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dissertation entitled

MECHANISMS OF SULINDAC/LPS-INDUCED LIVER INJURY IN RATS: AN ANIMAL MODEL OF DRUG-INDUCED IDIOSYNCRATIC HEPATOTOXICITY

presented by

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of the requirements for the

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MECHANISMS OF SULINDAC/LPS-INDUCED LIVER INJURY IN RATS: AN ANIMAL MODEL OF DRUG-INDUCED IDIOSYNCRATIC HEPATOTOXICITY

By

Wei Zou

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ABSTRACT

SULINDAC/LPS-INDUCED LIVER INJURY IN RATS: AN ANIMAL MODEL OF IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY

By

Wei Zou

Idiosyncratic adverse drug response is a type of adverse reaction that occurs in a minority of patients during drug therapy. Liver is one of the major organ targets. All of the nonsteroidal anti-inflammatory drugs (NSAIDs) have been associated with hepatic idiosyncratic adverse drug reactions (IADRs) in patients, and the risk from sulindac (SLD) is reported to be 5-10 fold greater than for NSAIDs as a class. However, the mechanism of SLD-induced hepatotoxicity has not been clarified because of the lack of experimental animal models. Previous studies suggest that inflammatory stress is a susceptibility factor for IADRs. The work in this dissertation supports this hypothesis. Cotreatment of rats with lipopolysaccharide (LPS), which induces modest inflammation, and SLD resulted in liver necrosis, whereas neither LPS nor SLD was hepatotoxic alone. After we developed a SLD- inflammation interaction model of idiosyncratic liver injury by treating rats with SLD and LPS, the mechanisms of SLD/LPS- induced liver injury were investigated. SLD/LPS cotreatment causes an increase in the production of tumor necrosis factor-α (TNF), activation of the hemostatic system and of neutrophils (PMNs) as well as oxidative stress in the liver. Neutralization of TNF, anticoagulant administration, PMN depletion or antioxidant treatment attenuated liver injury in this model. Results of neutralization or inhibition studies

in vivo and in vitro suggest roles for TNF, the hemostatic system, PMNs and oxidative stress in the pathogenesis of liver injury-induced by SLD/LPS. Moreover, these mediators are not independent players. They contribute to liver injury by interacting with each other and with the SLD toxic metabolite, SLD sulfide. The studies in this dissertation provide an understanding of mechanisms of liver injury resulting from SLD- inflammation interaction.

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It is my fortune to work with so many talented and warm-hearted people in our lab. I would like to thank Shawn Deng. He is the person I closely worked with during the rotation in the lab. He was always ready to answer all kinds of

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questions I came up with and never weary of showing me lab techniques. Erica Sparkenbaugh also deserves my gratitude. She helped me a lot with the in vitro experiments, including hepatocyte isolation. I also thank Kevin Beggs who worked side by side with me for months on this project. In addition to them, I would like to thank all the people in the lab who I worked with. They are Dr. Jane Maddox, Dr. Francis Tukov, Dr. Sachin Devi, Dr. Jesus Olivero-Verbel, Dr. Rohit Sinhal, Pat Shaw, Christine Dugan, Jingtao Lu, Aaron Fullerton, Kyle Poulsen, Nicole Crisp, Allen MacDonald, Theresa Eagle and Sandy Newport.

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TABLE OF CONTENTS

LIST OF TABLES.....x

LIST OF FIGURES.....xi

LIST OF ABBREVIATIONS.....xvi

CHAPTER 1
GENERAL INTRODUCTION.....1
1.1 Idiosyncratic adverse drug reactions (IADRs).....2
1.1.1 Overview of drug-induced idiosyncratic hepatotoxicity.....2
1.1.2 Conventional explanations for IADRs.....4
1.1.3 Inflammatory stress hypothesis as potential explanation for IADR.....9
1.2 Idiosyncratic adverse drug reactions (IADRs).....14
1.2.1 Overview of the inflammatory response.....14
1.2.2 Tumor necrosis factor-α (TNF).....18
1.2.3 The hemostatic system and hypoxia.....22
1.2.4 Neutrophils (PMNs).....23
1.2.5 Reactive oxygen species.....25
1.3 Models of drug-inflammation interaction.....28
1.3.1 Ranitidine/LPS-induced hepatotoxicity in rats.....28
1.3.2 Diclofenac/LPS-induced hepatotoxicity in rats.....30
1.3.3 Trovafloxacin-inflammation interaction model of idiosyncratic liver injury.....32
1.4 Sulindac-induced idiosyncratic liver injury.....35
1.5 Hypothesis and specific aims.....39

CHAPTER 2
HEPATOTOXIC INTERACTION OF SULINDAC WITH
LIPOPOLYSACCHARIDE: ROLE OF THE HEMOSTATIC SYSTEM.....41
2.1 Abstract.....42
2.2 Introduction.....43
2.3 Materials and methods.....44
2.3.1 Materials.....44
2.3.2 Animals.....44
2.3.3 Experimental protocol.....44
2.3.4 Evaluation of liver injury.....45
2.3.5 Evaluation of serum TNFα concentrations.....47
2.3.6 Evaluation of hemostasis and fibrin deposition.....47
2.3.7 Evaluation of liver hypoxia.....48
2.3.8 Hepatocyte (HPC) isolation and hepatocytotoxicity assessment in vitro.....48
2.3.9 Statistical analysis.....49
2.4 Results.....50
2.4.1 Dose-response and timecourse of liver injury.....50

242 H
243 E
244 A
245 E
246 E
vi
25 Disc

CHAPTER
SCLNDAC
NFAMMA

31 Abstra
32 Introd
33 Mate
331 M
332 A
333 E
334 E
335 D
336 L
337 E
338 C
339 S
34 Res
341 T
342 E
343 E
344 E
345 C
he
346 E
ra
35 Disc

CHAPTER
THE CRIT
ACTIVATIO
IDIOSYNC
41 Abst
42 Introc
43 Mate
431 M
432 A
433 A

2.4.2 Histopathological findings 50
2.4.3 Effect of SLD/LPS cotreatment on serum TNF concentration 57
2.4.4 Activation of the hemostatic system 57
2.4.5 Effect of heparin on liver injury induced by SLD/LPS cotreatment 62
2.4.6 Effect of low oxygen on hepatocytotoxicity induced by SLD sulfide in vitro 69
2.5 Discussion 74

CHAPTER 3
SULINDAC METABOLISM AND SYNERGY WITH TNF IN A DRUG-INFLAMMATION INTERACTION MODEL OF IDIOSYNCRATIC LIVER INJURY **81**
3.1 Abstract 82
3.2 Introduction 83
3.3 Materials and methods 84
3.3.1 Materials 84
3.3.2 Animals 84
3.3.3 Experimental protocol 84
3.3.4 Evaluation of liver injury 85
3.3.5 Determination of TNF concentration in serum 86
3.3.6 LC/MS/MS analysis 86
3.3.7 Evaluation of cytotoxicity of SLD and its metabolites in vitro 87
3.3.8 Cytotoxicity from TNF and SLD metabolites 88
3.3.9 Statistical analysis 89
3.4 Results 90
3.4.1 Timecourse of TNF concentration in plasma 90
3.4.2 Effect of TNF inhibition on liver injury 90
3.4.3 Effect of LPS on SLD metabolism in rat 94
3.4.4 Effect of etanercept on SLD metabolism in rats 101
3.4.5 Cytotoxicity of SLD and its metabolites in HepG2 cells and rat primary hepatocytes 101
3.4.6 Effect of TNF on cytotoxicity of SLD and its metabolites in HepG2 and rat primary hepatocytes 101
3.5 Discussion 109

CHAPTER 4
THE CRITICAL ROLE OF TNF AND PAI-1 MEDIATED NEUTROPHIL ACTIVATION IN A SULINDAC/ LIPOPOLYSACCHARIDE- MODEL OF IDIOSYNCRATIC LIVER INJURY IN RATS **115**
4.1 Abstract 116
4.2 Introduction 117
4.3 Materials and methods 118
4.3.1 Materials 118
4.3.2 Animals 118
4.3.3 Animal model and sample collection 118

434
435
436
437
439
439
44 Res
441
442
443
444
445
446
447
45 Dis

CHAPTE
OXIDAT
LIVER IN
51 Abs
52 Int
53 Ma
531
532
533
534
535
536
537
538
539
54 Res
541
542
543
544
545
546

4.3.4 Anti-PMN serum, eglin C, PAI039 and etanercept treatment protocol 119
4.3.5 Evaluation of hepatotoxicity 119
4.3.6 Determination of CINC-1, MIP-1α and PAI-1 concentrations in plasma 120
4.3.7 Evaluation of liver PMN accumulation and activation 120
4.3.8 Assessment of fibrin deposition in liver 121
4.3.9 Statistical analysis 121
4.4 Results 123
4.4.1 Evaluation of PMN accumulation and activation in livers 123
4.4.2 Time course of CINC-1 and MIP-1α concentrations in plasma 123
4.4.3 Effect of PMN depletion and PMN protease inhibition on SLD/LPS-induced liver injury 126
4.4.4 Effect of TNF on PMN accumulation and activation 131
4.4.5 Role of PAI-1 in liver injury and accumulation and activation of PMNs 131
4.4.6 Effect of TNF on plasma PAI-1 concentration 131
4.4.7 Effect of TNF and PAI-1 on fibrin deposition 136
4.5 Discussion 140

CHAPTER 5
OXIDATIVE STRESS IS A POTENTIAL PLAYER IN THE PATHOGENESIS OF LIVER INJURY INDUCED BY SULINDAC AND LIPOPOLYSACCHARIDE **147**
5.1 Abstract 148
5.2 Introduction 149
5.3 Materials and methods 150
5.3.1 Materials 150
5.3.2 Animals 150
5.3.3 Design of experiments in vivo 150
5.3.4 Gene expression analysis 151
5.3.5 Evaluation of liver injury and protein carbonyls in mitochondria 152
5.3.6 Evaluation of mitochondrial membrane potential 153
5.3.7 Evaluation of reactive oxygen species in HepG2 cells 153
5.3.8 Evaluation of cytotoxicity and capase 3 activity 154
5.3.9 Statistical analyses 154
5.4 Results 155
5.4.1 Gene expression changes regulated by treatment with SLD, LPS or SLD/LPS 155
5.4.2 Gene expression changes specifically regulated by SLD/LPS point to oxidative stress 155
5.4.3 Oxidative stress in SLD/LPS-cotreated rats 158
5.4.4 Effect of SLD sulfide on mitochondrial membrane potential 158
5.4.5 Effect of SLD sulfide on the production of reactive oxygen species in HepG2 cells 164
5.4.6 Cytotoxicity of hydrogen peroxide and TNF to HepG2 cells 164

5
5
5.5
CHA
SOL
6.1
6.2
6.3
APP
BIB

5.4.7 Effect of antioxidant treatment and caspase inhibition on cytotoxicity 164
5.4.8 Effect of antioxidant treatment on caspase 3/7 activity induced by SLD sulfide and TNF cotreatment 169
5.5 Discussion 172

CHAPTER 6
SUMMARY AND CONCLUSIONS **177**
6.1 Summary and conclusions 178
6.2 Commonality and differences among liver injury models of drug-inflammation interaction 183
6.3 Potential future studies 187

APPENDICES 189

BIBLIOGRAPHY **201**

LIST OF TABLES

TABLE 1.1 LIST OF IDIOSYNCRATIC DRUGS TO CAUSE LIVER INJURY IN DRUG/LPS MODELS IN RODENTS 13

TABLE 2.1 MIDZONAL HEPATIC NECROSIS IN LIVERS OF RATS TREATED WITH SLD/LPS 58

TABLE 2.2 SERUM TNFA CONCENTRATION IN SLD/LPS-TREATED RATS .59

TABLE 3.1 EFFECT OF ETANERCEPT ON SLD METABOLISM 102

TABLE 5.1 PATHWAYS ASSOCIATED WITH GENES SPECIFICALLY CHANGED BY SLD/LPS COTREATMENT 159

TABLE 6.1 CHARACTERISTICS AND UNDERLYING MECHANISMS OF IDIOSYNCRATIC LIVER INJURY MODELS OF DRUG-INFLAMMATION INTERACTION 184

LIST OF FIGURES

IMAGES IN THIS DISSERTATION ARE PRESENTED IN COLOR

FIGURE 1.1 INFLAMMATION MIGHT PRECIPITATE DRUG- INDUCED IADRS 11

FIGURE 1.2 SUMMARY OF THE INFLAMMATORY RESPONSE 15

FIGURE 1.3 TNF SIGNAL TRANSDUCTION PATHWAY 20

FIGURE 1.4 METABOLISM OF SLD 36

FIGURE 2.1 EXPERIMENTAL PROTOCOL FOR ANIMAL TREATMENT 46

FIGURE 2.2 SULINDAC DOSE-RESPONSE IN THE ABSENCE AND PRESENCE OF LPS 51

FIGURE 2.3 DEVELOPMENT OF HEPATOCELLULAR INJURY INDUCED BY SLD/LPS COTREATMENT 52

FIGURE 2.4 MARKERS OF HEPATIC CHOLESTASIS INDUCED BY SLD/LPS COTREATMENT 54

FIGURE 2.5 LIVER HISTOPATHOLOGY IN SLD/LPS-COTREATED RATS 56

FIGURE 2.6 ACTIVATION OF THE HEMOSTATIC SYSTEM 60

FIGURE 2.7 FIBRIN DEPOSITION IN LIVER 63

FIGURE 2.8 HYPOXIA STAINING IN LIVER 64

FIGURE 2.9 EVALUATION OF FIBRIN DEPOSITION AND HYPOXIA IN LIVER 65

FIGURE 2.10 EFFECT OF HEPARIN ON LIVER FIBRIN AND HYPOXIA 67

FIGURE 2.11 EFFECT OF HEPARIN ON SLD/LPS-INDUCED LIVER INJURY 70

FIGURE 2.12 EFFECT OF HEPARIN ON HEPATIC LESIONS INDUCED BY SLD/LPS 72

FIGURE 2.13 EFFECT OF HYPOXIA ON SLD SULFIDE-INDUCED CYTOTOXICITY 73

FIGURE 3.1 TIMECOURSE OF TNF CONCENTRATION IN RAT SERUM 91

FIGURE 3.2 EFFECT OF TNF INHIBITION ON LIVER INJURY INDUCED BY SLD/LPS 92

FIGURE 3.3 EFFECT OF LPS ON PLASMA CONCENTRATIONS OF SLD, SLD SULFONE AND SLD SULFIDE 95

FIGURE 3.4 EFFECT OF LPS ON LIVER CONCENTRATIONS OF SLD, SLD SULFONE AND SLD SULFIDE 97

FIGURE 3.5 CONCENTRATIONS OF SLD, SLD SULFONE AND SLD SULFIDE IN GI TRACT AND FECES 99

FIGURE 3.6 EVALUATION OF CYTOTOXICITY INDUCED BY SLD, SLD SULFONE OR SLD SULFIDE 103

FIGURE 3.7 CYTOTOXICITY INDUCED BY TNF AND SLD OR ITS METABOLITES 105

FIGURE 3.8 EFFECT OF TNF ON SLD SULFIDE-INDUCED INJURY TO RAT PRIMARY HEPATOCYTES 108

FIGURE 4.1 EVALUATON OF PMN ACCUMULATION AND ACTIVATION IN RAT LIVERS 124

FIGURE 4.2 CONCENTRATIONS OF PMN CHEMOKINES IN RAT PLASMA 127

FIGURE 4.3 EFFECT OF PMN DEPLETION ON SLD/LPS-INDUCED LIVER INJURY 129

FIGURE 4.4 EFFECT OF PMN PROTEASE INHIBITION ON SLD/LPS-INDUCED LIVER INJURY 130

FIGURE 4.5 EFFECT OF TNF INHIBITION ON PMN ACCUMULATION AND ACTIVATION 132

FIGURE 4.6 EFFECT OF PAI-1 INHIBITION ON LIVER INJURY AND PMN ..134

FIGURE 4.7 EFFECT OF TNF INHIBITION ON PLASMA PAI-1 CONCENTRATION 137

FIGURE 4.8 EFFECT OF TNF AND PAI-1 ON LIVER FIBRIN DEPOSITION .138

FIGURE 4.9 MECHANISMS OF SLD/LPS-INDUCED LIVER INJURY 146

FIGURE 5
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FIGURE 5
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FIGURE 5.1 VENN DIAGRAM OF PROBE SETS REGULATED BY SLD/VEH, VEH/LPS OR SLD/LPS 156

FIGURE 5.2 EVALUATION OF PROTEIN CARBONYL CONCENTRATION IN LIVER MITOCHONDRIA..... 161

FIGURE 5.3 EFFECT OF ANTIOXIDANTS ON LIVER INJURY 162

FIGURE 5.4 EFFECT OF SLD AND ITS METABOLITES ON MITOCHONDRIAL MEMBRANE POTENTIAL 165

FIGURE 5.5 EFFECT OF SLD SULFIDE AND TNF ON PRODUCTION OF REACTIVE OXYGEN SPECIES IN HEPG2 CELLS 166

FIGURE 5.6 CYTOTOXICITY INDUCED BY HYDROGEN PEROXIDE AND TNF 167

FIGURE 5.7 EFFECT OF ANTIOXIDANT ON CYTOTOXICITY INDUCED BY SLD SULFIDE AND TNF 168

FIGURE 5.8 EFFECT OF ANTIOXIDANT ON CASPASE 3/7 ACTIVITY 170

FIGURE 5.9 EFFECT OF PAN-CASPASE INHIBITOR ON CYTOTOXICITY INDUCED BY SLD SULFIDE AND TNF 171

FIGURE 6.1 PROPOSED PATHWAY IN THE PATHOGENESIS OF SLD/LPS-INDUCED LIVER INJURY 182

ALP-

ALT-

ANOV

AST-

CNC-

DMEM

DMSO

FBS-

RMO-

GGT-

H&E-

HCC-

IACUC

IDEL-

LDH-

LPS-

MPO-

MRM-

MSR-

NSAID

PAL-

PVN-

LIST OF ABBREVIATIONS

ALP - alkaline phosphatase

ALT- alanine aminotransferase

ANOVA- analysis of variance

AST- aspartate aminotransferase

CINC-1- cytokine-induced neutrophil chemoattractant-1

DMEM- Dulbecco's modified Eagle's medium

DMSO- dimethyl sulfoxide

FBS- fetal bovine serum

FMO- flavin containing monooxygenase

GGT- γ-glutamyltransferase

H&E- hematoxylin and eosin

HOCl- hypochlorous acid

IADRs- idiosyncratic adverse drug reactions

IDILI- idiosyncratic drug-induced liver injury

LDH- lactate dehydrogenase

LPS- lipopolysaccharide

MPO- myeloperoxidase

MRM- multiple reaction monitoring

MSR- methionine sulfoxide reductase

NSAID- nonsteroidal anti-inflammatory drug

PAI-1- plasminogen activator inhibitor-1

PMN- polymorphonuclear neutrophil

ROS- re

SLD- s

TAT - t

TNF- tu

UPLC- u

ROS- reactive oxygen species

SLD- sulindac

TAT - thrombin-antithrombin

TNF- tumor necrosis factor-α

UPLC- ultra performance liquid chromatography

CHAPTER 1

General introduction

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1.1 Idiosyncratic adverse drug reactions (IADRs)

1.1.1 Overview of drug-induced idiosyncratic hepatotoxicity

Adverse drug reactions (ADRs) remain a major issue for affected patients as well as a huge challenge to health providers. The safety of new compounds are sometimes not well understood until a drug has been on the market for many years. As a result, serious ADRs commonly emerge after approval of a drug by the Food and Drug Administration (FDA). More than 10% of newly approved drugs from 1975 to 2000 in the United States either had to be withdrawn from the market or received a warning due to adverse reactions (Lasser et al., 2002). The liver, which plays an important role in the metabolism of drugs, is a frequent target of IADRs.

There are two types of adverse drug reactions: dose-related reactions (Type A reactions) and idiosyncratic reactions (Type B reactions). Type A reactions occur during drug therapy and they are dose-dependent, and most likely occur in overdosed individuals. A typical example is acetaminophen-induced adverse reactions that are due to acetaminophen overdose (Amar and Schiff, 2007;Larson et al., 2005). IADRs differ from type A adverse drug reactions in that they are unpredictable and not apparently dose-dependent. Typically, IADRs occur only in a minority of patients who are treated with a specific drug. IADRs do not relate to the known pharmacologic effects of the drug (Kaplowitz, 2005; Uetrecht, 2006; Uetrecht, 2007).

Drug-induced idiosyncratic hepatotoxicity, which might result in permanent disability or death, has great importance to human health. In addition, these

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ADRs

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reactions are a major issue for the pharmaceutical industry, because they lead to a large number of withdrawals and restrictions to the use of efficient drugs on the markets. A typical example of a drug which induced idiosyncratic hepatotoxicity is troglitazone, which contributed 10% of overall idiosyncratic drug reactions between 1998 and 2001 and was withdrawn from the market by the U.S. FDA in 2000 (Ostapowicz et al., 2002). The main reason for the withdrawal was that troglitazone was associated with the development of acute liver failure (Chojkier, 2005). Troglitazone is a peroxisomal proliferator-activated receptor (PPAR)-γ agonist that is used to treat type 2 diabetes. Although troglitazone was beneficial at improving insulin resistance, among 1.92 million patients who took troglitazone, ninety-four cases of liver failure were reported (89 acute, 5 chronic) (Graham et al., 2003).

The risk of idiosyncratic adverse drug reactions is difficult to predict, and most idiosyncratic reactions are not discovered until a drug is on the market. That is because a clear mechanistic understanding of IADRs is still absent, and IADRs are generally not reproducible in traditional animal models. For example, oral administration of troglitazone to monkeys at large doses (60- to 120-fold larger than the therapeutic dose) for 52 weeks did not increase serum liver enzymes and had little gastrointestinal, hematologic or hepatic effects (Rothwell et al., 2002). The lack of effective preclinical animal models causes mechanisms of IADRs to be poorly understood, so that appropriate action cannot be applied to prevent or treat IADRs.

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1.1.2 Conventional explanations for IADRs

Although the mechanisms are still not fully understood, extensive studies on IADRs have been performed. Several hypotheses have been raised to explain mechanisms of IADRs, including the metabolic polymorphism hypothesis, the hapten hypothesis, the danger hypothesis, the mitochondrial abnormality hypothesis, the failure to adapt hypothesis and the multiple determinant hypothesis. The detailed hypotheses, their supporting evidence and limitations are described below.

Metabolic polymorphism hypothesis

The metabolic polymorphism hypothesis suggests that drug metabolites are responsible for the toxicity of IADRs. Drugs are metabolized into electrophiles or free radicals, which can covalently bind to proteins and/or unsaturated fatty acids, or induce lipid peroxidation (Kaplowitz et al., 1986). As a result, cell functions can be impaired, and cytotoxicity can be caused by inducing a cell death signaling pathway, causing impaired calcium homeostasis or decreased energy generation. Cytochrome P450 is a superfamily of enzymes that transform drugs into their reactive metabolites. Overexpression of P450 in a fraction of patients can lead to excessive reactive metabolite formation and accumulation in the liver that can result in hepatotoxicity. Therefore, polymorphism of P450 that is responsible for drug-induced hepatotoxicity is present in a specific fraction of patients.

