Cytokine-induced neutrophil chemoattractant in a rat model of lipopolysaccharide-induced acute lung injury. *Inflammation* **23**:263-274.

Yamazoe K, Inaba T, Bonkobara M, Matsuki N, Ono K and Kudo T (1998) Changes of hepatic tissue phospholipid peroxidation, malondialdehydes, and antioxidative enzyme activities in dogs with halothane inhalation. *J Vet Med Sci* **60**:15-21.

Yan SF, Lu J, Zou YS, Soh-Won J, Cohen DM, Buttrick PM, Cooper DR, Steinberg SF, Mackman N, Pinsky DJ and Stern DM (1999) Hypoxia-associated induction of early growth response-1 gene expression. *J Biol Chem* **274**:15030-15040.

Yang SQ, Lin HZ, Lane MD, Clemens M and Diehl AM (1997) Obesity increases sensitivity to endotoxin liver injury: implications for the pathogenesis of steatohepatitis. *Proc Natl Acad Sci U S A* **94**:2557-2562.

Yasar U, Eliasson E, Forslund-Bergengren C, Tybring G, Gadd M, Sjoqvist F and Dahl ML (2001) The role of CYP2C9 genotype in the metabolism of diclofenac in vivo and in vitro. *Eur J Clin Pharmacol* **57**:729-735.

Yee SB, Ganey PE and Roth RA (2003a) The role of Kupffer cells and TNF-alpha in monocrotaline and bacterial lipopolysaccharide-induced liver injury. *Toxicol Sci* **71**:124-132.

Yee SB, Hanumegowda UM, Copple BL, Shibuya M, Ganey PE and Roth RA (2003b) Endothelial cell injury and coagulation system activation during synergistic hepatotoxicity from monocrotaline and bacterial lipopolysaccharide coexposure. *Toxicol Sci* **74**:203-214.

Yee SB, Hanumegowda UM, Hotchkiss JA, Ganey PE and Roth RA (2003c) Role of neutrophils in the synergistic liver injury from monocrotaline and bacterial lipopolysaccharide exposure. *Toxicol Sci* **72**:43-56.

Yee SB, Harkema JR, Ganey PE and Roth RA (2003d) The coagulation system contributes to synergistic liver injury from exposure to monocrotaline and bacterial lipopolysaccharide. *Toxicol Sci* **74**:457-469.

Yee SB, Kinser S, Hill DA, Barton CC, Hotchkiss JA, Harkema JR, Ganey PE and Roth RA (2000) Synergistic hepatotoxicity from coexposure to bacterial endotoxin and the pyrrolizidine alkaloid monocrotaline. *Toxicol Appl Pharmacol* **166**:173-185.

Yokoyama M, Yokoyama A, Mori S, Takahashi HK, Yoshino T, Watanabe T, Watanabe T, Ohtsu H and Nishibori M (2004) Inducible histamine protects mice from P. acnes-primed and LPS-induced hepatitis through H2-receptor stimulation. *Gastroenterology* **127**:892-902.

Younes M, Heger B, Wilhelm KP and Siegers CP (1988) Enhanced in vivo-lipid peroxidation associated with halothane hepatotoxicity in rats. *Pharmacol Toxicol* **63**:52-56.

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

Zhao B, Stavchansky SA, Bowden RA and Bowman PD (2003) Effect of interleukin-1beta and tumor necrosis factor-alpha on gene expression in human endothelial cells. *Am J Physiol Cell Physiol* **284**:C1577-C1583.

Zhong H, Willard M and Simons J (2004) NS398 reduces hypoxia-inducible factor (HIF)-1alpha and HIF-1 activity: Multiple-level effects involving cyclooxygenase-2 dependent and independent mechanisms. *Int J Cancer* **112**:585.

Zhou HR, Harkema JR, Hotchkiss JA, Yan D, Roth RA and Pestka JJ (2000) Lipopolysaccharide and the trichothecene vomitoxin (deoxynivalenol) synergistically induce apoptosis in murine lymphoid organs. *Toxicol Sci* **53**:253-263.

Zimmerman HJ (1978) Drug-induced liver disease. *Drugs* **16**:25-45.

Zimmerman HJ (1993) Hepatotoxicity. *Dis Mon* **39**:675-787.

Zimmerman HJ, Jacob L, Bassan H, Gillespie J, Lukacs L and Abernathy CO (1986) Effects of H2-blocking agents on hepatocytes in vitro: correlation with potential for causing hepatic disease in patients. *Proc Soc Exp Biol Med* **182**:511-514.