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Take troglitazone (TGZ) as an example. TGZ is metabolized into reactive metabolites by CYP3A4, a member of the cytochrome P450 superfamily (He et al., 2004). CYP3A4 mediates oxidative cleavage of the thiazolidinedione ring, generating a highly electrophilic metabolite, TGZ quinine, which covalently binds to cellular macromolecules (Kassahun et al., 2001). This is supported by the evidence that administrating TGZ to HepG2 cells transfected with CYP3A4 led to increased cytotoxicity (Vignati et al., 2005). However, another study suggests that TGZ quinone is less toxic to hepatocytes and HepG2 cells than TGZ itself (Tettey et al., 2001). Moreover, the role of CYP3A4 in the TGZ-induced toxicity has not been tested in vivo.

Although many epidemiological studies have been performed attempting to link susceptibility to drug-induced toxicity with genes involved in drug metabolism (Kumashiro et al., 2003; Daly et al., 2007), there is no direct evidence proving that genetic/metabolic polymorphism contributes to idioscyncratic drug toxicity in vivo.

Hapten hypothesis

The hapten hypothesis is another prevalent theory of IADRs. It suggests that prodrugs or more likely their reactive metabolites form drug-protein adducts through covalent binding (Macher and Chase, 1969; Uetrecht, 2006). The drug-protein adducts are recognized as non-self antigens by the immune system and are taken up by antigen presenting cells. The processed adduct peptides are presented to helper T cells. Thus, an active immune response can be elicited

which causes the production of specific antibodies to adducts. The production of antibodies may target self proteins and lead to the destruction of host tissues.

This theory is supported by case reports in patients with liver injury. Antibodies have been detected after exposure to drugs causing idiosyncratic reactions, including halothane, diclofenac and trogalitazone (Sallie et al., 1991; Maniratanachote et al., 2005; Nguyen et al., 2008). However, evidence against this theory is also accumulating. Autoantibodies are present in only a portion of patients having drug-induced idiosyncratic hepatotoxicity. Moreover, IADRs are induced in some patients after the first exposure to drug (Clay et al., 2006), whereas a second exposure is required in this theory of adaptive immune response.

Several animal models of drug-induced autoimmunity have been developed, including penicillamine-induced autoimmune syndrome and nevirapine-induced skin rash in rats (Tournade et al., 1990; Shenton et al., 2003). Although antibodies against penicillin-modified proteins are present in rats treated with penicillin, liver injury is not induced in the animal model (Shenton et al., 2004). In the nevirapine-induced skin rash model, liver injury is not induced either. Therefore, the results in animal models and human patients suggest that the autoantibodies are not necessarily pathogenic, and additional evidence supporting this theory is needed.

Danger hypothesis

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The immune response induced by a foreign antigen is weak in the absence of adjuvant, which is a possible explanation of why the immune response induced by a drug itself is insufficient to cause liver injury (Uetrecht, 2006). The danger hypothesis was proposed as an alternative (Matzinger, 1994; Uetrecht, 1999). It suggests that necrosis or cell stress imposed by reactive drug metabolites provides a "danger signal" that activates macrophages, antigen presenting cells or other cells. The danger signals from stressed cells lead to upregulation of costimulatory molecules or production of cytokines which cause an enhanced antibody or T-cell-mediated specific immune response. However, it is not clear what these danger signals are. Activation of the innate immune system, e.g. inflammation, was proposed to be a potential danger signal (Kaplowitz, 2005).

Mitochondrial abnormality hypothesis

This theory proposes that the mitochondria are targeted by idiosyncratic drugs, and mitochondrial abnormality is the underlying mechanism of IADRs. Mitochondria are a critical player in mediating cell death and also a common target of xenobiotics (Wallace and Starkov, 2000). Numerous drugs associated with IADRs, such as troglitazone, tolcapone, nimesulide and valproic acid are reported to cause mitochondrial dysfunction in vitro (Bjorge and Baillie, 1991; Mingatto et al., 2000; Bedoucha et al., 2001; Haasio et al., 2002). There is also clinical evidence associating IADRs with mitochondrial dysfunction. In one case

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An inherited mitochondrial dysfunction or mutation in mitochondrial DNA might make people more susceptible to drug-induced toxicity. In one case of valproate-induced liver injury, an inherited dysfunction of mitochondrial electron transport chain complexes was observed in the patient (Krahenbuhl et al., 2000). For many unrelated drugs, age is one of the risk factors for IADRs. Interestingly, mitochondrial DNA mutations also increase with age, which could explain why aged people are more sensitive to IADRs (Kujoth et al., 2005).

Models have been developed using an animal model of silent genetic mitochondrial abnormality. Superoxide dismutase (SOD) eliminates superoxide in mitochondria, and heterozygous SOD2 mice have a decreased ability to manage oxidative stress in the liver. Either nimesulide or troglitazone induced hepatotoxicity in heterozygous SOD2 mice, whereas these drugs had no hepatotoxic effect on normal mice (Ong et al., 2006; Ong et al., 2007).

Failure to adapt hypothesis

It has been observed that the majority of patients with ALT elevations due to drugs associated with idiosyncratic liver injury will eventually recover from liver injury despite continued exposure to the drug (Watkins, 2005). Therefore, another hypothesis has been raised that a small fraction of patients fail to adapt to the initial injury, which then leads to the progression of severe liver injury.

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Few animal models have been developed to support this hypothesis, and the mechanisms underlying injury are not known. One possibility is that a single inherited defect in adaptation precipitates drug-induced liver injury.

Multiple determinant hypothesis

This theory proposed that idiosyncratic drug toxicity is a result of "the occurrence of multiple critical and discrete events, with the probability for the occurrence of idiosyncratic drug toxicity being a product of the probabilities of each event" (Li, 2002). This theory is not exclusive with some of the other hypotheses already discussed. The critical events could include chemical properties of the drug, exposure, environmental factors and genetic factors. Inflammation could be a critical event to take into account.

1.1.3 Inflammatory stress hypothesis as potential explanation for IADRs

In addition to the hypotheses mentioned above, a hypothesis has been proposed that inflammation may render an individual susceptible to IADRs (Roth et al., 2003; Ganey et al., 2004). Inflammatory episodes are commonplace in humans. Inflammation can be induced by infections, inflammatory diseases or exposure to endotoxin, which is a potent inducer of inflammation. Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is an inflammagen that can cause damage to several organs, including the liver (Hewett and Roth, 1993). Various conditions, including alcohol consumption, gastrointestinal distress, changes in diet, antibiotic treatment and surgery can

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increase LPS concentrations in human plasma (Roth et al., 1997). These conditions are not usually severe enough to cause overt illness, but they may potentiate the toxicity of xenobiotics through activating inflammatory cells and cytokines. Previous studies proved that an inflammatory stress precipitates hepatotoxicity of numerous xenobiotics, e.g. allyl alcohol and aflatoxin (Sneed et al., 1997; Yee et al., 2000; Barton et al., 2001).

The characteristics of inflammation described above led to the inflammatory stress hypothesis for IADRs. As illustrated in Fig. 1.1, the decrease in threshold for toxicity results from the inflammatory episodes that occur in the lifetime of normal humans. When an individual is undergoing drug therapy, the drug concentration reaches its therapeutic concentration without causing toxicity under normal conditions. However, if an inflammatory stress decreases the threshold of toxicity below the drug concentration, an IADR may occur. This hypothesis can provide a plausible explanation for the characteristics of IADRs. The occurrence and magnitude of a modest inflammatory episode can be unnoticeable in humans. Therefore, IADRs due to the interaction of an inflammatory episode with idiosyncratic drugs would be expected to have an inconsistent temporal relationship to exposure and not appear to be dose dependent.

Numerous drug-induced idiosyncratic liver injury models have been developed in rodents that support the inflammatory stress hypothesis. Specifically, drugs including diclofenac (DCLF), sulindac (SLD), halothane (HAL), chlorpromazine (CPZ), trovafloxacin (TVX) and ranitidine (RAN), when administered at nonhepatotoxic doses, induced significant liver injury in rodents

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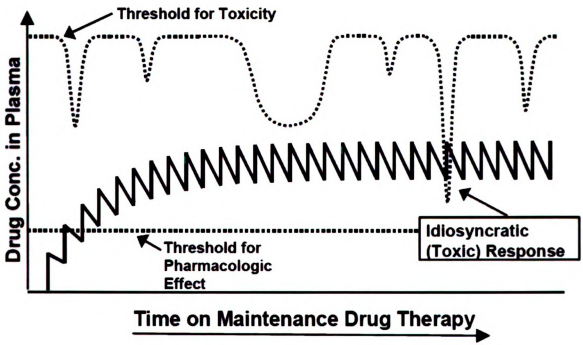
Fig. 1.1 Inflammation might precipitate drug- induced IADRs (Roth et al., 2003). The subcurve indicates the drug concentration in plasma increases and remains at a therapeutic concentration during drug therapy. However, the threshold for drug toxicity (upper dotted line) might be decreased as a result of inflammatory stress. IADRs occur if the threshold for toxicity drops below drug concentration.



pretreated with LPS (Table 1.1). Levofloxacin and famotidine are in the same pharmacological class with trovafloxacin and ranitidine, respectively, but they have far lower tendency to cause IADRs in humans and also failed to induce liver injury with LPS in rodents. Inflammatory stress induced by the viral RNA mimetic, poly (I:C) also precipitated hepatotoxicity caused by halothane in mice (Cheng et al., 2009). Therefore, these results suggest that inflammation may precipitate the toxicity of drugs that cause IADRs. Mechanisms underlying drug/LPS interaction models of idiosyncratic liver injury have been investigated. In the following sections of this chapter, the innate immune response and mechanisms of several drug/LPS interaction models are introduced.

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Table 1.1 List of Idiosyncratic drugs that cause liver injury in drug/LPS models in rodents (Deng et al., 2009).

Drug	Human IADRs?	LPS + Drug Hepatotoxicity in Rodents?
Diclofenac	Yes	Yes
Sulindac	Yes	Yes
Halothane	Yes	Yes
Chlorpromazine	Yes	Yes
Trovafloxacin	Yes	Yes
Levofloxacin	No	No
Ranitidine	Yes	Yes
Famotidine	No	No

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1.2 Inflammation

1.2.1 Overview of the inflammatory response

People are exposed to infectious microorganisms in everyday life. Inflammation is a first-line defense mechanism by the innate immune system against infections. Not only infections, but also tissue trauma and non-infectious disease can contribute to inflammation (Trunkey, 1988). Tissue injury leads to activation of the innate immune response after trauma, which presents as a systemic inflammatory response syndrome (Lenz et al., 2007). Inflammation is also associated with anemia (Buck et al., 2009), Parkinson's disease (Barcia et al., 2003), diabetes (Granic et al., 2009), obesity (Elmarakby and Imig, 2009; Olefsky, 2009), heart disease and other vasculopathies (Linde et al., 2006) and cancers (Sevinir et al., 2003; De Marzo et al., 2007).

Inflammation is a complex response coordinated by various inflammatory cells (Fig. 1.2). Neutrophils, macrophages, endothelial cells, epithelial cells and platelets are activated by inflammatory stimuli (Ganey et al., 2004). Transcriptional activation occurs, and several genes are upregulated in inflammatory cells. Proteases and reactive oxygen species are released by neutrophils and macrophages and can kill parenchymal cells directly. Proinflammatory cytokines, such as tumor necrosis factor-α (TNF) are also released by macrophages. These cytokines can either activate cell death signaling pathway or lead to other inflammatory events. The coagulation system as well as the complement system can also be activated as a result of inflammation.

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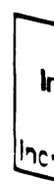
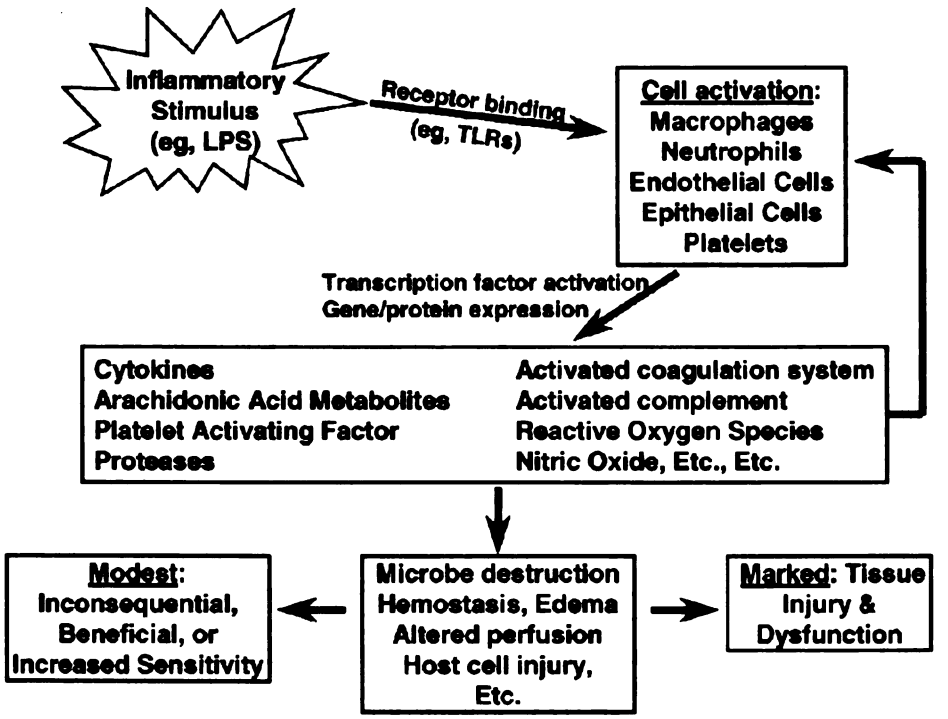


Fig. 1.2 Summary of the inflammatory response (Ganey et al., 2004). Inflammatory cells are activated by stimulus through receptor binding e.g. TLRs. As a result, numerous mediators of inflammation are released and cause homeostatic imbalance in the target tissue. These mediators might lead to tissue injury in aggravated conditions or be inconsequential, beneficial or increase tissue sensitivity when the response is modest.



Severe inflammation can lead to dramatic changes in physiology, such as redness, swelling, pain and heat at the site of inflammation. Moreover, severe tissue damage or organ failure can result from a marked inflammatory response. More commonly, modest inflammatory episodes occur in humans, which may be unnoticeable. Although modest inflammation resolves and may have no detrimental effect alone, it might potentiate the toxicity of xenobiotics (Sneed et al., 1997; Yee et al., 2000; Barton et al., 2001).

The Toll-like receptor (TLR) signaling pathway is a well studied pathway involved in the initiation of the inflammatory response (O'Neill, 2008). TLRs are expressed by inflammatory cells and recognize specific structures conserved among microorganisms (Takeda et al., 2003). LPS binds to TLR4, which results in signal transduction leading to a cascade of inflammatory events (Linde et al., 2006). Macrophages are the primary immune cells responding to LPS. TLR4 is also expressed on the membranes of hepatocytes, endothelium and mast cells (Migita et al., 2004). TLR4 signals through two intracellular Toll/IL-1 receptor (TIR) domain-containing adaptors, myeloid differentiation factor 88 (MyD88) and TIR-containing adapter molecule (TRIF). In the MyD88 dependent pathway, interleukin-1 receptor-associated kinase (IRAK) 1 and IRAK 4 are recruited to MyD88. Once phosphorylated, IRAK dissociates from MyD88 and activates TNF receptor associated Factor (TRAF) 6. TRAF6 binds to the complex of TGF-β-activated kinase 1 (TAK1) and TAK-1 binding protein (TAB). In turn, TAK1 is phosphorylated and leads to the activation of MAPK pathway or NFκB-regulated genes. In the TRIF dependent pathway, IκB kinase is activated and leads to the

activation of NFκB. TRIF also leads to the activation of interferon regulatory factor 3 (IRF3) and the expression of interferon-inducible genes.

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1.2.1 Tumor necrosis factor-α (TNF)

TNF is a proinflammatory cytokine that is produced by many cell types, e.g. macrophages, mast cells, endothelial cells and stellate cells in response to LPS (Gordon and Galli, 1990; Thirunavukkarasu et al., 2006). Kupffer cells are resident macrophages attached to the outer surface of endothelial cells in liver sinusoids (Hewett and Roth, 1993), and they are the major macrophage population exposed to inflammagens in blood. After LPS binding to TLR4, pro-TNF is produced by Kupffer cells as a result of the activation of NFκB signaling pathway. Pro-TNF is a 26 kDa membrane-bound precursor form which is not biologically active (Solomon et al., 1997). TNF converting enzyme (TACE), a membrane bound metalloprotease, recognizes a cleavable signal sequence on pro-TNF and leads to shedding of the active form of TNF from the cell membrane (Ishisaka et al., 1999; Wullaert et al., 2007).

TNF exerts its biological effects by binding two plasma membrane receptors, TNF-receptor 1 (TNF-R1) and TNF-R2. In a few cell types, e.g., T cells, the binding of TNF to TNF-R2 leads to proliferation, NFkB activation and cytokine production (Rothe et al., 1994; Vandenabeele et al., 1994). However, in most cells, TNF-R2 has no direct effect on signal transduction and plays an indirect role in TNF-R1 responses by delivering TNF to the low affinity TNF-R1. The ligand passing activity of TNF-R2 allows fine-tuning of TNF-R1 mediated signal transduction.

TNF plays an important role in regulating liver homeostasis (Wullaert et al., 2007). TNF-R1 activation leads to either hepatocyte proliferation via the

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activation of NFkB, the initiation of MAPK cascades or cell death (Fig. 1.3). Activated TNF-R1 recruits adapter proteins, TNF-R-associated death domain (TRADD) protein, TNF-R associated factor (TRAF) 2, and receptor interacting protein (RIP) to the cytoplasmic part of TNF-R1. These proteins form complex I. TRAF2 possesses ubiquitin ligase activity, which leads to the ubiquitination of RIP and itself. This serves as an important signal for the binding of TAK1-TAB2-TAB3 complex. TAK1 leads to the activation of the MAPK pathway as well as IKK complex, which leads to the expression of NFκB-inducible genes.

On the other hand, activated TNF-R1 can recruit adapter proteins TRADD and Fas-associated death domain (FADD) as well as pro-caspase 8, which leads to the formation of the death-inducing signaling complex (DISC). Autoactivation of procaspase 8 results in the generation of active caspase 8, which directly cleaves procaspase 3 into caspase 3 and leads to apoptosis (Hehlgans and Pfeffer, 2005). Caspase 8 converts Bid into truncated Bid (tBid). tBid contributes to mitochondrial dysfunction via the formation of permeability transition pores and Bak/Bax pores on the outer mitochondrial membrane, which leads to the release of cytochrome c and reactive oxygen species to cytosol. Cytochrome c activates caspase 9 and 3, which lead to apoptosis through activating caspase activated DNase (CAD). Thereby, cell death is induced as a result of TNF-R1 activation.

Whether TNF leads to cell survival or death is determined by NFkB (Wullaert et al., 2007). NFkB plays a protective role against cell death by inducing the expression of several antiapoptotic genes, which include caspase-8 inhibitor c-

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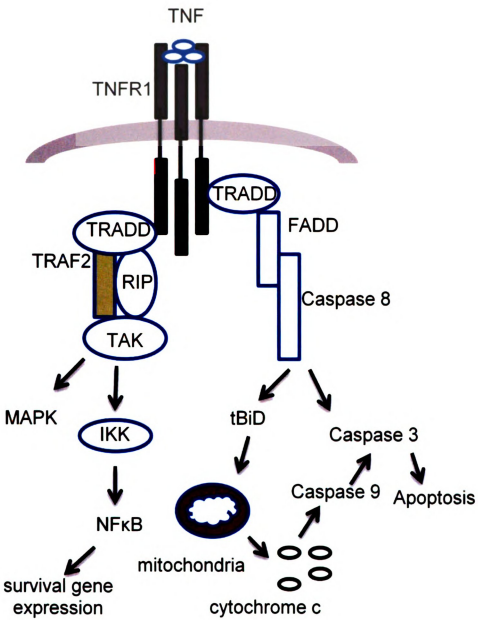
Fig. 1.3 TNF signal transduction pathway. TNF can either activate NFκB or induce cell death.



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FLIPL, the Bcl-2 family members Bcl-xL and A1/Bfl-1, and X-linked inhibitor of apoptosis (XIAP) (Zong et al., 1999; Chen et al., 2000; Micheau et al., 2001). Moreover, prolonged activation of JNK resulting from ROS accumulation or TNF signaling, causes cell death (Kamata et al., 2005). NFkB can rapidly terminate JNK activation by upregulating the expression of antioxidant manganese superoxide dismutase (MnSOD) and ferritin heavy chain (FHC) (Sakon et al., 2003).

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1.2.3 The hemostatic system and hypoxia

Vascular hemostasis is regulated by coagulation and fibrinolysis. The coagulation system is activated in many inflammatory diseases (Vrij et al., 2003). It can be activated through the extrinsic pathway or the intrinsic pathway. In the extrinsic pathway, tissue factor is exposed to blood and forms a complex with factor VII, which activates factor X through the activation cascade. Factor X cleaves prothrombin into thrombin, which converts fibrinogen into fibrin monomers by cleaving fibrinopeptides. Fibrin can cross-link to form fibrin clots in blood vessels (Mosesson et al., 2001). Fibrin clots are controlled and dissolved by the fibrinolytic system, in which plasmin plays an important role by degrading fibrin clots into D-dimers (Fay et al., 2007). Plasminogen activators (PAs) cleave plasminogen to active plasmin. PAs can be inhibited by active plasminogen activator inhibitor (PAI-1), which is synthesized by hepatocytes, endothelial cells and platelets in response to TNF or LPS (Levi et al., 2003; Westrick and Eitzman, 2007). Therefore, an increase in active PAI-1 can dampen the fibrinolytic system and enhance fibrin deposition.

Thrombin and PAI-1 are two critical factors in regulating the hemostatic system, but they also play a role in activating inflammatory cells. Thrombin cleaves and activates G protein-coupled receptors, including protease-activated receptor-1 (PAR-1), which can significantly increase production of pro-inflammatory cytokines (TNF, IL-1 and IL-6) from target cells (Fan et al., 2005). PAI-1 can potentiate LPS-induced neutrophil activation through a JNK-mediated pathway in vitro and enhance nuclear translocation of NFkB, which increases

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production of the proinflammatory cytokines IL-1, TNF and MIP-2 (Kwak et al., 2006). Previous studies in vivo also suggest that PAI-1 contributes to production of TNF, IL-10, KC and MCP-1 (Shaw et al., 2009c).

Fibrin deposition controls the magnitude and area of infection, through which fibrin deposition plays a protective role against inflammagen exposure. However, detrimental effects can also be induced by fibrin deposition in liver sinusoids by impairing blood flow and thereby causing tissue hypoxia. Hypoxia exerts various effects on hepatocytes. It activates numerous intracellular signaling pathways related to transcription of hypoxia-responsive genes, mostly mediated by hypoxia inducible factor-1α. Reactive oxygen species that accumulate after exposure to hypoxia lead to the MPT (Qu et al., 2001; Schild and Reiser, 2005). Energy deficit (loss of ATP) and inhibition of aerobic metabolism are also caused by the decrease of available oxygen. Hepatic metabolism and function are highly oxygen-dependent. As a consequence of decreased cellular ATP level, physiological function is impaired and liver damage can occur (Semenza, 2004). Furthermore, hypoxia enhances LPS-induced liver damage (Shibayama, 1987) and potentiates the toxic effects of PMN-derived proteases on hepatocytes *in vitro* (Luyendyk et al., 2005).

1.2.4 Neutrophils (PMNs)

PMNs are abundant blood leukocytes and play an important role in the innate immune response. PMNs are a contributor to tissue injury induced by ischemia-perfusion and alcohol. (Jaeschke et al., 1990; Hewett et al., 1992;

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Jaeschke, 2002). PMNs are involved in several models of drug-induced liver injury (e.g., acetaminophen) and in drug-LPS interaction models of idiosyncratic liver injury (Deng et al., 2006; Deng et al., 2007b; Jaeschke and Liu, 2007; Ramaiah and Jaeschke, 2007; Shaw et al., 2009d).

PMNs are primed and activated by systemic or local exposure to proinflammatory mediators. TNF, IL-1 or CXC chemokines (CINC-1, MIP-2) may increase the expression of integrin on the surface of PMNs as well as ICAM-1 and VCAM-1 expression on the surface of endothelial cells (Jaeschke and Smith, 1997; Jaeschke et al., 1998). The binding of an adhesion molecule (ICAM-1 or VCAM-1) to its counter-receptor (integrin) results in PMNs adhering tightly to endothelial cells. However, in the RAN/LPS model, neutralization of integrin did not reduce PMN numbers in the liver (Deng et al., 2007b). Thus, accumulation of PMNs in vasculature is not necessarily dependent on the binding of adhesion molecules.

PMN migration from the vasculature and infiltration into the parenchyma is a prerequisite for neutrophil cytotoxicity (Jaeschke and Smith, 1997). During this process, a signal from parenchymal cells is required. A chemotactic gradient of CXC chemokines in liver can lead to PMN infiltration, which contributes to liver injury (Okaya and Lentsch, 2003). Necrotic cells can release a mediator called high mobility group box 1 (HMGB1) which can signal to PMNs and cause their migration (Tsung et al., 2005). Anti-HMGB1 antibody significantly reduced the production of proinflammatory cytokines and PMN infiltration in an ischemia-reperfusion model of liver injury (Tsung et al., 2005). Interestingly, apoptotic cells might also trigger PMN migration (Jaeschke, 2006). It was observed that

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engulfment of apoptotic bodies by Kupffer cells promotes cytokine expression and PMN activation (Canbay et al., 2003). Alternatively, it has been proposed that gaps in the sinusoidal endothelial cells may facilitate the direct contact of PMNs with altered membranes of apoptotic cells (Jaeschke, 2006).

After PMNs cross the endothelial cell barrier, they localize close to hepatocytes and degranulate, leading to the release of proteases and reactive oxygen species (ROS) including hydrogen peroxide and hypochlorous acid (Dahlgren and Karlsson, 1999). Both proteases and ROS contribute to liver injury in animal models (Jaeschke, 2006). Cathepsin G and elastase are two important proteases which are released by activated PMNs. They are important mediators of hepatic parenchymal cell killing (Ho et al., 1996). Inhibition of cathepsin G and elastase protected against liver injury in a drug-inflammation interaction model (Luyendyk et al., 2005). Besides direct effect on hepatocytes, proteases released by PMNs can also contribute to fibrin deposition by activating plasminogen activator inhibitor-1 (PAI-1) (Deng et al., 2007a). The role of ROS in cell death is discussed in the following section.

1.2.5 Reactive oxygen species

Reactive oxygen species (ROS) are oxygen free radical and nonradical (but reactive) oxygen species. They include hydrogen peroxide, superoxide, hydroxyl radicals, etc. ROS are produced by several sources within the cell. Mitochondria, in which the electron transport chain transfers electrons to oxygen, are a major source of ROS. During normal cellular respiration, about 2% of electrons escape

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and lead to production of superoxide anion (Boveris and Cadenas, 1975). As described in previous sections, TNF and hypoxia have been shown to cause increased ROS generation in a mitochondria-dependent manner. Another major source of ROS is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. The NADPH oxidase complex assembles on phagosomal, plasma and granule membranes of activated PMNs and macrophages and generates ROS (Dahlgren and Karlsson, 1999). NADPH oxidase reduces oxygen to superoxide at the same time it oxidizes NADPH. Cytochrome b from NADPH is responsible for the transfer of electrons to oxygen present in intracellular compartments (Babior, 1999). In addition, many other enzymes, including xanthine oxidase, cyclooxygenases, lipoxygenases, myeloperoxidases, hemeoxygenase, monoamine oxidases, aldehyde oxidase, and cytochrome P450, can cause ROS accumulation. However, the capacity of these enzymes to generate ROS is less robust than the mitochondrial electron chain complex or NADPH oxidase (Morgan et al., 2008).

Excessive ROS may tilt the prooxidant-antioxidant balance, causing oxidative stress in cells. ROS can directly oxidize proteins and lipids as well as nucleic acids, leading to cellular damage and dysfunction (Morgan et al., 2008). ROS may induce cell death through a JNK-dependent pathway (Schwabe and Brenner, 2006). ROS directly inactivate JNK phosphatase, which leads to prolonged activation of JNK (Kamata et al., 2005). Activated JNK phosphorylates antiapoptotic Bcl-2 family proteins (Bcl-2, Bcl-X_L) and inactivates them (Yamamoto et al., 1999a; Fan et al., 2000).

Oxidative stress plays a role in several models of liver injury. It occurs and contributes to ischemia/reperfusion injury in mice (Serteser et al., 2002). Involvement of oxygen free radicals and consequently of oxidative stress has also been proven in alcoholic liver disease (Koch et al., 2004). ROS are involved in the pathogenesis of inflammatory liver diseases (Jaeschke, 2000). In an animal model of LPS-induced organ injury, a significant decrease in reduced glutathione and an increase in lipid peroxidation were observed in the lungs and livers of rats (Suntres and Shek, 1996). The administration of antioxidants after challenge with LPS resulted in a significant alleviation of both lung and liver injuries. Mice deficient in antioxidant enzyme showed greater susceptibility to PMN-mediated liver injury (Jaeschke et al., 1999). An NADPH oxidase inhibitor protected against endotoxin-induced PMN-mediated liver injury, suggesting a critical role for ROS (Gujral et al., 2004).

Oxidative stress has also been proposed as a potential mechanism of NSAID-induced hepatotoxicity (Boelsterli, 2002). Although the role of ROS in SLD-induced liver injury has not been determined in vivo, SLD and its toxic metabolite were reported to increase ROS production in several cell lines (Galati et al., 2002; Adachi et al., 2007).

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1.3 Models of drug-inflammation interaction

There are few animal models to study the mechanisms of drug-induced idiosyncratic hepatotoxicity. The main reason for this is that idiosyncratic hepatotoxicity-induced by drugs may only occur in a small fraction of animals, as it often does in humans. However, based on the hypothesis that inflammation precipitates drug toxicity, several idiosyncratic liver injury models based on drug-inflammation interaction have been developed.

1.3.1 Ranitidine/LPS- induced hepatotoxicity in rats

Ranitidine (RAN) is a histamine-2 (H2)-receptor antagonist used for the treatment of duodenal ulcers, gastric hypersecretory diseases and gastroesophageal reflux disease. RAN is associated with idiosyncratic hepatotoxicity with an incidence of less than 1 in 1000 patients taking the drug (Vial et al., 1991). Famotidine (FAM), although in the same pharmacological class with RAN, has a decreased propensity to cause idiosyncratic reactions.

An animal model of RAN-induced idiosyncratic liver injury was developed by pretreating rats with LPS (Luyendyk et al., 2003). In this model, LPS ($44.4X10^6$ EU/kg) or its saline vehicle was administered to rats via a tail vein. Two hours later, RAN (30 mg/kg), FAM (6 mg/kg), or their vehicle (sterile phosphate-buffered saline) was administered i.v. at a rate of approximately 0.15 ml/min. Neither RAN nor LPS given alone had a significant hepatotoxic effect as measured by ALT activity compared to control animals. FAM was also not hepatotoxic to rats in the presence or absence of LPS. In contrast, cotreatment of

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rats with RAN/LPS led to a significant increase in markers of liver injury (e.g., ALT, AST and GGT) in serum at 6, 12 and 24 hr after RAN treatment. Histopathology demonstrated the presence of midzonal hepatic necrosis in animals receiving RAN/LPS cotreatment.

RAN but not FAM enhanced the LPS-induced TNF increase before the onset of hepatocellular injury (Tukov et al., 2007b). It was also observed that a large concentration of RAN enhanced LPS-induced TNF release in a Kupffer cell-hepatocyte coculture system. RAN enhanced the activation of p38 induced by LPS, which led to increased TACE activation (Deng et al., 2008). TACE cleaved pro-TNF into TNF and led to an increase in TNF concentration in the plasma. TNF plays a critical role in the pathogenesis of liver injury, which is supported by the evidence that TNF neutralization protected against liver injury induced by RAN/LPS. RAN also enhanced the increase in serum interleukin (IL)-1beta, IL-6 and IL-10 induced by LPS.

In plasma of rats treated with RAN/LPS, a decrease in fibrinogen and increases in thrombin-antithrombin (TAT) dimers and PAI-1 occurred before the onset of liver injury, suggesting that the hemostatic system was activated by the cotreatment (Luyendyk et al., 2004). Hepatic fibrin deposition was observed in livers of rats cotreated with RAN/LPS at 3 after RAN (ie, before the onset of liver injury). The anticoagulant heparin or the fibrinolytic agent streptokinase significantly reduced liver injury induced by RAN/LPS. Hypoxia probably resulting from sinusoidal fibrin deposition was observed in livers of RAN/LPS-treated rats at 3 hr, and this was significantly attenuated by heparin.

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PMNs are also critical to RAN/LPS-induced liver injury (Luyendyk et al., 2006). PMN accumulation occurred in livers of rats treated with LPS/RAN. Depletion of PMNs using anti-PMN serum protected against liver injury, suggesting that PMNs are involved in the pathogenesis.

TNF, the hemostatic system and PMNs do not act independently of each other. TNF contributes to RAN/LPS-induced liver injury by enhancing production of inflammatory cytokines, chemokines (MIP-2) and hemostatic factors including TAT and PAI-1 (Tukov et al., 2007b). However, hepatic PMN accumulation was not affected by TNF. Heparin had little effect on liver PMN accumulation or plasma chemokine concentration, indicating that PMN accumulation is not affected by fibrin deposition in the liver (Luyendyk et al., 2006). However, both TNF and PAI-1 contribute to PMN activation (Deng et al., 2007b; Deng et al., 2008). PMN depletion reduced the plasma concentration of active PAI-1 and fibrin deposition in livers of rats treated with RAN/LPS, which suggests that PMNs promote fibrin deposition by increasing PAI-1concentration (Deng et al., 2007b). As a result, PMNs also promote hypoxia in the liver. These studies suggest that mediators involved in the pathogenesis of liver injury induced by RAN/LPS are not isolated, but interact with each other.

1.3.2 Diclofenac/LPS-induced hepatotoxicity in rats

Diclofenac (DCLF) is a nonsteroidal anti-inflammatory drug (NSAID) associated with serious idiosyncratic hepatotoxicity in humans (Aithal, 2004). The incidence of DCLF-induced liver injury is approximately one to five cases per

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100,000 persons exposed (Garcia Rodriguez et al., 1994). Liver failure has been recorded after the administration of DCLF (Greaves et al., 2001). Several hypotheses of DCLF-induced liver injury have been proposed, including metabolic polymorphism and allergic hypersensitivity (Daly et al., 2007). However, in vivo evidence that supports these hypotheses is still lacking.

As in the RAN/LPS-induced liver injury model, a model of DCLF-induced liver injury in rats was developed by treating rats with a nonhepatotoxic dose of DCLF, LPS or their vehicles (Deng et al., 2006). Generally, LPS (29 $X10^6$ EU/kg) or its saline vehicle was administered to rats via a tail vein. Two hours later, rats were given DCLF (20 mg/kg, i.p.) or sterile saline. Neither LPS nor DCLF alone had an effect on ALT activity. However, cotreatment with LPS and DCLF caused a significant increase in serum ALT activity. Hepatocellular apoptosis, parenchymal edema, and hemorrhage induced by LPS were also significantly increased by DCLF cotreatment.

A gene array study was performed to compare the gene expression patterns among LPS, DCLF and cotreatment groups. Genes encoding the neutrophil chemokines, such as MIP-2 and MIP-1, and the adhesion molecule ICAM-1, were greatly increased by DCLF/LPS cotreatment compared to LPS or DCLF alone. Both LPS alone and DCLF/LPS treatment led to hepatic PMN accumulation. DCLF did not enhance the effect of LPS on PMN accumulation, although the increase in MIP-2 concentration in serum of rats treated with LPS was enhanced by DCLF. Anti-PMN serum reduced PMN accumulation in liver

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and attenuated liver injury induced by DCLF/LPS. This result suggests that PMNs play a critical role in the pathogenesis.

Interestingly, a larger dose of DCLF (100 mg/kg, i.p.) caused liver injury in rats, which was attenuated by treatment with nonabsorbable antibiotics (polymyxin B and neomycin) for 4 days before DCLF administration. This suggests that bacterial translocation from intestine to liver plays a critical role in DCLF-induced hepatotoxicity though interacting with DCLF.

1.3.3 Trovafloxacin-inflammation interaction model of idiosyncratic liver injury

Trovafloxacin (TVX) is a broad spectrum antibiotic which functions through inhibiting bacterial topoisomerase IV. TVX was approved for marketing in 1997. Two years later, its use was severely limited due to the risk of hepatotoxicity. TVX is associated with idiosyncratic hepatotoxicity with an incidence of 1 in 18,000 prescriptions (Stahlmann, 2002). Another fluoroquinolone antibiotic, levofloxacin (LVX), has not been associated with idiosyncratic liver injury and was used in model development as a control (Shaw et al., 2007).

Both a rat and mouse model of liver injury induced by TVX/LPS cotreatment was developed (Shaw et al., 2009d). In the mouse model (Shaw et al., 2007), TVX (150 mg/kg), LVX (375 mg/kg) or their vehicle (saline) was administered to mice by oral gavage. LPS or its vehicle was administered intraperitoneally to mice 3 hr after the drug. TVX, LVX, LPS alone or LVX/LPS cotreatment did not increase plasma ALT activity, whereas TVX/LPS cotreatment significantly

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increased this biomarker of hepatocellular injury at 9 hr and peaked at 15-21 hr after LPS administration. Hepatocellular necrosis was observed in livers of mice treated with TVX/LPS, but not in those treated with TVX, LVX or LPS alone. The necrotic foci observed in the TVX/LPS-treated group were found in midzonal and centrilobular regions.

TVX prolonged the appearance of TNF induced by LPS in the plasma (Shaw et al., 2007). TNF neutralization using etanercept attenuated TVX/LPS-induced liver injury, suggesting that TNF is an important mediator. This was further supported by evidence that TVX and TNF cotreatment caused significant liver injury in mice, whereas neither TVX nor TNF was hepatotoxic (Shaw et al., 2009a). Interestingly, TVX prolonged the appearance of TNF in the plasma of mice cotreated with TVX/TNF compared to mice given only TNF. This prolonged appearance of TNF was caused by both enhanced production and decreased clearance of this cytokine. Comparison of hepatic gene expression profiles from mice treated with TVX/LPS to those treated with LPS or TVX alone suggested that the interferon γ (IFNγ) signaling pathway was selectively activated by TVX/LPS (Shaw et al., 2009b). TVX enhanced the appearance of interleukin (IL)-18 that contributes to the production of IFNγ. In turn, IFNγ can feedback to increase IL-18. TVX/LPS–induced liver injury was attenuated in either IFNγ -/- or IL-18 -/- mice, which indicate both IFNγ and IL-18 are important mediators in the pathogenesis of TVX/LPS–induced liver injury.

Compared to LPS, TVX, LVX alone or LVX/LPS cotreatment, TVX/LPS caused a significant increase in plasma concentration of TAT dimers and PAI-1,

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which was accompanied by fibrin deposition in the liver (Shaw et al., 2009c). Either PAI-1 knockout or heparin treatment reduced liver injury caused by TVX/LPS, indicating that PAI-1 and fibrin deposition contributed to liver injury in this model.

PMNs also contribute to TVX/LPS-induced liver injury (Shaw et al., 2009d). TNF is responsible for the production of PMN chemokines including MIP-1, MIP-2 and KC in this model (Shaw et al., 2009e).

In addition to the TVX/LPS interaction model of idiosyncratic liver injury, inflammation induced by a Gram-positive stimulus, a peptidoglycan-lipoteichoic acid (PGN-LTA) mixture isolated from *Staphylococcus aureus*, also precipitates TVX-induced liver injury in mice (Shaw et al., 2009d). PGN and LTA activate TLR2 to induce inflammation, which indicates that liver injury induced by drug-inflammation interaction is not necessarily dependent on TLR4 pathway.

Besides the three animal models introduced here, there are other models of IADRs, in which liver injury is induced by the cotreatment of chlorpromazine/LPS, halothane/LPS and halothane/poly I:C. However, the mechanisms underlying these models are not yet fully understood.

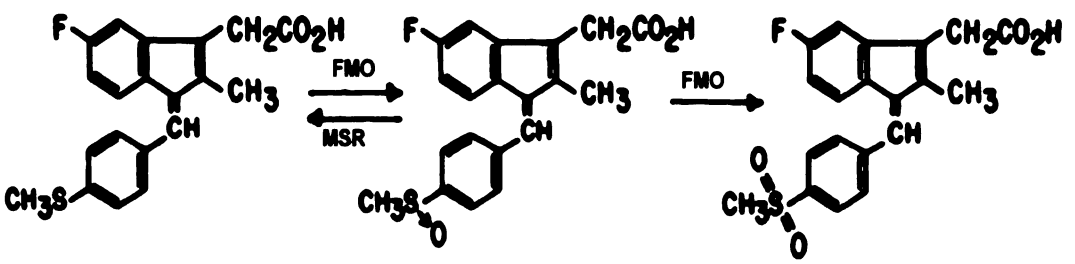
1.4 Sulindac-induced idiosyncratic liver injury

Sulindac (SLD) is a prodrug in the therapeutic class of NSAIDs. SLD was introduced into the market in 1978 by Merck under the brand name Clinoril to relieve pain, tenderness, swelling, and stiffness caused by osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. Typically, the dose of SLD for human patients is 150-200 mg, twice per day as a result of its 8 hr half life. The bioavailability of SLD is more than 90%. SLD is absorbed rapidly upon oral administration, and reaches a peak in human plasma in 2-4 hr (Davies and Watson, 1997). SLD and its metabolites are secreted into bile and undergo enterohepatic circulation (Bolder et al., 1999). SLD and its metabolites are excreted in urine and feces.

Unlike prodrugs that are irreversibly bioactivated to active metabolites, SLD can be reversibly converted to the active metabolite, SLD sulfide, and irreversibly converted to SLD sulfone (Fig. 1.4). According to previous studies, two enzymes are responsible for SLD metabolism: methionine sulfoxide reductase (MSR) in both liver and gut flora reduces SLD to SLD sulfide, and a flavin-containing monooxygenase (FMO) converts SLD to SLD sulfone and also catalyzes the conversion of SLD sulfide to SLD (Etienne et al., 2003b).

The SLD active metabolite, SLD sulfide, performs its pharmacological function by inhibiting cyclooxygenases (COX) -1 and -2 (Lin et al., 1985). Both COX-1 and COX-2 are responsible for the synthesis of prostaglandins. COX-1 is constitutively expressed in normal cells and plays a beneficial role, e.g.,maintain the normal function of GI tract, renal tract, platelet function. COX-2 is inducible

Fig. 1.4 Metabolism of SLD (Duggan et al., 1980). SLD can be reversibly converted to the active metabolite, SLD sulfide by methionine sulfoxide reductase (MSR), and irreversibly converted to SLD sulfone by flavin-containing monooxygenase (FMO).



SULFIDE SULINDAC (SULFOXIDE) SULFONE

enzyme expressed by macrophages and contributes to inflammation. Therefore, the inhibition of COX-2 is effective to resolve inflammation.

SLD has been used in the United State for over a decade, during which it has been associated with increased risk of heart attack, ulcer, stroke and liver injury (Tarazi et al., 1993). The Food and Drug Administration (FDA) Arthritis Advisory Committee wrote that "the potential for producing liver injury is a class characteristic of NSAIDs" (Paulus, 1982; Tarazi et al., 1993). SLD was associated with a 5–10 fold higher incidence of hepatic injury than other NSAIDs, which induced liver injury at an incidence of about 1 in 100,000 (Walker, 1997). According to the analysis of cases reported to FDA, SLD-induced liver injury often occurred within 8 weeks of taking the drug, whereas about 20% of the reported reactions occurred after 8 weeks of treatment (Tarazi et al., 1993). Females are more susceptible to toxicity of SLD than males, and two thirds of the patients were over 50 years of age. Histopathology showed that the pattern of SLD-associated liver injury can be cholestatic, hepatocellular or mixed. In the cases of hepatocellular injury, the lesions were spotty and panacinar in most cases (8 out of 9). Portal inflammation and eosinophil infiltration was observed in a portion of patients.

Mechanisms of SLD-induced idiosyncratic liver injury are not well understood. Because clinical characteristics consistent with hypersensitivity were observed in some patients, it was proposed that hypersensitivity accounts for a significant proportion of SLD-induced liver injury. However, no direct evidence for this has been found, and the mechanisms of pathogenesis still require further

investigation. Another hypothesis for liver injury induced by NSAIDs including SLD is through mitochondrial injury. A variety of NSAIDs or their metabolites (e.g., nimesulide, DCLF and SLD) have a toxic effect to the mitochondria of hepatocytes in vitro. There is little evidence in vivo supporting this hypothesis, and an idiosyncratic liver injury model for SLD has not been developed on the basis of this hypothesis.

1.5 Hypothesis and specific aims

The overall hypothesis of this dissertation is that an inflammatory episode induced by LPS precipitates SLD-induced liver injury in rats. According to previous studies on the mechanism of LPS- or drug/LPS- induced liver injury, we hypothesize that TNF, the hemostatic system, PMNs and ROS are elevated by SLD/LPS cotreatment and play a critical role in the pathogenesis of liver injury. The SLD toxic metabolite, SLD sulfide also contributes to liver injury by synergistically interacting with those inflammatory mediators.

Aim 1 Hypothesis

Cotreatment with nonhepatotoxic doses of SLD and LPS causes idiosyncrasy-like liver injury in rats. (Chapter 2)

Aim 2 Hypothesis

The hemostatic system is activated by SLD/LPS cotreatment in rats and contributes to liver injury by causing hypoxia. (Chapter 2)

Aim 3 Hypothesis

TNF, which is increased by SLD/LPS cotreatment, plays an important role in liver injury by interacting with SLD sulfide. (Chapter 3)

Aim 4 Hypothesis

PMNs are activated in livers of rats treated with SLD/LPS and contribute to liver injury by releasing toxic proteases. (Chapter 4)

Aim 5 Hypothesis

ROS production is increased in livers of rats treated with SLD/LPS. ROS are involved in the pathogenesis of liver injury by enhancing the cytotoxicity of TNF. (Chapter 5)

CHAPTER 2

Zou W, Devi SS, Sparkenbaugh E, Younis HS, Roth RA and Ganey PE (2009) Hepatotoxic interaction of sulindac with lipopolysaccharide: role of the hemostatic system. *Toxicol Sci* 108:184-193.

2.1 Abstract

Sulindac (SLD) is a nonsteroidal anti-inflammatory drug (NSAID) that has been associated with a greater incidence of idiosyncratic hepatotoxicity in human patients than other NSAIDs. One hypothesis regarding idiosyncratic adverse drug reaction (IADRs) is that interaction of a drug with a modest inflammatory episode precipitates liver injury. In this study, we tested the hypothesis that lipopolysaccharide (LPS) interacts with SLD to cause liver injury in rats. SLD (50 mg/kg) or its vehicle was administered to rats by gavage 15.5 hr before LPS ($8.3X10^5$ EU/kg) or its saline vehicle (i.v.). Thirty min after LPS treatment, SLD or vehicle administration was repeated. Rats were killed at various times after treatment, and serum, plasma and liver samples were taken. Neither SLD nor LPS alone caused liver injury. Cotreatment with SLD/LPS led to increases in serum biomarkers of both hepatocellular injury and cholestasis. Histological evidence of liver damage was found only after SLD/LPS cotreatment. As a result of activation of hemostasis induced by SLD/LPS cotreatment, fibrin and hypoxia were present in liver tissue before the onset of the hepatotoxicity. Heparin treatment reduced hepatic fibrin deposition and hypoxia and protected against liver injury induced by SLD/LPS cotreatment. These results indicate that cotreatment with nontoxic doses of LPS and SLD causes liver injury in rats, and this could serve as a model of human idiosyncratic liver injury. The hemostatic system is activated by SLD/LPS cotreatment and plays an important role in the development of SLD/LPS-induced liver injury.

2.2 Introduction

Previous studies suggested that mild inflammation induced by bacterial lipopolysaccharide (LPS) could potentiate hepatotoxicity in rodents from IADR-associated drugs such as chlorpromazine (Buchweitz et al., 2002), ranitidine (Luyendyk et al., 2003) and trovafloxacin (Shaw et al., 2007). In one of these LPS/drug interaction models, the hemostatic system proved to be important in liver pathogenesis (Luyendyk et al., 2005). This system comprises coagulation and fibrinolytic components. In coagulation, thrombin plays a critical role by cleaving fibrinogen to fibrin that can form occlusive clots in sinusoids. Meanwhile, plasminogen activator inhibitor-1 (PAI-1) can inhibit fibrin clearance by the fibrinolytic system by inhibiting the generation of plasmin from plasminogen.

To evaluate the utility of LPS/drug interaction models to study mechanism(s) of idiosyncratic liver injury, a broad range of drugs needs to be tested. Therefore, the purpose of this study was to test the hypothesis that modest inflammation induced by LPS interacts with SLD to cause liver injury in rats. When the results demonstrated a hepatotoxic interaction, the role of the hemostatic system was explored.

2.3 Materials and methods

2.3.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The activity of lipopolysaccharide (Lot 075K4038) derived from *Eschericia coli* serotype O55:B5 was 3.3 X 10^6 endotoxin units (EU)/mg as determined by a Limulus amebocyte lysate endpoint assay kit purchased from Cambrex Corp. (Kit 50-650U; East Rutherford, NJ). The reagents for alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT) and total bilirubin were purchased from Thermo Corp. (Waltham, MA). Alkaline phosphatase (ALP) reagent was purchased from BioAssay Systems (Hayward, CA), and the kit for total bile acid determination was purchased from Diazyme Laboratories (Poway, CA).

2.3.2 Animals

Male, Sprague-Dawley rats (Crl:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250 to 370 g or 150 to 200 g were used for *in vivo* or *in vitro* studies, respectively. Animals were allowed to acclimate for 1 week in a 12-hr light/dark cycle prior to use in experiments. They were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum.

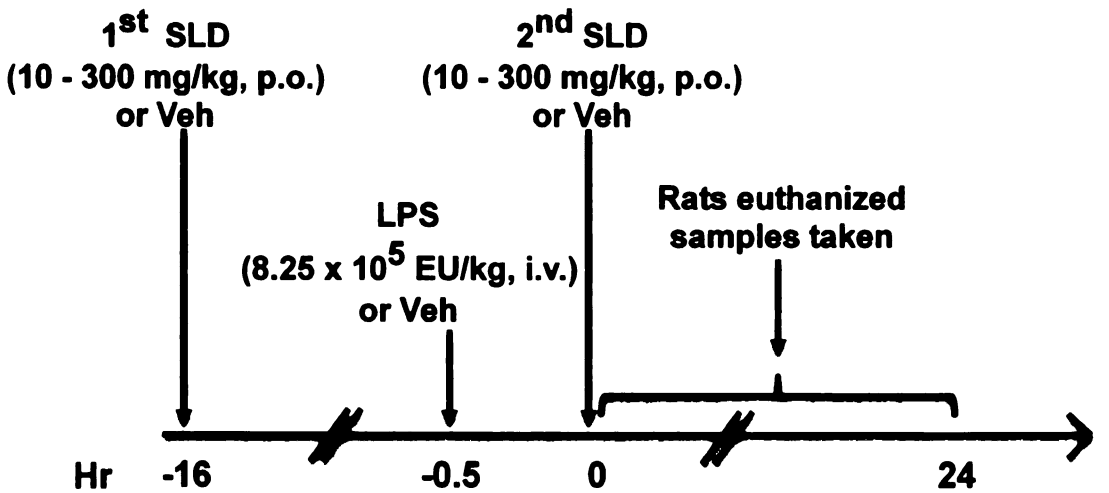
2.3.3 Experimental protocol

Two administrations of SLD were used in the studies (Fig. 2.1). In a dose-response study, rats were given the first administration of SLD (10, 20, 50, 100 or 300 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose), and food was removed for 24 hr. 15.5 hr after the first administration of SLD, LPS (8.25X 10^5 EU/kg, i.v.) or its saline vehicle was administered. Half an hour later, a second administration of SLD (same dose) or its vehicle was given. Rats were anesthetized with isoflurane and killed at various times after the second administration of SLD. For subsequent studies, 50 mg/kg was chosen as the dose of SLD. For all studies, blood was drawn from the vena cava of anesthetized rats, and part was transferred into vacutainer tubes (Becton Dickinson) containing sodium citrate for preparation of plasma. The rest of the blood was allowed to clot at room temperature for preparation of serum. The exterior of the liver was rinsed with saline, and a portion of the left medial lobe was snap frozen in cooled methylbutane for immunohistochemistry. Three slices of the left lateral lobe about 3-4 mm thick were fixed in 10% buffered formalin for histological analysis. In experiments designed to evaluate the role of the hemostatic system, anticoagulant heparin (3000 Units/kg, s.c.) or its saline vehicle was given to rats 0 and 6 hr after the second administration of SLD.

2.3.4 Evaluation of liver injury

Hepatic parenchymal cell injury was assessed by measuring the activities of ALT and AST in serum. Cholestatic injury markers, including the activities of ALP and GGT, as well as the concentrations of total bilirubin and bile acids in serum, were

Fig. 2.1. Experimental protocol for animal treatment. Rats were given SLD or its vehicle (0.5% methyl cellulose) at -16 hr, and food was removed. At -0.5 hr rats received LPS ($8.25X10^5$ EU/kg, i.v.) or its vehicle (saline), and 30 min later they were given a second administration (same dose as 1st administration) of SLD.



also assessed (see above).

Formalin-fixed liver slices were embedded in paraffin and cut into 6 um sections. Hematoxylin and eosin (H&E) staining was performed, and sections were examined under 100X magnification using a light microscope. Eight, randomly chosen microscope fields for each slide were evaluated for midzonal necrosis and assigned a score of 0-5. 0 represents no liver injury, and 1-5 represents lesions ranging from single cell necrosis (1) to necrotic area encompassing greater than 30% of the field (5). The average score was determined for each rat.

2.3.5 Evaluation of serum TNFα concentrations

The concentration of TNFα in serum taken at 1 hr after the second administration of SLD was measured using an ELISA kit purchased from BD Biosciences (San Diego, CA).

2.3.6 Evaluation of hemostasis and fibrin deposition

Thrombin-antithrombin dimer (TAT) concentration in plasma was used as a marker of thrombin activation and evaluated using an ELISA kit (catalog number OWMG15) purchased from Dade Behring, Inc. (Deerfield, Illinois). The concentration of the active form of plasminogen activator inhibitor-1 (active PAI-1) was determined using a kit from Molecular Innovations, Inc. (Southfield, MI).

The immunohistochemistry and quantification for cross-linked fibrin in liver were performed as described previously (Copple et al., 2002). Fibrin monomer is

solubilized in this protocol, and only cross-linked fibrin in liver is stained. To investigate whether fibrin deposition occurs before the onset of injury, livers were collected at 4 hr and fixed for immunohistochemistry. The fraction of positive pixels averaged from 10 randomly chosen microscope fields was determined for each animal.

2.3.7 Evaluation of liver hypoxia

Liver hypoxia was evaluated by quantifying pimonidazole (PIM)-protein adducts. PIM is a hypoxia probe which is rapidly reduced under low pO_2 conditions to a reactive intermediate that forms PIM-protein adducts. PIM hydrochloride (Hypoxyprobe-1, 120 mg/kg; Chemicon International, Temecula, CA) was given to rats 2 hr before sacrifice. Four hr after the second administration of SLD, livers were collected and fixed for immunohistochemistry. The fraction of positive pixels averaged from 10 randomly chosen microscope fields was determined for each rat (Copple et al., 2004).

2.3.8 Hepatocyte (HPC) isolation and hepatocytotoxicity assessment *in vitro*

HPCs were isolated from rat liver as previously described (Tukov et al., 2006). Isolated cells were suspended in Williams' Medium E (Gibco BRL, Rockville, MD) with 10% fetal bovine serum, and cell viability was evaluated using trypan blue exclusion. The cell viability was always above 80%. The HPCs were suspended and plated randomly at a density of 2.5×10^5 cells/well in 12-

well plates (Corning Inc., Corning, NY). After 2.5 to 3 hr incubation which allowed HPCs to attach to the plate, serum-containing medium was removed, and serum-free medium was added. HPCs were treated with 60 uM sulindac sulfide or its vehicle (0.06% DMSO) and incubated in the presence of 20% or 5% O_2 (with 5% CO_2 and balance N_2). After 8 hr incubation, the medium was collected, and the unattached cells were isolated by centrifugation. Both the remaining attached cells and unattached cells were lysed with 1% Triton X-100. ALT activity in the medium, attached cell lysate and unattached cell lysate was determined. Hepatocytotoxicity was assessed by calculating the ALT activity in the medium plus unattached cells as a percentage of the total ALT activity in the well (medium + unattached cell lysate + attached cell lysate).

2.3.9 Statistical analysis

One way or two way analysis of variance (ANOVA) was used for data analysis, and Tukey's test was employed as a *post hoc* test. For GGT activity and necrotic lesion score data, an ANOVA on ranks was performed, and Dunn's test was used for multiple comparisons. $P < 0.05$ was set as the criterion for statistical significance.

2.4 Results

2.4.1 Dose-response and timecourse of liver injury

SLD alone did not induce liver injury in rats at any of the doses given. SLD (2 administrations) at doses of 10 or 20 mg/kg did not cause hepatotoxicity in LPS-treated rats; however, rats had significant liver injury after cotreatment with 50, 100 or 300 mg/kg SLD plus LPS (Fig. 2.2). Fifty mg/kg was chosen as the SLD dose for further study.

In a time course study, SLD or LPS given alone did not increase serum ALT activity at any time examined (Fig. 2.3A). In the SLD/LPS-cotreated group, ALT activity remained normal for 4 hr but began to increase by 8 hr after the second administration of SLD. By 12 hr, a significant increase in serum ALT activity was observed. By 24 hr, ALT activity had decreased to near normal. The activity of serum AST in rats also reached its peak at 12 hr and showed a pattern similar to ALT activity (Fig. 2.3B).

At 12 hr, the activities of ALP and GGT as well as the concentrations of total bilirubin and bile acids were also elevated significantly in the sera of SLD/LPS-cotreated rats compared to those of rats treated with SLD or LPS alone (Fig. 2.4).

2.4.2 Histopathological findings

Hepatocellular lesions were not found in livers of rats treated with Veh/Veh, SLD/Veh or Veh/LPS (Fig 2.5A, 2.5B and 2.5C). In livers of SLD/LPS-cotreated rats, necrotic foci were present in the midzonal regions (Fig. 2.5D). These were

Fig. 2.2. Sulindac dose-response in the absence and presence of LPS. Rats were treated with various doses of SLD (10, 20, 50, 100 or 300 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) at -16 hr, and food was removed. At -0.5 hr rats received LPS ($8.25X10^5$ EU/kg, i.v.) or its vehicle (saline), and 30 min later they were given a second administration (same dose as 1st administration) of SLD. Blood samples were taken at 12 hr after the second administration of SLD, and ALT activity was measured. *significantly different from SLD/Veh group at the same dose. $P< 0.05$, n=3.

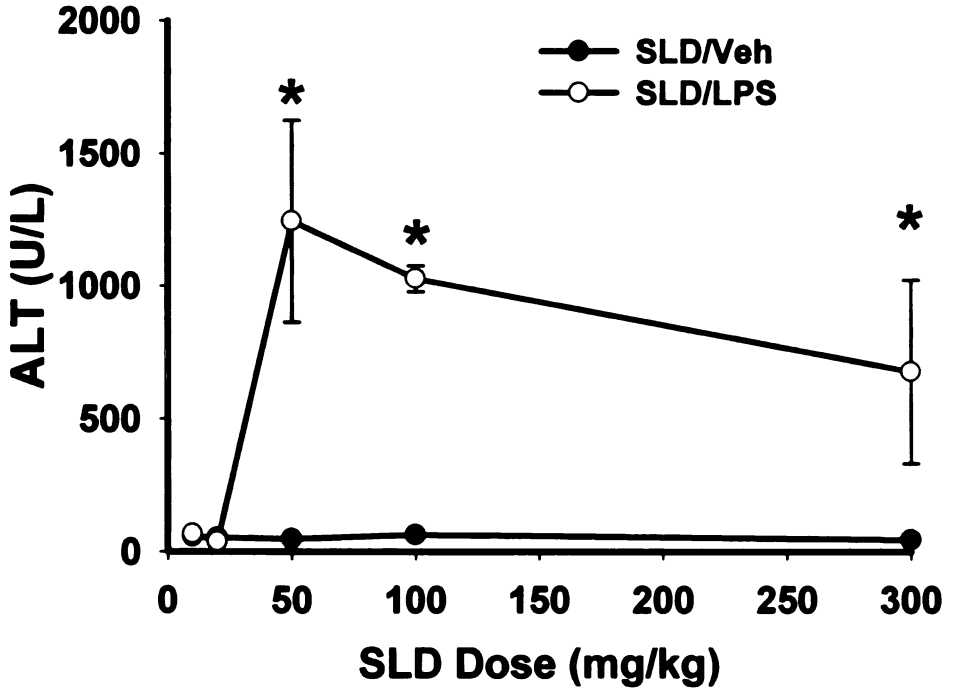
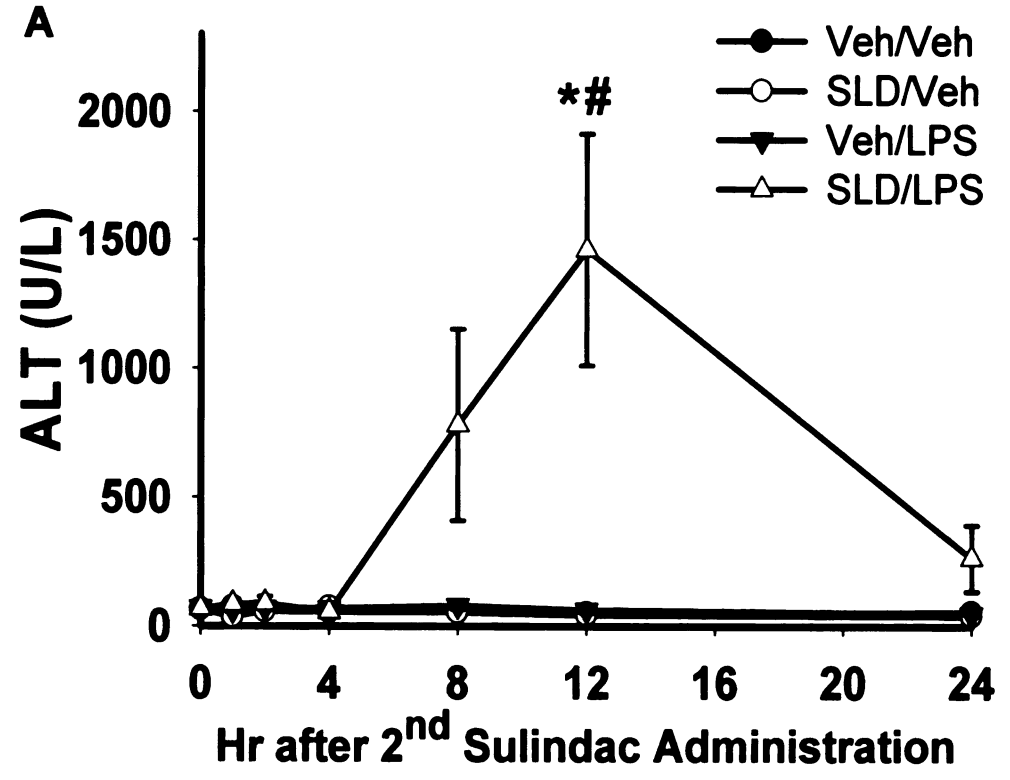


Fig. 2.3. Development of hepatocellular injury induced by SLD/LPS cotreatment. Rats were treated with SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) and LPS or its vehicle as described in Fig. 2.2. Blood samples were taken at various times (0, 1, 2, 4, 8, 12 or 24 hr), and ALT (A) and AST (B) activities in serum were measured. *significantly different from all other groups at the same time. #significantly different from SLD/LPS group at 0 hr. $P<0.05$, n=5-10.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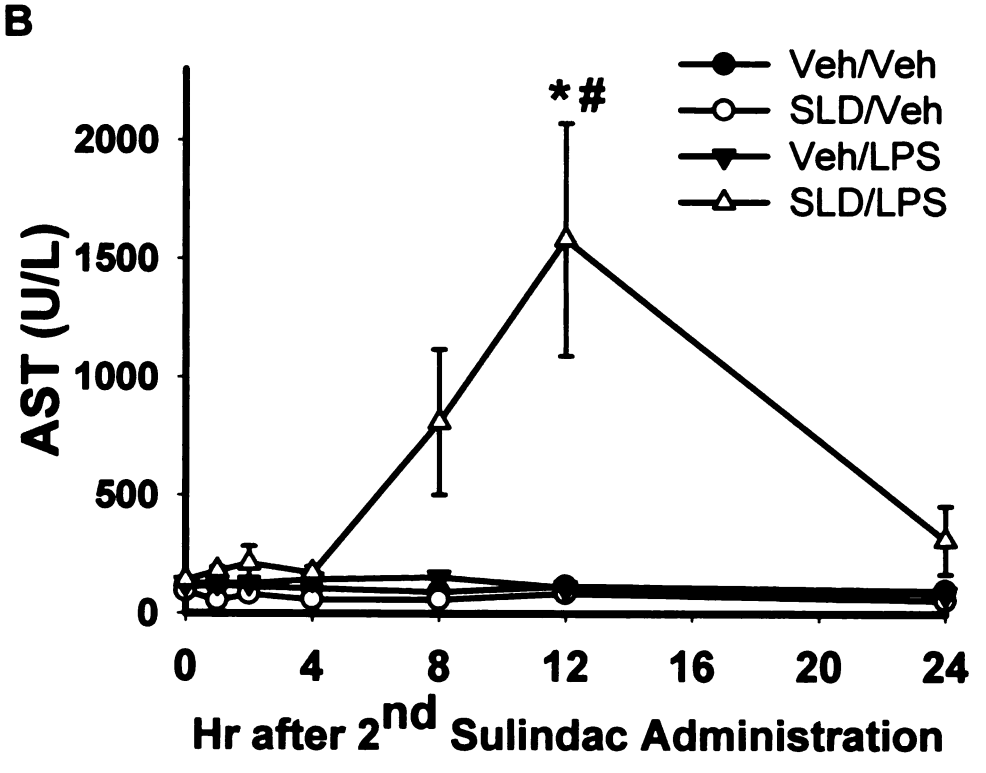
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24
Hr after 2nd Sulindac Administration

Fig. 2.4. Markers of hepatic cholestasis induced by SLD/LPS cotreatment. Rats were treated as described in Fig. 2.3, and serum samples were collected at 12 hr. Activities of alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) as well as concentrations of total bilirubin and bile acids in serum at 12 hr were evaluated. *significantly different from Veh/LPS group. #significantly different from SLD/Veh group. [a]significantly different from Veh/Veh group. $p<0.05$, n=4-11.

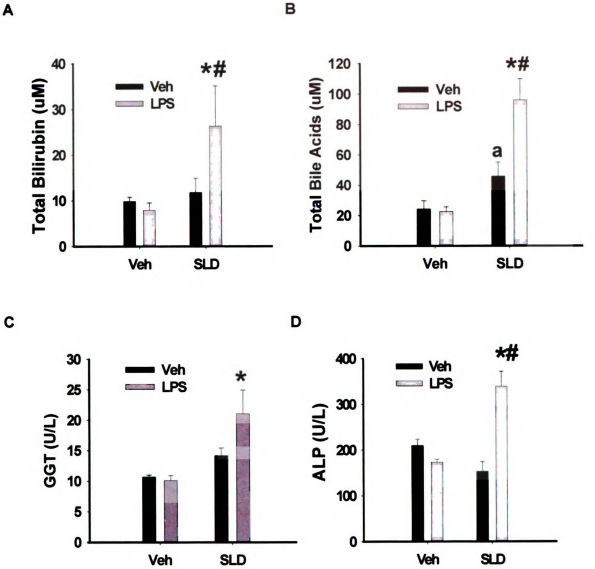
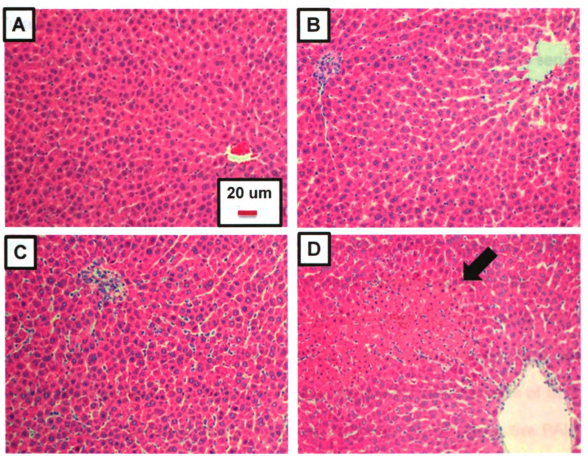

A
Total Bilirubin (uM)
40
30
20
10
0
Veh
LPS
*#
Veh
SLD
B
Total Bile Acids (uM)
120
100
80
60
40
20
0
Veh
LPS
*#
a
Veh
SLD
C
GGT (U/L)
30
25
20
15
10
5
0
Veh
LPS
*
Veh
SLD
D
ALP (U/L)
400
300
200
100
0
Veh
LPS
*#
Veh
SLD

Fig. 2.5. Liver histopathology in SLD/LPS-cotreated rats. Rats were treated with Veh/Veh (A), SLD/Veh (B), Veh/LPS (C) or SLD/LPS (D) as described in Fig. 2.3. Liver sections were collected at 12 hr and were stained with hematoxylin and eosin. The pictures were taken under 200 X maginification, and a necrotic area is indicated with an arrow.



associated with hemorrhage and neutrophil infiltration. The numbers and sizes of necrotic foci in livers from SLD/LPS-treated rats progressed with time and were consistent with the elevated ALT and AST activities in rat serum (Table 2.1). Significant liver lesions were observed beginning at 8 hr after the second administration of SLD and persisted through 24 hr.

2.4.3 Effect of SLD/LPS cotreatment on serum TNFα concentration

SLD alone had no effect on the concentration of TNFα in serum (Table 2.2). Treatment with LPS alone caused a significant increase in the serum TNFα concentration within 1.5 hr. SLD significantly enhanced the increase in serum TNFα induced by LPS.

2.4.4 Activation of the hemostatic system

Thrombin functions as a vital activator of the coagulation system, whereas active PAI-1 is the major endogenous down-regulator of the fibrinolytic system. The concentrations of these two regulators were evaluated using ELISA at 1, 4 and 8 hr after the second administration of SLD. Both TAT and active PAI-1 concentrations were elevated by LPS alone at 1, 4 and 8 hr, whereas SLD was without effect by itself (Fig. 2.6). At 4 hr after the second administration of SLD, SLD significantly enhanced the LPS-induced increases in TAT and active PAI-1 in plasma. Compared to LPS alone, SLD/LPS cotreatment also tended to prolong the elevation in thrombin and active PAI-1 in plasma (Fig. 2.6A, 2.6B).

Table 2.1. Midzonal hepatic necrosis in livers of rats treated with SLD/LPS

Liver sections from rats killed at 4, 8, 12 and 24 hr after the second administration of SLD were evaluated and assigned a score of 0-5 as described under *Materials and Methods*. Data are expressed as median score and 25th and 75th quartiles. *significantly different from Veh/Veh group at the same time. $P<0.05$, n=5-10.

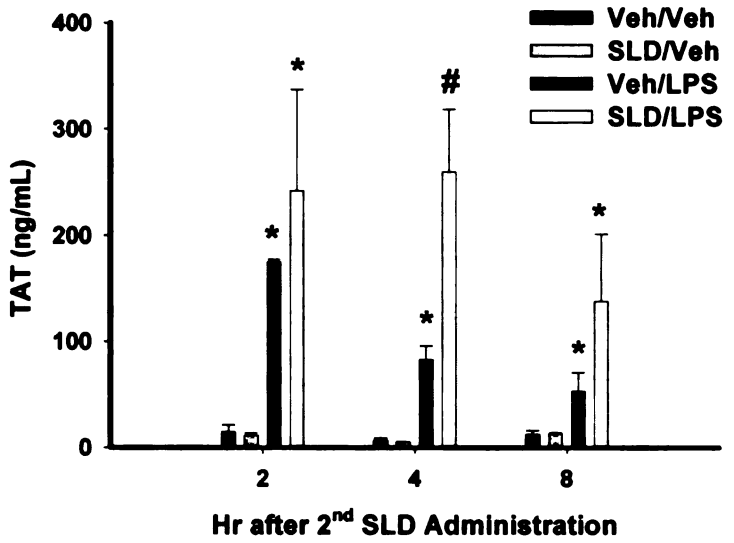
Treatment	Time after 2nd SLD (hr)			
	4	8	12	24
Veh/Veh	0.50 (0.34-0.53)	0.50 (0.31-0.66)	0.44 (0.25-0.63)	0.63 (0.47-0.66)
SLD/Veh	0.38 (0.34-0.88)	0.88 (0.47-1.03)	0.75 (0.63-1.00)	0.75 (0.44-0.88)
Veh/LPS	0.75 (0.47-0.97)	0.75 (0.56-1.19)	0.63 (0.44-1.09)	1.00 (0.81-1.26)
SLD/LPS	0.88 (0.72-1.40)	1.88 (1.63-3.59)*	3.37 (2.25-3.88)*	2.88 (2.25-3.38)*

Table 2.2. Serum TNFα concentration in SLD/LPS-treated Rats. Rats were treated with SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) at -16 hr, and food was removed. At -0.5 hr rats received LPS ($8.25X10^5$ EU/kg, i.v.) or its vehicle (saline), and 30 min later they were given a second administration (50 mg/kg) of SLD. Serum samples were collected at 1 hr after the second administration of SLD. The concentration of TNFα (ng/mL) in serum was determined using ELISA. *significantly different from corresponding group not treated with LPS. #significantly different from Veh/LPS group. $P<0.05$, n=3-7.

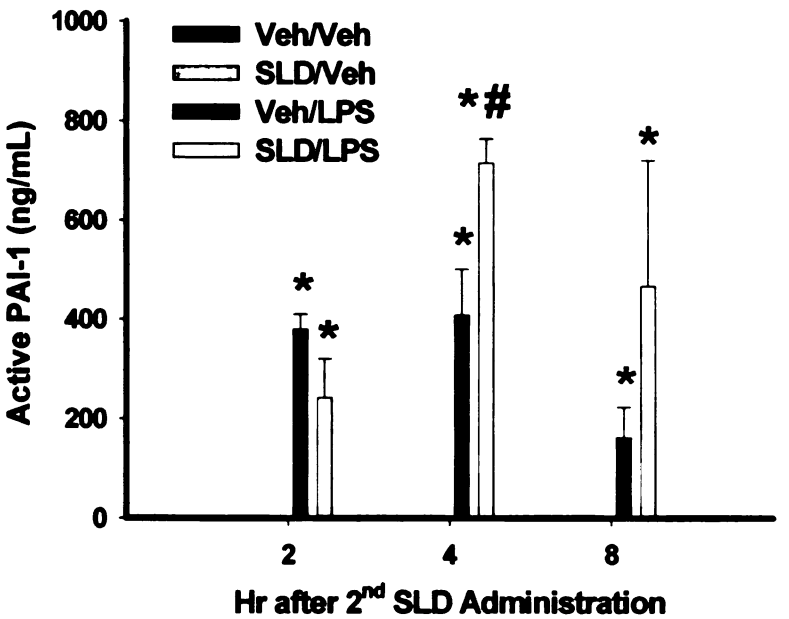
Treatment	Veh	SLD
Veh	0.024 ± 0.005	0.057 ± 0.018
LPS	16.9 ± 6.5*	50.2 ± 10.7*#

Fig. 2.6. Activation of the hemostatic system. Rats were treated with SLD, LPS or their vehicles as described in Fig.2.3. They were killed 2, 4 or 8 hr after the second administration of SLD and plasma was collected. Concentrations of TAT (A) and active PAI-1 (B) in plasma were measured. *significantly different from Veh/Veh group at the same time. #significantly different from Veh/LPS group at the same time. $P<0.05$, n=3-5.

A


Veh/Veh
SLD/Veh
Veh/LPS
SLD/LPS
TAT (ng/mL)
400
300
200
100
0
2
4
8
Hr after 2nd SLD Administration

B


Veh/Veh
SLD/Veh
Veh/LPS
SLD/LPS
Active PAI-1 (ng/mL)
1000
800
600
400
200
0
2
4
8
Hr after 2nd SLD Administration

Hepatic fibrin deposition was evaluated 4 hr after the second administration of SLD because that is a time just before the onset of liver injury as reflected by serum ALT and AST activities (Fig. 2.3). SLD alone and LPS alone were associated with small increases in fibrin that were not statistically significant. In contrast, SLD/LPS cotreatment led to a pronounced elevation in fibrin staining that was panlobular in distribution (Fig. 2.7 and 2.9A).

As mentioned above, fibrin deposition can lead to hypoxia, which was evaluated by quantifying PIM-protein adducts in livers. PIM-protein adducts were at control levels in livers of SLD-treated rats. LPS treatment caused a modest increase, and SLD/LPS cotreatment led to a greater elevation of PIM-protein adducts, predominantly in midzonal regions of liver lobules by 4 hr after the second administration of SLD (Fig. 2.7 and 2.8B).

2.4.5 Effect of heparin on liver injury induced by SLD/LPS cotreatment

Anticoagulant heparin (3000 Units/kg, s.c.), administered concurrently with the second administration of SLD, caused a marked decrease in fibrin deposition in liver as expected (Fig. 2.10A). PIM-protein adduct staining was also significantly reduced by heparin at 4 hr after the second SLD administration (Fig. 2.10B).

To evaluate the role of hemostatic system in liver injury, heparin was given to rats concurrently with and 6 h after the second administration of SLD, and liver injury was evaluated at 12 hr. Heparin was without effect on ALT activity in Veh/Veh-treated rats but significantly attenuated the increase in ALT activity in

Fig. 2.7 Fibrin deposition in liver. Rats treated with Veh/Veh (A), SLD/Veh (B), Veh/LPS (C) or SLD/LPS (D) as described in Fig. 2.4 were killed at 4 hr, and immunohistochemistry for fibrin was performed.

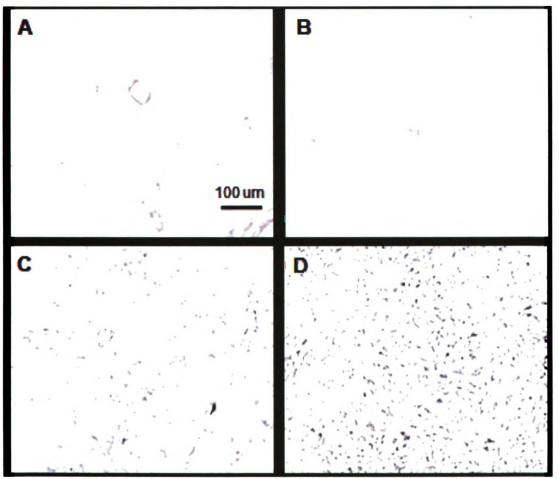


Fig. 2.8. Hypoxia staining in liver. Rats were treated with Veh/Veh (A), SLD/Veh (B), Veh/LPS (C) or SLD/LPS (D) as described in Fig. 2.4 except that PIM hydrochloride (120 mg/kg) was administered 2 hr after the second administration of SLD. At 4 hr after the second administration of SLD, livers were collected and fixed for immunohistochemistry.

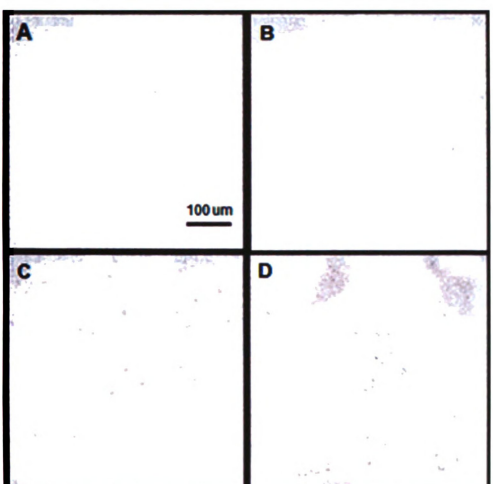
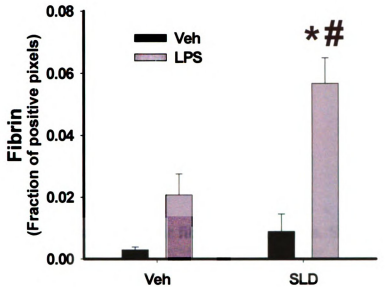


Fig. 2.9. Evaluation of fibrin deposition and hypoxia in liver. Rats were treated as described in Fig. 2.3. A) Animals were killed at 4 hr, and livers were processed for immunohistochemical determination of fibrin deposition. B) Rats received an additional treatment with PIM hydrochloride (120 mg/kg) 2 hr after the second administration of SLD. They were then killed at 4 hrs, and livers were processed for immunohistochemical determination of hypoxia. In both panels the fraction of positive pixels averaged from 10 randomly chosen microscope fields (100 X) was determined for each animal. *significantly different from Veh/LPS group. #significantly different from SLD/Veh group. [a]significantly different from Veh/Veh group. $P<0.05$, n=4-6.

A


Fibrin
(Fraction of positive pixels)
0.08
0.06
0.04
0.02
0.00
Veh
LPS
*#
Veh
SLD

B

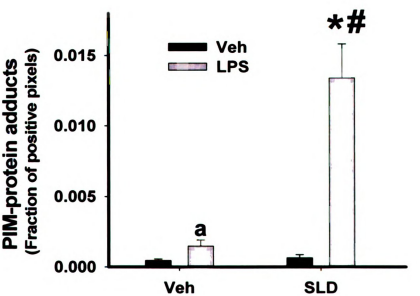
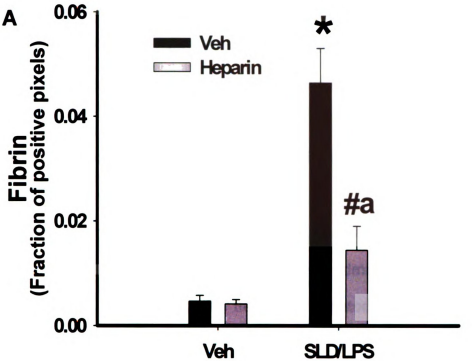
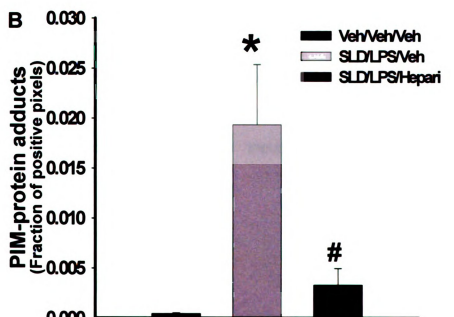

PIM-protein adducts
(Fraction of positive pixels)
0.015
0.010
0.005
0.000
Veh
LPS
*#
a
Veh
SLD

Fig. 2.10. Effect of heparin on liver fibrin and hypoxia. SLD and/or LPS were given to rats as described in Fig. 2.3. Heparin (3000 units/kg, s.c.) was given to rats at the same time as the second administration of SLD. Rats were killed at 4hr, and liver sections were fixed and stained immunohistochemically for fibrin (A) or PIM-protein adducts (B). The fraction of positive pixels was determined as described under *Materials and Methods*. *significantly different from Veh/Veh/Veh group. #significantly different from SLD/LPS/Veh group. [a]significantly different from Veh/Veh/heparin group. $P<0.05$, n=4-6.


A
Fibrin
(Fraction of positive pixels)
0.06
0.04
0.02
0.00
Veh
Heparin
*
#a
Veh
SLD/LPS

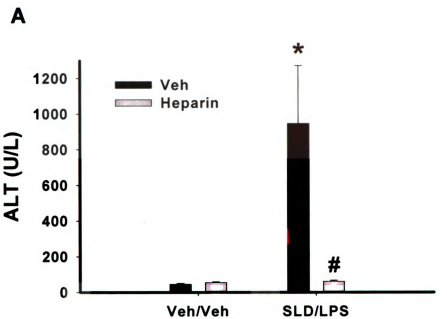

B
PIM-protein adducts
(Fraction of positive pixels)
0.030
0.025
0.020
0.015
0.010
0.005
0.000
*
#
Veh/Veh/Veh
SLD/LPS/Veh
SLD/LPS/Hepari

the sera of SLD/LPS-treated rats (Fig. 2.11A). The SLD/LPS-induced elevation in total bile acid concentration in serum was also attenuated by heparin (Fig. 2.11B). Necrotic foci were observed in livers of rats treated with SLD/LPS/Veh, but not in livers of rats treated with SLD/LPS/heparin (Fig. 2.12).

2.4.6 Effect of low oxygen on hepatocytotoxicity induced by SLD sulfide *in vitro*

To assess whether hypoxia can affect the killing of hepatocytes by SLD, the active metabolite of SLD, SLD sulfide (60 uM) was administered to primary rat hepatocytes *in vitro*. Immediately after treatment, hepatocytes were incubated in oxygen replete (20% O_2) or hypoxic (5% O_2) atmospheres. The 5% O_2 is equivalent to a nominal pO_2 of 30 mm Hg, which is the smallest reported oxygen concentration in blood around hepatic central vein *in vivo* (Jungermann and Kietzmann, 2000). This degree of hypoxia had no effect on the viability of hepatocytes after 8 hr of incubation. SLD sulfide alone increased ALT activity in the medium, indicating hepatocellular injury. When HPCs treated with SLD sulfide were exposed to 5% O_2, cell death caused by SLD sulfide was significantly enhanced compared to incubation in 20% O_2 (Fig. 2.13).

Fig. 2.11. Effect of heparin on SLD/LPS-induced liver injury. Rats were treated with SLD/LPS as described in Fig. 2.3. Heparin (3000 units/kg, s.c.) or its vehicle (saline) was given to rats at 0 and 6 h. Rats were killed at 12 hr, and ALT activity (A) and concentration of total bile acids (B) in serum was determined. *significantly different from Veh/Veh/heparin group. #significantly different from SLD/LPS/Veh group. $P<0.05$, n=3-13.


A
ALT (U/L)
1200
1000
800
600
400
200
0
Veh
Heparin
*
#
Veh/Veh
SLD/LPS

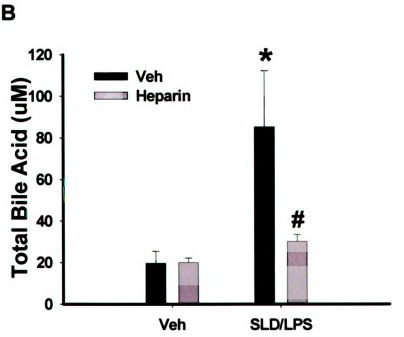

B
Total Bile Acid (uM)
120
100
80
60
40
20
0
Veh
Heparin
*
#
Veh
SLD/LPS

Fig. 2.12. Effect of heparin on hepatic lesions induced by SLD/LPS. Rats were treated with SLD/LPS as described in Fig. 2.4. Heparin (3000 units/kg, s.c.) or its vehicle (saline) was given to rats at 0 and 6 h. Liver sections were collected at 12 hr and stained with hematoxylin and eosin. Liver sections of rats treated with SLD/LPS/Veh (A) and SLD/LPS/heparin (B) were examined under 200X magnification. A necrotic area is indicated with an arrow.

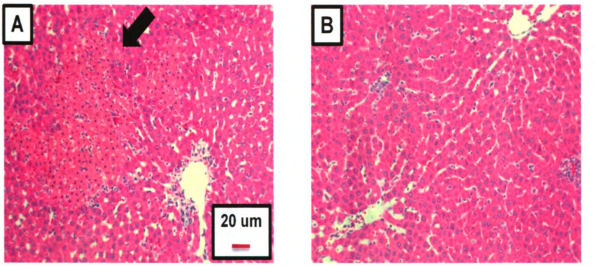
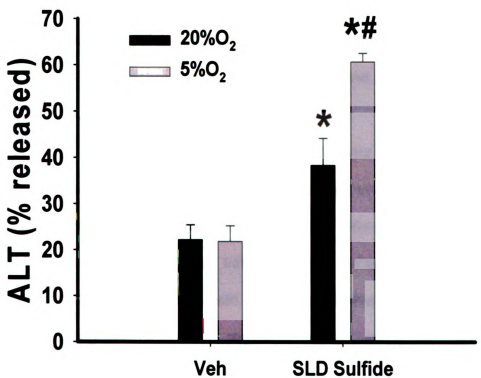


Fig. 2.13. Effect of hypoxia on SLD sulfide-induced cytotoxicity. Rat primary hepatocytes were isolated as described under *Materials and Methods.* They were treated with 60uM SLD sulfide and kept in 20% or 5% oxygen. After 8 hr, ALT release was determined. *significantly different from Veh group at the same oxygen level. #significantly different from SLD sulfide treatment group at 20% oxygen. $P<0.05$, n=3.



2.5 Discussion

SLD is an NSAID that is used for the treatment of arthritis. All of the NSAIDs have been associated with hepatic IADRs in patients (O'Connor et al., 2003). The average risk of serious hepatic injury for NSAIDs is approximately 1 case in 10,000 patient-years of use, and the risk from SLD is reported to be 5-10 fold greater than for NSAIDs as a class (Walker, 1997). The mechanism of NSAID-induced hepatic IADRs is not clearly understood, and several hypotheses have been proposed. It is commonly accepted that large amounts of active metabolites form after exposure to large doses of NSAIDs, and these might lead to protein adduct formation, oxidative stress and mitochondrial injury that could contribute to tissue damage (Boelsterli, 2002). However, evidence supporting these hypothses is mainly obtained from studies *in vitro*, and animal models of SLD-induced liver injury are lacking.

Previous studies in rodents suggested that there may be a connection between inflammation and hepatic IADRs for at least some drugs (Buchweitz et al., 2002; Luyendyk et al., 2003; Roth et al., 2003; Shaw et al., 2007). In these drug-LPS interaction models, one administration of the drug was sufficient to produce a hepatotoxic interaction with LPS. In preliminary studies with SLD, we tried several single-administration regimens using various doses and times between SLD and LPS treatment. Although hepatotoxic signals (i.e., increased serum ALT activity) were observed in some rats, these were inconsistent. Changing to a two-administration protocol in which the time between SLD administrations approximated 3 half lives (Hucker et al., 1973) provided relatively

consistent and statistically significant liver injury; moreover, this protocol corresponds to the twice per day treatment regimen typically used therapeutically in human patients. Using this protocol, SLD or LPS alone did not cause any lesions in the liver or increase the clinical chemical biomarkers of liver injury. However, in SLD/LPS-cotreated rats serum markers of both hepatocellular injury and cholestasis were increased significantly, and foci of necrotic parenchymal cells were found in livers. Interestingly, in a study of 91 cases reported to the U.S. Food and Drug Administration, SLD caused hepatocellular injury, cholestatic injury or mixed liver injury in human patients (Tarazi et al., 1993), consistent with observations presented here for rats treated with SLD/LPS. These results raise the possibility that a mild episode of inflammation might render human patients susceptible to SLD-induced liver injury.

Judging by the timecourse of serum ALT and AST activities (Fig. 2.3) and histological changes (Table 2.1), the onset of liver injury was between 4 and 8 hr. The serum markers of hepatocellular injury rose until 12 hr and declined by 24 hr. Despite this decline, histologic evidence of liver injury persisted at 24 hr (Table 2.1). This decline in serum transaminases suggests that injury occurred in the first 12 hrs, and ALT and AST released from hepatocytes were cleared from the blood thereafter. The short initial half life of ALT/AST in rat plasma (approximately 5 hr) is consistent with this interpretation (Saheki et al., 1990).

LPS has the potential to influence toxicity in a number of ways. For example, it is capable of downregulating the expression of several drug metabolizing enzymes (Morgan, 1989). SLD is bioactivated by hepatocytes to a

more toxic sulfide metabolite by methionine sulfoxide reductase (Kitamura et al., 1980; Kitamura and Tatsumi, 1982; Etienne et al., 2003a). It is not known if LPS downregulates the expression of this enzyme; however, in a preliminary study, LPS treatment did not increase the liver concentration of SLD sulfide (unpublished observation).

Cytokines such as tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1) and interleukin-6 (IL-6) are upregulated after the activation of Toll-like receptor 4 on Kupffer cells by LPS (Luster et al., 1994; Su, 2002). Some of these changes have been linked to liver injury from drug/LPS interaction (Shaw et al., 2007; Tukov et al., 2007b). The observation that fibrin deposited in livers of rats cotreated with SLD and LPS led us to explore the hemostatic system in this study. Cytokines are potent modulators of the hemostatic system. For example, TNFα and IL-1 activate coagulation by upregulating tissue factor expression and by reducing the fibrinolytic activity of endothelial cells through an increase in PAI-1 (Schleef et al., 1988; Salgado et al., 1994). The plasma concentrations of TAT and active PAI-1 increased rapidly in LPS-treated rats, confirming that LPS induces the activation of the coagulation system and provides conditions for inhibition of the fibrinolytic system (Fig. 2.6). Although SLD had no effect on these factors when it was given alone, it enhanced the LPS-mediated changes and tended to prolong the activation of the hemostatic system. This could be due to enhanced TNFα release by SLD (Table 2.2). In the liver injury induced by ranitidine/LPS cotreatment, ranitidine enhanced the LPS-induced increase in TNFα concentration through p38-dependent activation of TNFα converting

enzyme (TACE), which cleaves membrane-bound pro-TNFα to form mature TNFα (Deng et al., 2008). Whether a similar mechanism is at play in SLD/LPS-cotreated rats is yet to be determined.

SLD/LPS cotreatment significantly increased cross-linked fibrin in livers at 4 hr– i.e., a time before the onset of liver injury. Although LPS activated the hemostatic system in rats, it alone was not sufficient to induce marked fibrin deposition in the liver (Fig. 2.9A). The hemostatic system is activated in endotoxemia-induced liver injury (Hewett and Roth, 1995), and one possible consequence is hypoxia in the liver resulting from disrupted blood flow in the sinusoids. PIM-protein adducts were slightly elevated in livers of rats treated with LPS alone, indicating that mild hypoxia occurred. In contrast, SLD/LPS led to a pronounced increase in PIM-protein adducts, suggesting marked hypoxia (Fig. 2.9B). Unlike the distribution of fibrin which was panlobular, hypoxia occurred only in the midzonal regions of livers (Fig. 2.3, 2.4). Interestingly, necrotic foci were also present predominantly in this region (Fig.2.5).

It has been reported that hypoxia causes hepatocelluar injury in isolated, perfused rat livers (Lemasters et al., 1981), and liver injury was induced *in vivo* in rats exposed for a brief period to a low concentration of oxygen (Fassoulaki et al., 1984). In studies presented here, the anticoagulant heparin reduced hepatic fibrin deposition and hypoxia induced by SLD/LPS cotreatment (Fig. 2.10). This suggests that hypoxia might be caused by fibrin clots in liver sinusoids. Moreover, both hepatocellular and bile ductular injury in SLD/LPS-treated rats

was significantly attenuated by heparin, supporting the hypothesis that hypoxia induced by fibrin clots plays an important role in the pathogenesis (Fig. 2.11).

Previous studies suggested that hypoxia can potentiate the toxicity of some xenobiotics towards hepatocytes (Shen et al., 1982). Compared to SLD or its sulfone metabolite, SLD sulfide is more cytotoxic (Leite et al., 2006). Therefore, we treated rat primary hepatocytes with SLD sulfide. Hypoxia enhanced its ability to kill these cells (Fig. 2.13). SLD sulfide, but not SLD or SLD sulfone uncouples the mitochondria of HepG2 cells (Leite et al., 2006). Thus, hypoxia could exacerbate SLD sulfide-mediated mitochondrial dysfunction by diminishing the aerobic metabolism of hepatocytes. Moreover, hypoxia inducible factor 1 (HIF-1) accumulated in cells under hypoxic stress might induce the expression of proapoptotic proteins and cause the stabilization of p53, which increases permeability of the mitochondrial membrane and causes cell death (Greijer and van der Wall, 2004). In the SLD/LPS idiosyncratic liver injury model, hypoxia might enhance the mitochondrial toxicity of SLD, providing a synergistic effect on a mitochondrial pathway to cell death. Although further study is required to test this, SLD/LPS cotreatment in rats could be an animal model supporting the mitochondrial injury hypothesis of NSAID-induced IADRs described above.

The mitochondrial pathway might not be the only contributor to liver injury. Hypoxia can also interplay with inflammatory factors. For example, when stimulated by LPS, neutrophils accumulate in sinusoids and transmigrate through blood vessels to liver parenchyma. At the site of inflammation, activated neutrophils release proteases that can kill rat hepatocytes (Ho et al., 1996), and

hypoxia enhanced neutrophil protease-mediated hepatocyte killing (Luyendyk et al., 2005).

Results with SLD are similar to those observed with diclofenac (DCLF), another NSAID that causes hepatic IADRs in people, in the sense that both drugs interacted with LPS to cause liver injury in rats. However, a large dose of DCLF (100 mg/kg) caused liver injury by itself in rats in the absence of LPS-cotreatment. This was associated with intestinal injury and translocation of bacteria to the liver and was prevented by sterilization of the GI tract (Deng et al., 2006). These results suggested that GI irritation and translocation of bacteria or LPS caused by DCLF contribute to DCLF-induced hepatotoxicity in rats, and a similar mechanism might underlie DCLF IADRs in humans. In contrast, large doses of SLD (up to 300 mg/kg) did not cause liver injury in rats by themselves (Fig. 2.2). This suggests that, at least in rats, SLD is less irritating to the intestine than DCLF, although the relative gastrointestinal toxicity of SLD and DCLF in humans is still controversial. One study indicated that SLD is less associated with gastrointestinal hospitalizations than DCLF (Garcia Rodriguez et al., 1992), whereas others suggested the opposite (Henry et al., 1993; Savage et al., 1993). The potential implication for human IADRs is that DCLF may be able to provide its own inflammatory stress through GI irritation, whereas SLD toxicity might require an additional inflammatory stress that arises independently of drug treatment. It seems possible that the latter could arise from the very condition that the drug is used to treat-- e.g., an inflammatory flare of rheumatoid arthritis. Alternatively, an independently occurring inflammatory episode might interact

with SLD. The finding that viral hepatitis may predispose patients to NSAID hepatotoxicity supports this possibility (Teoh and Farrell, 2003).

In summary, SLD/LPS cotreatment caused liver injury in rats, which was not produced by SLD or LPS alone. The coagulation system was activated while the fibrinolytic system was inhibited in the cotreated rats. As a consequence, fibrin clots formed in sinusoids and hypoxia occurred selectively in livers of rats treated with SLD/LPS. The anticoagulant heparin protected rats against liver injury and also attenuated fibrin deposition and liver hypoxia. Hypoxia enhanced the cytotoxicity of SLD sulfide *in vitro*. The results support the hypothesis that NSAIDs that cause hepatic IADRs in humans interact with an inflammatory stress to cause liver injury in animals. Hypoxia may play an important role in this SLD/LPS idiosyncratic liver injury model through synergistic interplay with the toxic SLD sulfide metabolite. These observations do not exclude a role for other factors, such as cytokines, that are a focus of ongoing investigation.

CHAPTER 3

Zou W, Beggs KM, Sparkenbaugh EM, Jones AD, Younis HS, Roth RA and Ganey PE (2009). Sulindac metabolism and synergy with TNF in a drug-inflammation interaction model of idiosyncratic liver injury. *J Pharmacol Exp Ther*.

3.1 Abstract

Sulindac (SLD) is a nonsteroidal anti-inflammatory drug (NSAID) that has been associated with a greater incidence of idiosyncratic hepatotoxicity in human patients than other NSAIDs. In previous studies, cotreatment of rats with SLD and a modestly inflammatory dose of lipopolysaccharide (LPS) led to liver injury, whereas neither SLD nor LPS alone caused liver damage. In studies presented here, further investigation of this animal model revealed that the concentration of tumor necrosis factor-α (TNF) in plasma was significantly increased by LPS at 1 hr, and SLD enhanced this response. Etanercept, a soluble TNF receptor, reduced SLD/LPS-induced liver injury, suggesting a role for TNF. SLD metabolites in plasma and liver were determined by LC/MS/MS. Cotreatment with LPS did not increase the concentrations of SLD or its metabolites, excluding the possibility that LPS contributed to liver injury through enhanced exposure to SLD or its metabolites. The cytotoxicities of SLD and its sulfide and sulfone metabolites were compared in primary rat hepatocytes and HepG2 cells; SLD sulfide was more toxic in both types of cells than SLD or SLD sulfone. TNF augmented the cytotoxicity of SLD sulfide in primary hepatocytes and HepG2 cells. These results suggest that TNF can enhance SLD sulfide-induced hepatotoxicity, thereby contributing to liver injury in SLD/LPS-cotreated rats.

3.2 Introduction

Several hypotheses have been put forward to explain the basis for IADRs; however, the modes of action are still unclear, in part because of the lack of animal models. One hypothesis is that inflammatory stress precipitates hepatic IADRs in humans (Roth et al., 2003; Ganey et al., 2004). In concert with this hypothesis, cotreatment of rats with lipopolysaccharide (LPS), which induces modest inflammation, and SLD resulted in liver necrosis, whereas neither LPS nor SLD was hepatotoxic alone (Zou et al., 2009b).

In this study, we examined factors that could contribute to the pathogenesis of liver injury in rats cotreated with LPS and SLD. In vivo, SLD can be metabolized either irreversibly to SLD sulfone or reversibly to SLD sulfide, which is more cytotoxic than SLD itself. Since LPS can regulate drug metabolism (Renton, 2001), we tested whether LPS coexposure enhances bioactivation of SLD. Moreover, we determined the effect of SLD on LPS-induced tumor necrosis factor-α (TNF) production and its role in the development of liver injury.

3.3 Materials and methods

3.31 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). LPS (Lot 075K4038) derived from *Escherichia coli* serotype O55:B5 with an activity of 3.3 X 10^6 endotoxin units (EU)/mg was used in experiments. Etanercept was purchased from Amgen Pharmaceuticals (Thousand Oaks, CA). HepG2/C3A cells for in vitro studies were obtained from American Type Culture Collection (Manassas, VA).

3.3.2 Animals

Male, Sprague-Dawley rats (Crl:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250 to 370 g were used for studies in vivo (rats weighing 290 to 300g were used to evaluate SLD and its metabolites in GI and feces), and rats weighing 150 to 200 g were used for primary hepatocyte isolation. Animals were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to spring water. They were allowed to acclimate for 1 week in a 12-hr light/dark cycle prior to use in experiments.

3.3.3 Experimental protocol

As described in previous studies (Zou et al., 2009b), rats were given two administrations of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) with a 16 hr interval, and food was removed after the first administration. Half an hour before the second administration of SLD, LPS (8.25X 10^5 EU/kg, i.v.) or its

vehicle (saline) was administered via a tail vein. Depending on the purpose of experiments, rats were anesthetized with isoflurane and euthanized at various times (0, 1, 2, 4, 8 and 12 hr) after the second administration of SLD. For the collection of plasma, a portion of blood drawn from anesthetized rats was transferred into vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium citrate (final concentration 0.38%). The rest of the blood was allowed to clot at room temperature for preparation of serum. Collected plasma and serum were stored at – 80 ^{0}C until use. Three slices (3-4 mm thick) of the left lateral liver lobe were collected and fixed in 10% buffered formalin for histological analysis. A portion of the right medial lobe of the liver was flash-frozen in liquid nitrogen for pharmacokinetic study of SLD and its metabolites. For determining drug concentration in the gastrointestinal (GI) tract and feces, each rat was housed in a separate cage after LPS or vehicle injection and euthanized at 2 hr. The entire GI tract and its contents were collected. Feces were retrieved from the cages and were homogenized with the GI tract and its contents for each rat. In the TNF inhibition study, rats were given etanercept (8mg/kg) or vehicle (sterile water) subcutaneously an hour before LPS (8.25X 10^{5} EU/kg, i.v.) or its saline vehicle. We have demonstrated that etanercept inactivates TNF activity induced by LPS administration in rats using this treatment protocol (Tukov et al., 2007b).

3.3.4 Evaluation of liver injury

The activity of alanine aminotransferase (ALT), a marker of hepatic parenchymal cell injury, was assessed in serum using a diagnostic kit from

Thermo Corp. (Waltham, MA). Liver slices fixed in 10% buffered formalin were embedded in paraffin, cut into 6 um sections and stained with hematoxylin and eosin (H&E) for histological evaluation.

3.3.5 Determination of TNF concentration in serum

The concentration of TNF in serum collected at 0, 1, 2, 4 and 8 hr after the second administration of SLD was measured by ELISA (BD Biosciences; San Diego, CA).

3.3.6 LC/MS/MS analysis

Plasma samples, liver homogenates, GI and fecal homogenates or HepG2 cells in culture medium were mixed with acetonitrile containing diclofenac as internal standard. After vortex and centrifugation, protein was removed, and the supernatant was diluted and transferred to ultra performance liquid chromatography (UPLC) sample vials for LC/MS/MS analysis.

LC/MS/MS analysis was performed using a Waters ACQUITY UPLC System coupled to a Quattro Premier XE tandem quadrupole mass spectrometer. An extract volume of 2 µL was injected into the UPLC system and eluted with a gradient mixture (0-99%) of formic acid and acetonitrile. Electrospray ionization in positive ion mode was performed for analyses of plasma samples, and the collision and source cone voltages were optimized independently for each analyte. Multiple reaction monitoring (MRM) of the following m/z transitions was used for the quantitative analysis of diclofenac (296.2->214.2), SLD (357.2-

>333.2), SLD sulfone (373.2->233.2), SLD sulfide (341.2->234.2), SLD acyl glucuronide (533.1->339.1), SLD sulfone acyl glucuronide (549.1->355.1) and SLD sulfide acyl glucuronide (517.1->323.1).

For samples other than plasma, electrospray ionization was performed in negative ion mode, and metabolite concentrations were determined by the MRM of transition of diclofenac (294.2->250.0), SLD (311.2->296.2), SLD sulfone (327.2->264.2), SLD sulfide (295.2->280.2), SLD acyl glucuronide (531.1->355.1), SLD sulfone acyl glucuronide (547.1->371.1) and SLD sulfide acyl glucuronide (515.1->339.1).

The LC/MS/MS method achieved low limits of quantification (LLOQ) of 30 ng/mL or less for all three forms of sulindac (sulfoxide, sulfide, and sulfone) using both positive and negative ion modes. Analytical reproducibility was judged to be ± 12% in the middle of the calibrated range of concentrations.

3.3.7 Evaluation of cytotoxicity of SLD and its metabolites in vitro

HepG2 cells were plated at a density of 4×10^4 cells/well in 96-well plates. After overnight incubation in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), medium was renewed and SLD, SLD sulfone, SLD sulfide (0- 500 μM) or its vehicle (0.5% dimethyl sulfoxide [DMSO]) was added to the wells. After a 24-hr incubation, lactate dehydrogenase (LDH) released into the medium and total cellular LDH were evaluated using a kit from Promega Corporation (Madison, WI). Cytotoxicity was assessed as the

percentage of LDH released into the medium relative to the total LDH in the well (medium plus lysed cells).

For primary rat hepatocytes, isolation was performed as described previously (Tukov et al., 2006). Briefly, rat liver was first perfused *in situ* through the portal vein and then digested with Liver Digest Medium (Invitrogen Corp, Carlsbad, CA). The digested liver was combed gently, and hepatocytes were obtained after centrifugation (100 x g, 30 s).

Hepatocytes were suspended in Williams' Medium E (Invitrogen Corp, Carlsbad, CA) with 10% FBS, and the cell viability was always above 80%. Hepatocytes were plated at a density of 2.5×10^5 cells/well in 12-well plates and incubated for 3 hr to attach to the plate. Serum-containing medium was replaced by serum-free medium, and SLD, SLD sulfone, SLD sulfide (0- 120 μM) or vehicle (0.1% DMSO) was added to the culture wells. After 8 hr incubation, cytotoxicity was assessed by calculating the ALT activity in the medium plus unattached cells as a percentage of the total ALT activity in the well as described previously (Zou et al., 2009b).

3.3.8 Cytotoxicity from TNF and SLD metabolites

SLD sulfide or its vehicle (0.5% DMSO) was administrated to HepG2 cells with recombinant human TNF (200 ng/mL) or its vehicle (medium). After 24 hr incubation, the percentage of LDH released was evaluated. To determine the remaining concentration of SLD sulfide in each well, HepG2 cells were scraped,

and acetonitrile was added to precipitate protein. After centrifugation, the concentration of SLD sulfide in supernatant was determined using LC/MS/MS.

To assess further whether TNF can affect the cytotoxicity of SLD metabolites, isolated primary rat hepatocytes were treated with SLD sulfide (60 μM) in the presence or absence of recombinant rat TNF (2 μg/mL), and the percentage of ALT released was evaluated 8 hr later.

3.3.9 Statistical analysis

Results are expressed as means ± SEM. One-way or two-way analysis of variance (ANOVA) was applied for data analysis as appropriate, and Tukey's test was employed as a *post hoc* test. Student's t-test was performed when only two groups were compared. For all studies, $P < 0.05$ was considered as the criterion for statistical significance.

3.4 Results

3.4.1 Timecourse of TNF concentration in plasma

Rats were treated with LPS and two administrations of SLD or their vehicles as described in *Methods,* and TNF concentration in serum was evaluated at various times up to 8 hr after the second administration of SLD. SLD had no effect on serum TNF concentration in rats. LPS alone led to a significant increase in TNF serum concentration at 0 and 1 hr (i.e., 0.5 and 1.5 hr after LPS). The elevation of TNF concentration induced by LPS was significantly increased by SLD at 1 hr after the second administration of the drug (Fig. 3.1).

3.4.2 Effect of TNF inhibition on liver injury

Etanercept is a soluble TNF receptor that neutralizes the biological activity of TNF. To investigate the role of TNF in liver injury, rats were treated with etanercept 1 hr before LPS administration. This treatment protocol inhibits the activity of TNF in rats (Geier et al., 2003). We have reported previously that neither LPS nor SLD produces liver injury when given alone at the doses used in these studies (Zou et al., 2009b). Also consistent with our previous report, SLD/LPS cotreatment increased serum ALT activity significantly (Fig. 3.2). Etanercept significantly attenuated this increase, whereas etanercept alone had no effect on serum ALT activity. Histological examination of H&E-stained livers of rats revealed a pattern consistent with the ALT activity. That is, midzonal necrotic foci were present in livers of rats treated with SLD/LPS but were found infrequently in livers of rats treated with etanercept/SLD/LPS.

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Fig. 3.1. Timecourse of TNF concentration in rat serum. Rats were treated with two administrations of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) with a 16 hr interval. Half an hour before the second administration of SLD, LPS (8.25X 10^5 EU/kg, i.v.) or its saline vehicle was administered via a tail vein. TNF was evaluated by ELISA in serum samples obtained from rats at 0, 1, 2, 4 or 8 hr after the second administration of SLD. *significantly different from Veh/Veh group at the same time. #significantly different from Veh/LPS group at the same time. $P<0.05$, n=4-5 for all points except 8 hr group (n=3), Veh/LPS and SLD/LPS at 1 hr (n=8 and 9, respectively), and SLD/LPS at 0 hr (n=8).

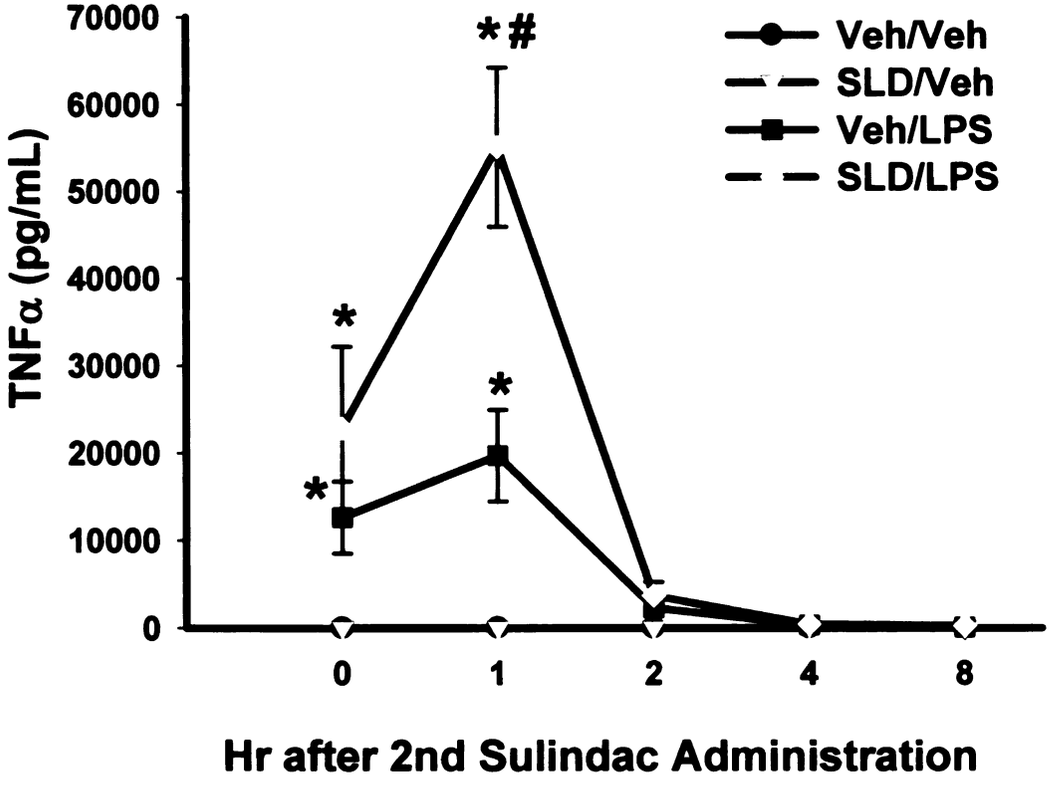

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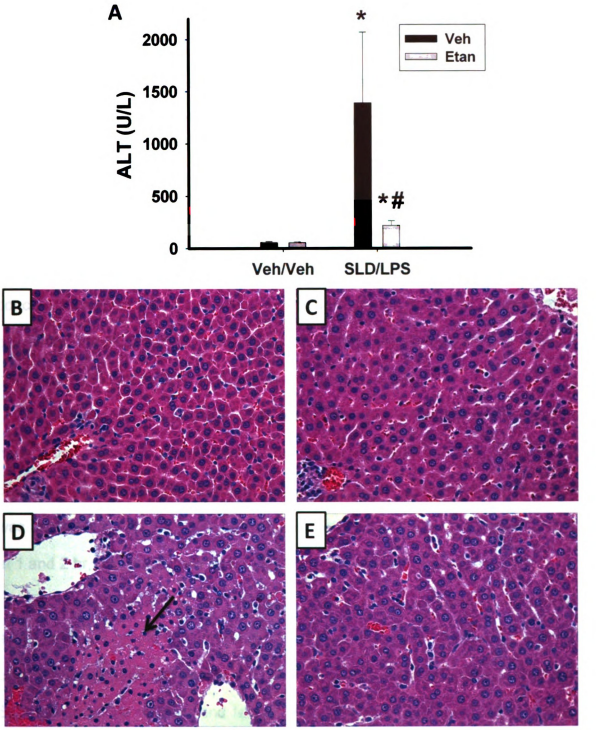
Fig. 3.2. Effect of TNF inhibition on liver injury induced by SLD/LPS. Rats were treated with etanercept (8 mg/kg, s.c.) or its vehicle 1 hr before LPS. SLD and LPS or their vehicles were administered to rats as described in Methods. ALT activity was determined at 12 hr (A). *significantly different from respective Veh/Veh group. #significantly different from Veh/ SLD/LPS group. $P<0.05$, n=4 for all groups except SLD/LPS/Etan (n=6). Liver sections from rats treated with Veh/Veh/Veh (B), Etan/Veh/Veh (C), Veh/SLD/LPS (D), and Etan/SLD/LPS (E) were examined. A necrotic area is indicated with an arrow.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3.4.3 Effect of LPS on SLD metabolism in rats

SLD and its sulfone and sulfide metabolites were determined in rat plasma at various times after the second administration of SLD. Plasma SLD concentration reached a peak 1 hr after administration and decreased gradually over 8 hr (Fig. 3.3A). In LPS-treated rats, plasma SLD concentration was significantly smaller. SLD treatment increased SLD sulfone concentration in plasma steadily between 2- 8 hr (Fig. 3.3B). This increase was not observed after SLD/LPS cotreatment, so that the plasma concentration of SLD sulfone was significantly less in SLD/LPS-cotreated rats by 8 hr. Plasma SLD sulfide concentration reached a peak within 4 hr in both groups, and LPS administration decreased the SLD sulfide concentration in plasma significantly at 1, 2, 4 and 8 hr compared to that of SLD/vehicle-treated rats (Fig. 3.3C)

In livers of rats treated with SLD alone, the concentrations of SLD and its metabolites showed trends similar to those in plasma. LPS cotreatment decreased SLD and SLD sulfide concentrations, but SLD sulfone concentration was unaffected (Fig. 3.4). LPS selectively lowered the SLD concentration in liver at 1 and 2 hr, and decreased SLD sulfide concentration in liver at 2 and 4 hr.

To investigate further the effect of LPS on SLD metabolism, rats were euthanized at 2 hr and SLD metabolite concentrations were determined in the GI tract and feces collected between -0.5 and 2 hr. The concentrations of SLD and SLD sulfide in the GI tract and feces were significantly increased by LPS (Fig. 3.5). However, the SLD sulfone concentration was not affected by LPS.

Fig. 3.3. Effect of LPS on plasma concentrations of SLD, SLD sulfone and SLD sulfide. Rats were treated with SLD and with LPS or its saline vehicle as described in Fig. 3.1. They were euthanized, and plasma was collected at 0, 1, 2, 4 and 8 hr after the second administration of SLD. The plasma concentrations of SLD, SLD sulfone or SLD sulfide were determined as described in Methods. *significantly different from SLD/Veh group at the same time. $P<0.05$, n=5 for all groups except SLD/LPS at 4 hr (n=7).

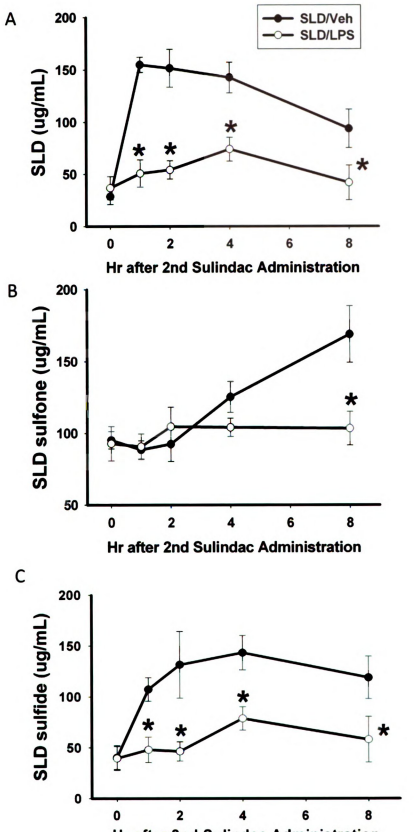
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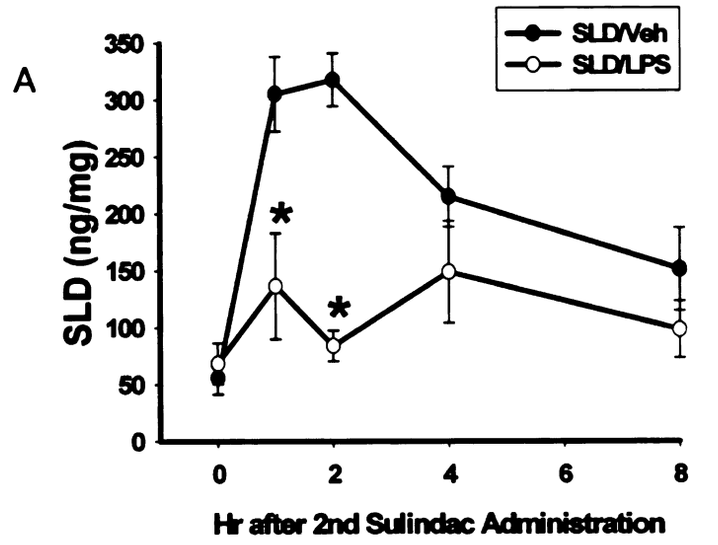
Fig. 3.4. Effect of LPS on liver concentrations of SLD, SLD sulfone and SLD sulfide. Rats were treated with SLD and with either LPS or its saline vehicle as described in Fig. 3.1. Liver concentrations of SLD, SLD sulfone and SLD sulfide were determined at 0, 1, 2, 4 and 8 hr after the second administration of SLD. *significantly different from SLD/Veh group at the same time. $P<0.05$, n=5 for all groups except SLD/LPS at 4 hr (n=8).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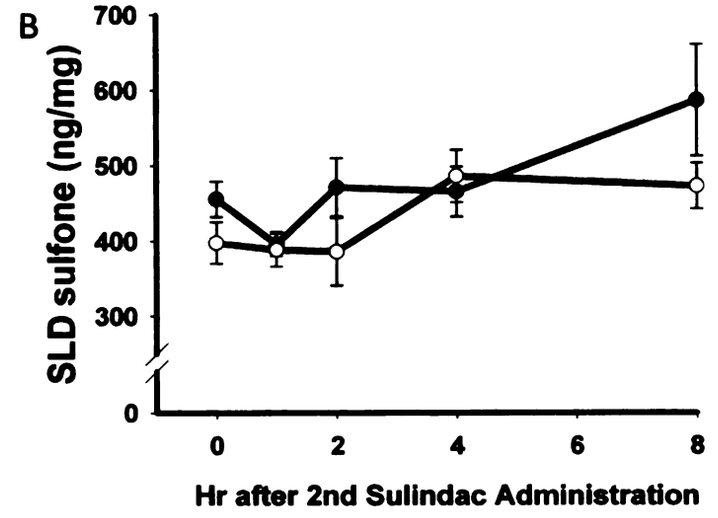
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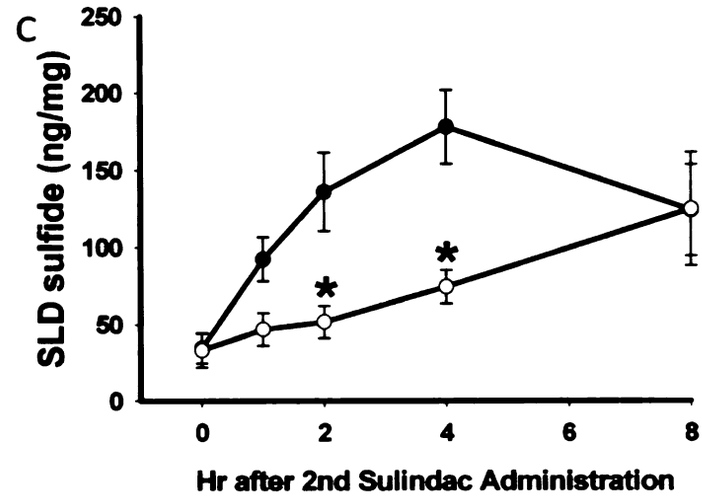
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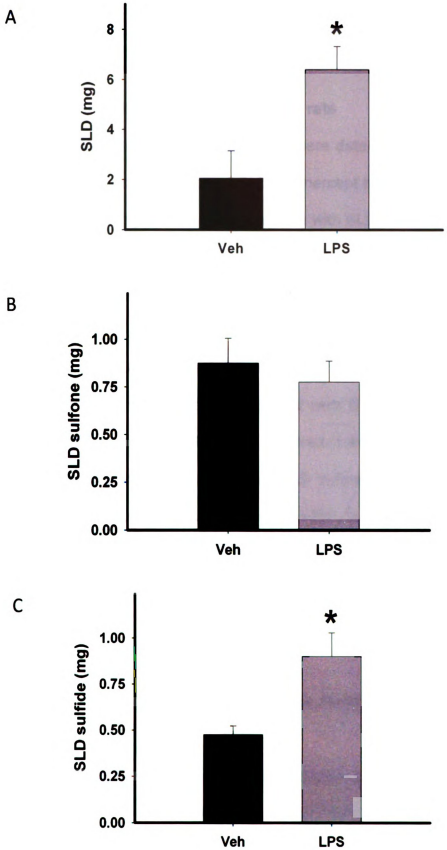
Fig. 3.5. Concentrations of SLD, SLD sulfone and SLD sulfide in GI tract and feces. Rats were treated with SLD and with either LPS or its saline vehicle as described in Fig. 3.1. Each rat was housed in a different cage after the LPS injection. Two hours after the second administration of SLD, feces in the cage and the whole GI tract and its contents were collected for each rat. The mixture was homogenized with acetonitrile, and the concentrations of SLD, SLD sulfone and SLD sulfide were determined by LC/MS/MS. *significantly different from SLD/Veh group. $P<0.05$, n=4.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The concentrations of acyl glucuronide conjugates of SLD, SLD sulfone and SLD sulfide were below the limit of detection in all of the samples measured.

3.4.4 Effect of etanercept on SLD metabolism in rats

SLD and its sulfone and sulfide metabolites were determined in rat plasma at 8 hr after the second administration of SLD. Etanercept had no effect on SLD metabolite concentration in plasma of rats cotreated with SLD/LPS (Table 3.1).

3.4.5 Cytotoxicity of SLD and its metabolites in HepG2 cells and rat primary hepatocytes

Neither SLD nor SLD sulfone at concentrations up to 500 μM led to an increase in released LDH when applied to HepG2 cells (Fig. 3.6A). In contrast, SLD sulfide induced significant LDH release at concentrations greater than 125 μM. In rat primary hepatocytes, SLD and SLD sulfone also produced no cytotoxicity at the concentrations examined (Fig. 3.6B), but SLD sulfide caused cell death at concentrations as small as 30 μM. The cytotoxicity of SLD sulfide was concentration-dependent, and 120 μM SLD sulfide killed almost all of the hepatocytes.

3.4.6 Effect of TNF on cytotoxicity of SLD and its metabolites in HepG2 and rat primary hepatocytes

TNF alone did not affect the release of LDH from HepG2 cells (Fig. 3.7). Neither SLD nor SLD sulfone was cytotoxic in the presence or absence of TNF.

Table 3.1. Effect of etanercept on SLD metabolism. Rats were killed and plasma was collected at 8 hr after the second SLD administration. Metabolite concentrations were determined as described under *Methods*. *n= 4-5.*

Treatment	Concentration in plasma (ug/mL)		
	SLD	SLD sulfone	SLD sulfide
Veh/SLD/LPS	59.6 ± 12.2	139.3 ± 16.7	62.2 ± 12.1
Etan/SLD/LPS	52.8 ± 10.8	153.8 ± 13.8	39.8 ± 13.3

Fig. 3.6. Evaluation of cytotoxicity induced by SLD, SLD sulfone or SLD sulfide. SLD, SLD sulfone or SLD sulfide was administered at various concentrations to HepG2 cells (A). The percentage of LDH released in the medium after 24 hr was determined as a marker of cytotoxicity. (B) Rat primary hepatocytes were treated with SLD, SLD sulfone or SLD sulfide for 8 hr, and the percentage of ALT activity released into medium was determined as described in Methods. *significantly different from vehicle (0 concentration). #significantly different from SLD or SLD sulfone at the same concentration. $P<0.05$, n=3.

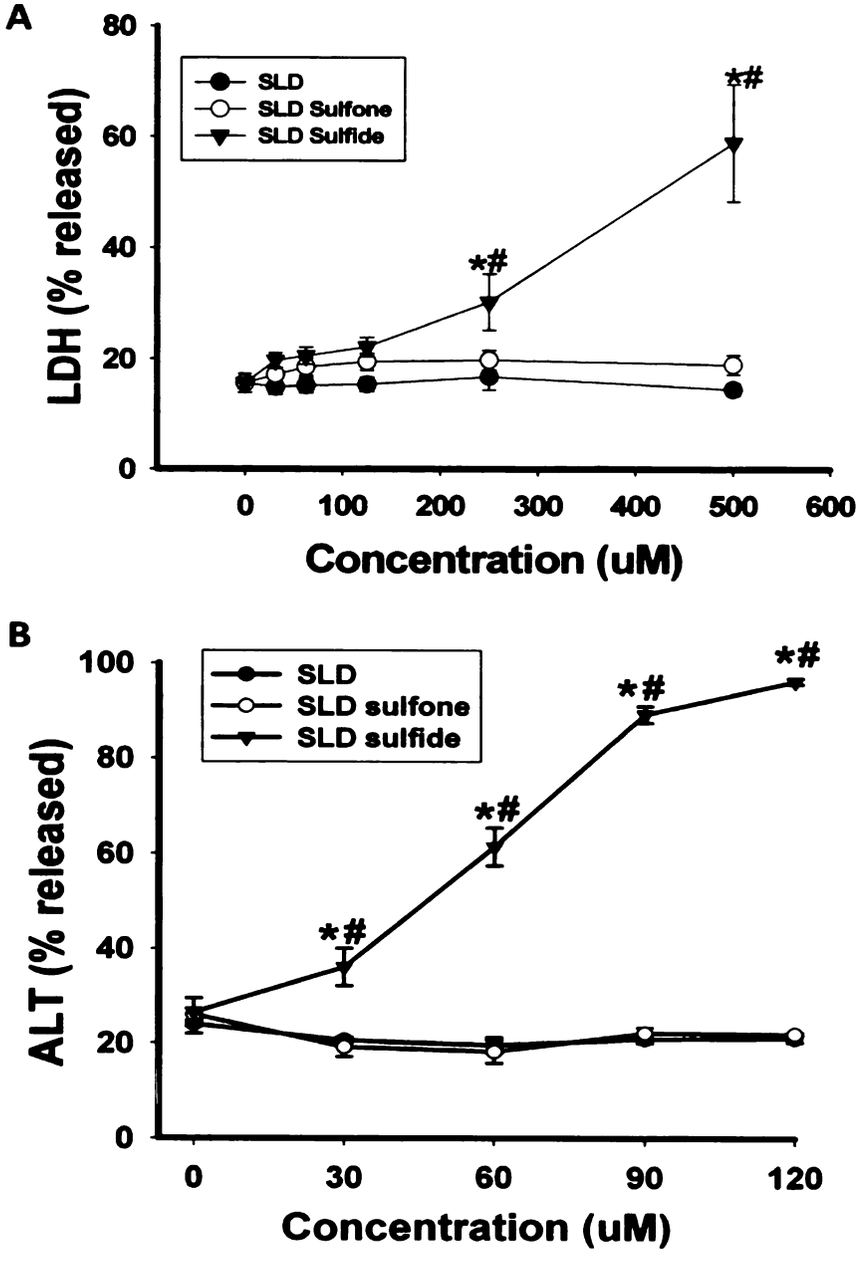
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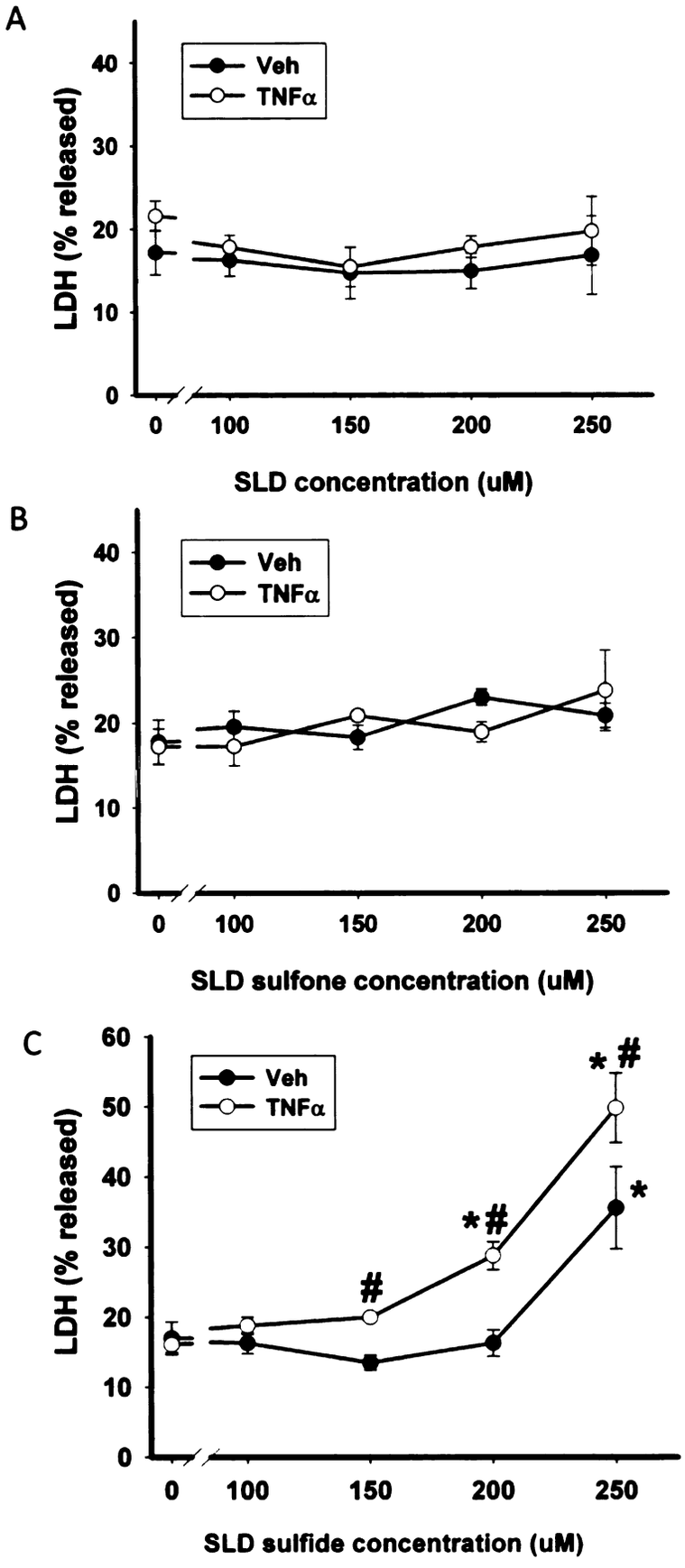
Fig. 3.7. Cytotoxicity induced by TNF and SLD or its metabolites. HepG2 cells were treated with SLD (A), SLD sulfone (B) or SLD sulfide (C) in the presence or absence of TNF (200ng/mL). The percentage of LDH released was determined after 24 hr as described in Methods. *significantly different from vehicle (0 concentration). #significantly different from value in the absence of TNF at the same concentration of SLD or metabolite. $P<0.05$, n=3.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Smaller concentrations (150, 200 μM) of SLD sulfide, which were not cytotoxic alone, induced cell death in the presence of TNF. TNF also enhanced the cytotoxicity of a larger concentration of SLD sulfide (250 μM).

The conversion of SLD to SLD sulfide is a reversible reaction (Duggan, 1981). To evaluate whether TNF affects metabolism of SLD sulfide in HepG2 cells, the amount of SLD sulfide in medium plus HepG2 cells was measured 24 hr after SLD sulfide application to the cells. This amount in wells with TNF (5.4±0.2 μg) was not significantly different from wells that received vehicle (5.3±0.1μg).

A potentiating effect of TNF was also observed on the cytotoxicity of SLD sulfide in primary hepatocytes. TNF alone did not affect ALT activity released into the culture medium compared to vehicle treatment. SLD sulfide alone caused significant release of ALT activity into the medium (Fig. 3.8). When hepatocytes were treated with SLD sulfide and TNF together, TNF significantly enhanced the cell injury induced by SLD sulfide.

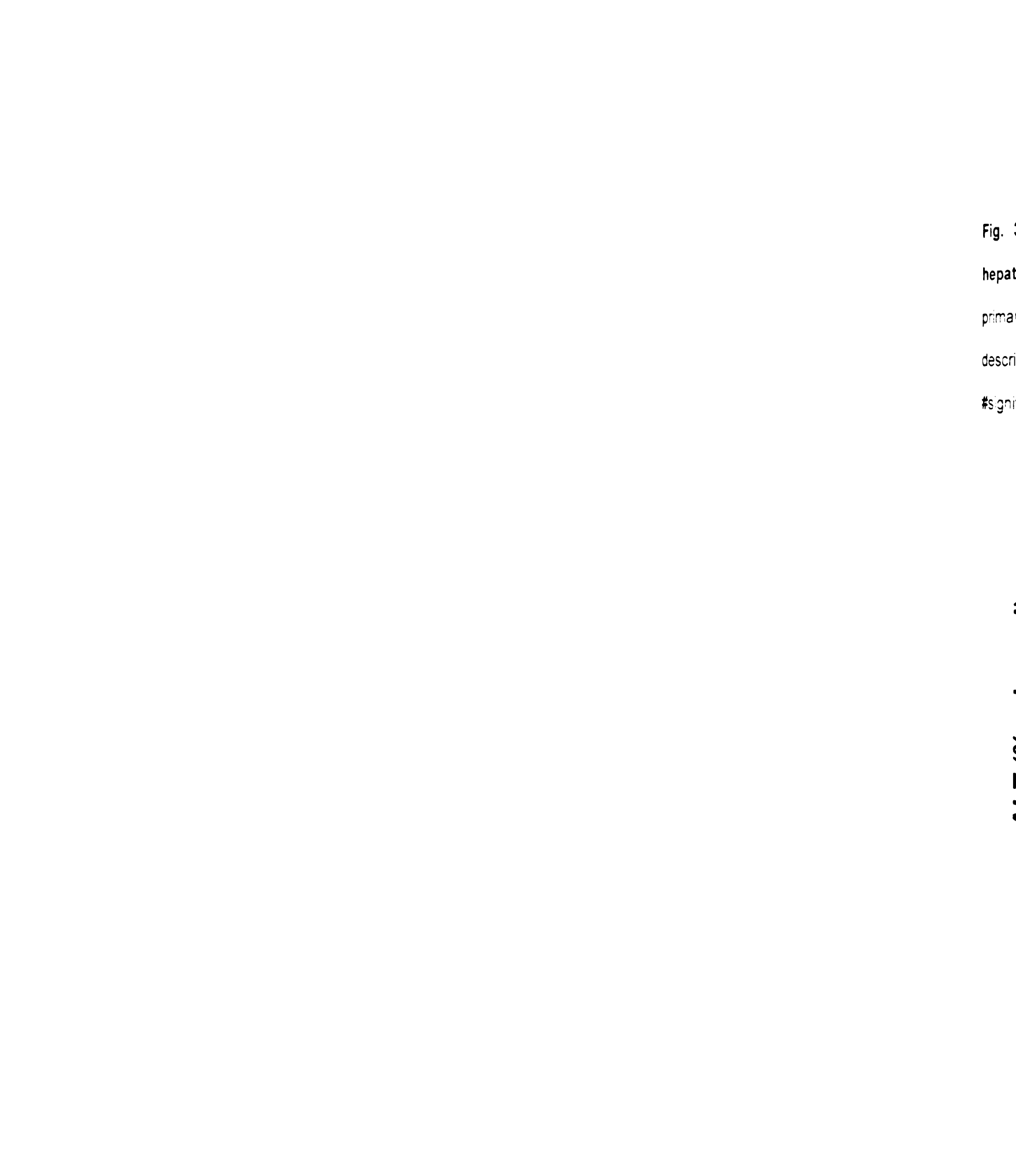

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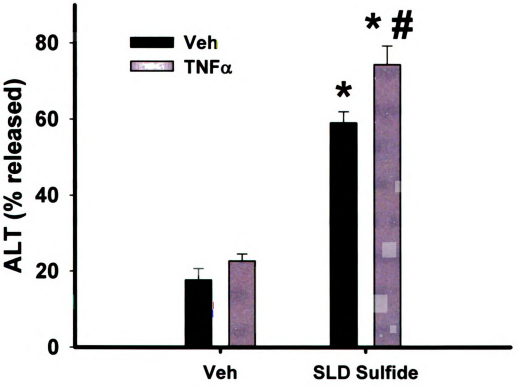
Fig. 3.8. Effect of TNF on SLD sulfide-induced injury to rat primary hepatocytes. SLD sulfide (60 μM) and/or TNF (2 μg/mL) was administered to rat primary hepatocytes. The percentage of LDH released was determined as described in Methods. *significantly different from corresponding vehicle group. #significantly different from SLD sulfide alone group. P<0.05, n=3.



3.5 Discussion

As reported previously (Zou et al., 2009b), SLD/LPS cotreatment induced severe liver injury in rats. Pro-inflammatory cytokines, especially TNF, have proved to play a critical role in other drug/LPS-induced liver injury models (Shaw et al., 2007; Tukov et al., 2007b). Moreover, studies suggest that reactive drug metabolites produced in liver are critical for idiosyncratic hepatotoxicity from some drugs (Kaplowitz, 2005). Therefore, we focused in this study on the roles of TNF and the toxic metabolite of SLD as well as their interaction in SLD/LPS-induced liver injury.

The concentration of TNF in serum was elevated in rats after exposure to LPS, and SLD significantly enhanced the LPS-mediated increase in TNF as early as 1 hr. Besides SLD, other drugs associated with idiosyncratic hepatotoxicity in humans, such as ranitidine and trovafloxacin, also had a synergistic effect on the LPS-mediated increase in TNF in rodents (Shaw et al., 2007; Tukov et al., 2007b). Sulindac and other NSAIDs enhanced TNF release from LPS-pretreated, macrophage-derived RAW264.7 cells at concentrations achieved clinically in humans (Cho, 2007). These findings suggest that enhancement of serum TNF concentration might be a common characteristic of drugs that induce idiosyncratic liver injury. The source of TNF and the mechanism by which SLD enhances TNF appearance are unknown. After LPS exposure, the increase in plasma TNF concentration is mirrored by elevated liver concentration (Fernandez-Martinez et al., 2004). Therefore, the source of TNF after LPS exposure is likely liver. However, the source of enhanced TNF in serum after

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SLD-cotreatment is not known. TNF-converting enzyme (TACE), which is required for release of biologically active TNF, is a possible contributor, since some NSAIDs can enhance the activity of this enzyme (Gomez-Gaviro et al., 2002). It is also possible that SLD or its metabolites enhances TNF transcription or translation or interferes with TNF clearance.

The importance of TNF in SLD/LPS hepatotoxicity was explored by pretreating rats with etanercept, a soluble receptor that neutralizes TNF. TNF inhibition protected against SLD/LPS-induced liver injury, suggesting a critical role for TNF in this model. However, elevation in TNF concentration alone is not sufficient to cause liver damage, since much larger TNF concentrations have failed to induced liver injury (Deng et al., 2008). Thus, additional factors are likely involved in liver toxicity in SLD/LPS-cotreated rats.

The requirement for bioactivation of SLD raises the possibility that LPS treatment leads to liver injury in SLD-treated rats by increasing the conversion of SLD to a toxic metabolite. To study the effect of LPS on SLD metabolism, we examined the concentration of SLD and its metabolites in plasma, liver, and GI tract plus feces. According to previous studies, two enzymes are responsible for SLD metabolism; methionine sulfoxide reductase (MSR) in both liver and gut flora reduces SLD to SLD sulfide, and a flavin-containing monooxygenase (FMO) converts SLD to SLD sulfone and also catalyzes the conversion of SLD sulfide to SLD. SLD was maximally absorbed in 1 hr, and SLD as well as its sulfone metabolite accumulated in liver, a result consistent with previous findings (Duggan et al., 1980). LPS can significantly down-regulate the expression of

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hepatic FMO in mice (Zhang et al., 2008). Oxidative stress, a possible consequence of LPS exposure, can increase the expression of MSR in bacteria (Vattanaviboon et al., 2005). Therefore, LPS might have an effect on shifting the metabolism of SLD towards SLD sulfide by regulating the expression of these two enzymes. However, LPS decreased the concentrations of SLD and SLD sulfide in plasma after the second administration of SLD. The liver concentrations of SLD and SLD sulfide were also decreased by LPS at 1 and 2 hr and 2 and 4 hr, respectively. These results suggested that LPS might decrease absorption of SLD from the GI tract. To address this possibility, we measured metabolite concentrations in the GI tract and feces at 2 hr, a time at which we found a significant decrease in both SLD and SLD sulfide in plasma and liver after LPS exposure (Fig. 3.5). LPS increased the concentration of SLD in the GI tract and feces, suggesting that LPS decreased the bioavailability of SLD by reducing its absorption. This result does not rule out the possibility that LPS has an effect on the expression of enzymes that metabolize SLD. Moreover, the SLD metabolite concentrations in the plasma of cotreated rats were not changed at 8hr by etanercept pretreatment, suggesting that TNF does not play a role in the ability of LPS to reduce SLD absorption.

The cytotoxicity of SLD and its metabolites were compared in both HepG2 cells and primary rat hepatocytes. SLD and SLD sulfone were not toxic to HepG2 cells even up to 500 μM, yet SLD sulfide showed significant toxicity. This result is consistent with previous findings, although different medium was used and a different cytotoxicity assay was performed (Leite et al., 2006). It also has been

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widely reported that SLD sulfide can induce apoptosis of other cancer cell lines (Kim et al., 2005; Bock et al., 2007), which raised interest in treating cancer with SLD. However, in this study, we found that the active metabolite of SLD was also cytotoxic to primary hepatocytes and that primary rat hepatocytes were more sensitive than HepG2 cells (Fig. 3.6). This might have implications for the use of SLD as an anticancer agent if normal host cells are more sensitive to the cytotoxic effects of SLD than are cancer cells.

Although the mechanisms of drug-induced idiosyncratic liver injury are still not clear, it is believed that accumulation of active metabolites in liver is an essential first step for many drugs (Watkins, 2005). Accordingly, excessive SLD sulfide in liver might be critical for SLD- induced idiosyncratic liver injury. This might be why two administrations of SLD were required in this model to effect hepatotoxicity. Interestingly, LPS decreased the concentration of SLD sulfide in the livers of rats, suggesting that SLD sulfide accumulation alone was not sufficient to induce liver injury, and that LPS might be activating pathways that enhance the toxicity of SLD sulfide, instead of increasing the concentration of SLD toxic metabolite.

Since TNF and SLD or its metabolites are both indispensable for the development of SLD/LPS-induced liver injury, we explored whether TNF acted synergistically with SLD or its metabolites using an in vitro system. Both HepG2 and primary rat hepatocytes were resistant to TNF toxicity. Even a much greater concentration of TNF than we used failed to kill HepG2 cells and primary rat hepatocytes (Adamson and Billings, 1992). SLD or SLD sulfone in combination

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with TNF was not cytotoxic; in contrast, this cytokine enhanced the toxicity of SLD sulfide to both cell types. There is evidence that SLD and TNF act synergistically to kill tumor cells in mice, which raised the possibility of using this combination of agents as a new anticancer therapy (Hiroshi Yasui, 2003). However, our results suggest that this therapy might also increase the chance of liver injury. The mechanism of SLD sulfide and TNF interaction is under investigation. TNF can lead either to hepatocyte proliferation through NF-kappaB activation or to activation of cell death signaling (Wullaert et al., 2007). SLD, and particularly SLD sulfide, are potent inhibitors of the NF-kappaB pathway through inhibition of IKappa kinase activity (Yamamoto et al., 1999b). It was reported that NF-kB plays an essential role in preventing TNF- induced cell death (Beg and Baltimore, 1996). As a result, it is possible that SLD sulfide sensitizes hepatocytes to TNF-induced cell death through inhibition of NF-kappaB prosurvival signaling. Moreover, SLD sulfide and TNF share a common toxic effect, which may add to enhance cell death. It has been reported that SLD sulfide can induce reactive oxygen species (ROS) in vitro (Sun et al., 2009) and lead to mitochondrial uncoupling (Leite et al., 2006). TNF can also cause the production of ROS (Schwabe and Brenner, 2006) and mitochondrial injury (Bradham et al., 1998). Therefore, ROS and mitochondria are two potential targets of interaction of SLD and TNF.

SLD sulfide and TNF are not the only mediators that contribute to hepatotoxicity in this model. Previously, we found that liver hypoxia is induced through the activation of the hemostatic system in SLD/LPS-cotreated rats and

that inhibition of coagulation protects from liver damage (Zou et al., 2009b). Hypoxia might contribute to liver injury through synergistic interplay with SLD sulfide. Furthermore, we cannot exclude the possible roles of other mediators. For example, proteases released from neutrophils are important in other drug/LPS models (Luyendyk et al., 2005; Deng et al., 2007a). The proinflammatory cytokine, interferon-gamma, has been shown to exacerbate TNF-induced cytotoxicity in hepatocytes (Adamson and Billings, 1993). These mediators might interact with SLD sulfide, TNF and/or hypoxia to promote liver injury.

In summary, SLD and LPS interact to produce liver injury in rats. The LPS-stimulated increase in the concentration of TNF in rat serum was enhanced by SLD, and this cytokine plays a critical role in the pathogenesis. SLD sulfide was more toxic than SLD or SLD sulfone in vitro. Although LPS cotreatment reduced the bioavailability of SLD and the production of toxic SLD sulfide, the synergy of this toxic metabolite with TNF was sufficient to cause liver injury in rats. Such synergistic interactions might be a trigger for idiosyncratic liver injury from SLD in humans.

CHAPTER 4

Wei Zou, Robert A. Roth, Husam S. Younis, Ernst Malle, and Patricia E. Ganey. The critical role of tumor necrosis factor-α- and plasminogen activator inhibitor-1- mediated neutrophil activation in a sulindac/lipopolysaccharide-model of idiosyncratic liver injury in the rat. (Submitted)

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4.1 Abstract

Previous studies indicated that lipopolysaccharide (LPS) interacts with the nonsteroidal anti-inflammatory drug sulindac (SLD) to produce liver injury in rats. In this study, the mechanism of SLD/LPS-induced liver injury was further investigated. Accumulation of polymorphonuclear neutrophils (PMNs) in the liver was greater in SLD/LPS-cotreated rats compared to those treated with SLD or LPS alone. In addition, PMN activation occurred specifically in livers of rats cotreated with SLD/LPS. We tested the hypothesis that PMNs and proteases released from them play critical roles in the hepatotoxicity. SLD/LPS-induced liver injury was attenuated by prior depletion of PMNs or by pretreatment with the PMN protease inhibitor, eglin C. Previous studies suggested that tumor necrosis factor-α (TNF) and the hemostatic system play critical roles in the pathogenesis of liver injury induced by SLD/LPS. TNF and plasminogen activator inhibitor-1 (PAI-1) can contribute to hepatotoxicity by affecting PMN activation and fibrin deposition. Therefore, we tested the role of TNF and PAI-1 in PMN activation and fibrin deposition in the SLD/LPS-induced liver injury model. Neutralization of TNF or inhibition of PAI-1 attenuated PMN activation. TNF had no effect on PAI-1 production or fibrin deposition. In contrast, PAI-1 contributed to fibrin deposition in livers of rats treated with SLD/LPS. In summary, PMNs, TNF and PAI-1 contribute to the liver injury induced by SLD/LPS cotreatment. TNF and PAI-1 independently led to PMN activation, which is critical to the pathogenesis of liver injury in SLD/LPS-treated rats. Moreover, PAI-1 contributed to liver injury by promoting fibrin deposition.

4.2 Introduction

We reported previously that SLD enhanced the LPS-induced elevation of serum TNF and plasma PAI-1 in rats (Zou et al., 2009b). TNF neutralization protected against liver injury in this model, suggesting that TNF plays an important role in the pathogenesis (Zou et al., 2009a). Fibrin deposition in liver sinusoids resulted from cotreatment and contributed to SLD/LPS-induced hepatotoxicity (Zou et al., 2009b). TNF and PAI-1 participate in other liver injury models by causing PMN activation and fibrin deposition (Deng et al., 2008). Therefore, we investigated the role of TNF and PAI-1 in mediating PMN accumulation and activation as well as fibrin deposition in livers of SLD/LPS-treated rats.

4.3 Materials and methods

4.3.1 Materials

LPS (Lot 075K4038) derived from *Escherichia coli* serotype O55:B5 with an activity of 3.3 X 10^6 endotoxin units (EU)/mg as well as SLD and its metabolites were purchased from Sigma-Aldrich (St. Louis, MO). Eglin C was provided by Novartis Pharm (Basel, Switzerland). PAI039 was purchased from Axon Medchem BV (Groningen, Netherlands).

4.3.2 Animals

Male, Sprague-Dawley rats (Crl:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250 to 370 g were used. Animals were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum. They were allowed to acclimate for 1 week in a 12 hr light/dark cycle prior to use in experiments. All procedures were approved by the MSU Committee on Animal Use and Care and complied with "Guide for the Care and Use of Laboratory Animals" published by the National Academy of Sciences.

4.3.3 Animal model and sample collection

The SLD/LPS-induced liver injury model was described previously (Zou et al., 2009b). Food was removed, and rats were given the first administration of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) 16 hr before the second administration of the same dose. LPS (8.25X 10^5 EU/kg, i.v.) or its

vehicle (saline) was administered half an hour before the second administration of SLD. Rats were anesthetized at various times after the second administration of SLD. Serum and plasma was prepared from blood withdrawn from the vena cava. Liver tissue from the left lateral lobe was collected and fixed in 10% buffered formalin for PMN staining. A portion of the left medial lobe of the liver was flash-frozen in isopentane for determination of hypochlorous acid (HOCl)-protein adduct staining as well as for fibrin deposition analysis.

4.3.4 Anti-PMN serum, eglin C, PAI039 and etanercept treatment protocols

In PMN depletion experiments, rabbit anti-PMN serum or normal rabbit serum control was diluted 1:1 in sterile saline and given to rats (0.5 ml per rat, i.v.) half an hour before the first administration of SLD. The efficacy of the anti-PMN serum in depleting PMNs has been demonstrated in previous studies (Deng et al., 2007b). A PMN protease inhibitor, eglin C (8 mg/kg, i.v.; kindly provided by Novartis Pharm AG, Basel, Switzerland) or its saline vehicle, was administered to rats 4, 6 and 8 h after the second administration of SLD. A PAI-1 inhibitor, PAI039 [{1-benzyl-5-[4-(trifluoromethoxy)phenyl]-1H-indol-3-yl}(oxo)acetic acid] (6 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) was administered to rats 1 hr after the second administration of SLD. Etanercept (8 mg/kg) or vehicle (sterile water) was given to rats subcutaneously one hour before LPS or its saline vehicle.

4.3.5 Evaluation of hepatotoxicity

The activity of alanine aminotransferase (ALT) in serum was used as a marker to assess injury to hepatic parenchymal cells. The assay was performed using a diagnostic kit from Thermo Fisher Scientific (Waltham, MA).

4.3.6 Determination of CINC-1, MIP-1α and PAI-1 concentrations in plasma

The concentrations of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-1α (MIP-1α) in plasma were estimated by multiplex ELISA. Specific antibody-coupled beads were purchased from Millipore Corp. (Billerica, MA). Functionally active PAI-1 was measured by ELISA using a commercially available test kit from Molecular Innovations, Inc (Southfield, MI).

4.3.7 Evaluation of liver PMN accumulation and activation

Paraffin-embedded liver tissue was cut into 6 μm-thick sections on which PMN immunohistochemistry was performed as described previously (Yee et al., 2003). Briefly, paraffin was removed with xylene, and liver sections were incubated with polyclonal rabbit anti-PMN IgG as first antibody, and then incubated with biotinylated goat anti-rabbit IgG, avidin-conjugated alkaline phosphatase, and Vector Red substrate to stain PMNs. The numbers of PMNs enumerated in 10 randomly selected, 400 X high power fields were averaged to assess PMN accumulation in the liver.

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reacts with proteins to form chloramines. These HOCl-protein adducts can be used as fingerprints (Malle et al., 2006) to directly assess activation of PMNs in liver tissues (Hasegawa et al., 2005; Deng et al., 2007b). Frozen liver sections fixed in 4% formalin for 10 min at room temperature were washed with phosphate-buffered saline (PBS) 3 times for 5 min each. The sections were blocked for 1 hr at room temperature with 3% [v/v] goat serum (Molecular Probes, Carlsbad, CA) in PBS, and then incubated for 2 hr at room temperature with a monoclonal antibody (clone 2D10G9, subtype IgG2bk; diluted 1:1 in 3% [v/v] goat serum) specific for HOCl-modified epitopes generated *in vivo* (Malle et al., 2006) and *in vitro* (Malle et al., 1995). After another 3 washes with PBS, slides were incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (diluted 1:500 in 3% [v/v] goat serum, Molecular Probes, Carlsbad, California). Ten pictures were taken of 200 X power, randomly selected fields using a fluorescence microscope, and the fraction of positive pixels was averaged for each slide (Deng et al., 2008).

4.3.8 Assessment of fibrin deposition in liver

The immunohistochemistry for cross-linked fibrin in liver was performed as described previously (Zou et al., 2009b).

4.3.9 Statistical analyses

Results are presented as means $\pm$ SEM. Student's t-test was performed on fibrin deposition data. For the rest of the studies, one way or two way analysis of

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variance (ANOVA) was applied for data analysis, as appropriate, and Student-Newman-Keuls test was used as a *post hoc* test to compare means. The criterion for statistical significance was $P < 0.05$.

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4.4 Results

4.4.1 Evaluation of PMN accumulation and activation in livers

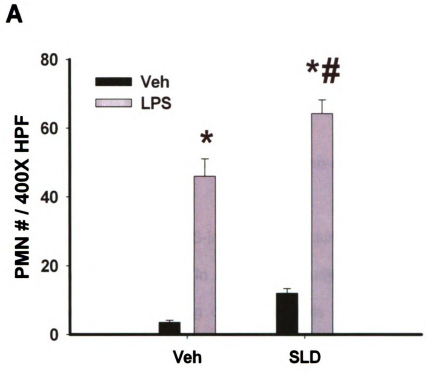
Liver injury induced by SLD/LPS occurs between 4 and 8 hr after the second administration of SLD, and ALT activity in rats significantly increases by 12 hr (Zou et al., 2009b). Accordingly, PMN accumulation was assessed in livers collected at 4 hr, a time before the onset of hepatocellular injury induced by SLD/LPS. SLD given alone had no significant effect on hepatic PMN number (Fig. 4.1A). An increase in PMN number was observed in livers of rats treated with LPS. PMN numbers were significantly greater in livers of rats cotreated with LPS and SLD compared to those treated with LPS alone.

HOCl-protein adducts are generated by the MPO-hydrogen peroxide-chloride system of activated PMNs (Malle et al., 2006), cells containing up to 5% MPO of total cell protein content. Adducts were not elevated in liver sections at 4 hr (not shown). However, at 8 hr pronounced formation of HOCl-modified epitopes was found in livers of rats treated with SLD/LPS, but not in rats treated with SLD or LPS alone (Fig. 4.1B). This result indicates that the MPO-hydrogen peroxide-halide system of PMNs was activated between 4 and 8 hr in the livers of rats treated with SLD/LPS.

4.4.2 Time course of changes in CINC-1 and MIP-1α concentrations in plasma

Plasma was collected from rats euthanized at various times (1, 4 and 12 hr), and the concentrations of CINC-1 and MIP-1α were measured. LPS increased

Fig. 4.1. Evaluaton of PMN accumulation and activation in rat livers. Rats were treated with two administrations of SLD (50 mg/kg, p.o.) or its vehicle (Veh, 0.5% methyl cellulose) with a 16 hr interval. LPS (8.25 X 10^5 EU/kg, i.v.) or its saline vehicle was administered half an hour before the second administration of SLD. (A) PMN staining was performed on livers collected 4 hr after the second administration of SLD. PMN number in 400 X high power fields (HPF) was counted to evaluate PMN accumulation. (B) HOCl-protein adduct staining was performed on slides of frozen liver collected at 8 hr. Ten random fields were photographed for every section, and the fraction of positive pixels was determined. *significantly different from respective group without LPS. #significantly different from Veh/LPS group. $P<0.05$, n=4-5.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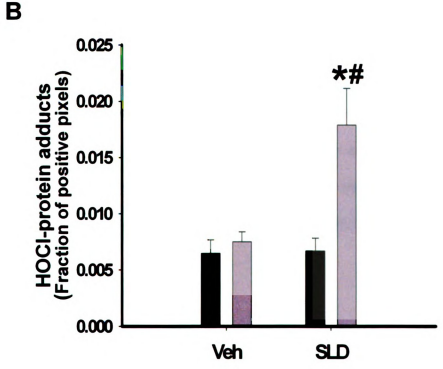
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CINC-1 and MIP-1α concentrations at 1 and 4 hr (Fig. 4.2). The concentrations of both chemokines had returned to baseline by 12 hr. SLD treatment had no effect on CINC-1 or MIP-1α concentrations in vehicle- or LPS-cotreated rats.

4.4.3 Effect of PMN depletion and PMN protease inhibition on SLD/LPS-induced liver injury

To assess the role of PMNs in SLD/LPS-induced liver injury, rabbit anti-PMN serum or normal serum was given to rats. In a previous study, anti-PMN serum selectively reduced PMNs without affecting other leukocyte numbers in blood (Deng et al., 2007b). Blood PMN number in the anti-PMN serum/SLD/LPS group (499 ± 23) was significantly smaller than that in the normal serum/SLD/LPS group (2726 ± 144) at 12 hr. Cotreatment with normal serum/SLD/LPS led to increased serum ALT activity (Fig. 4.3). Pretreatment with anti-PMN serum abolished the SLD/LPS-induced increase in ALT activity.

Eglin C is a potent and selective inhibitor of elastase and cathepsin G released by activated PMNs (Schnebli et al., 1985; Braun et al., 1987). Eglin C had no effect on ALT activity in serum of rats treated with vehicle but attenuated the elevation in serum ALT activity of rats treated with SLD/LPS (Fig. 4.4).

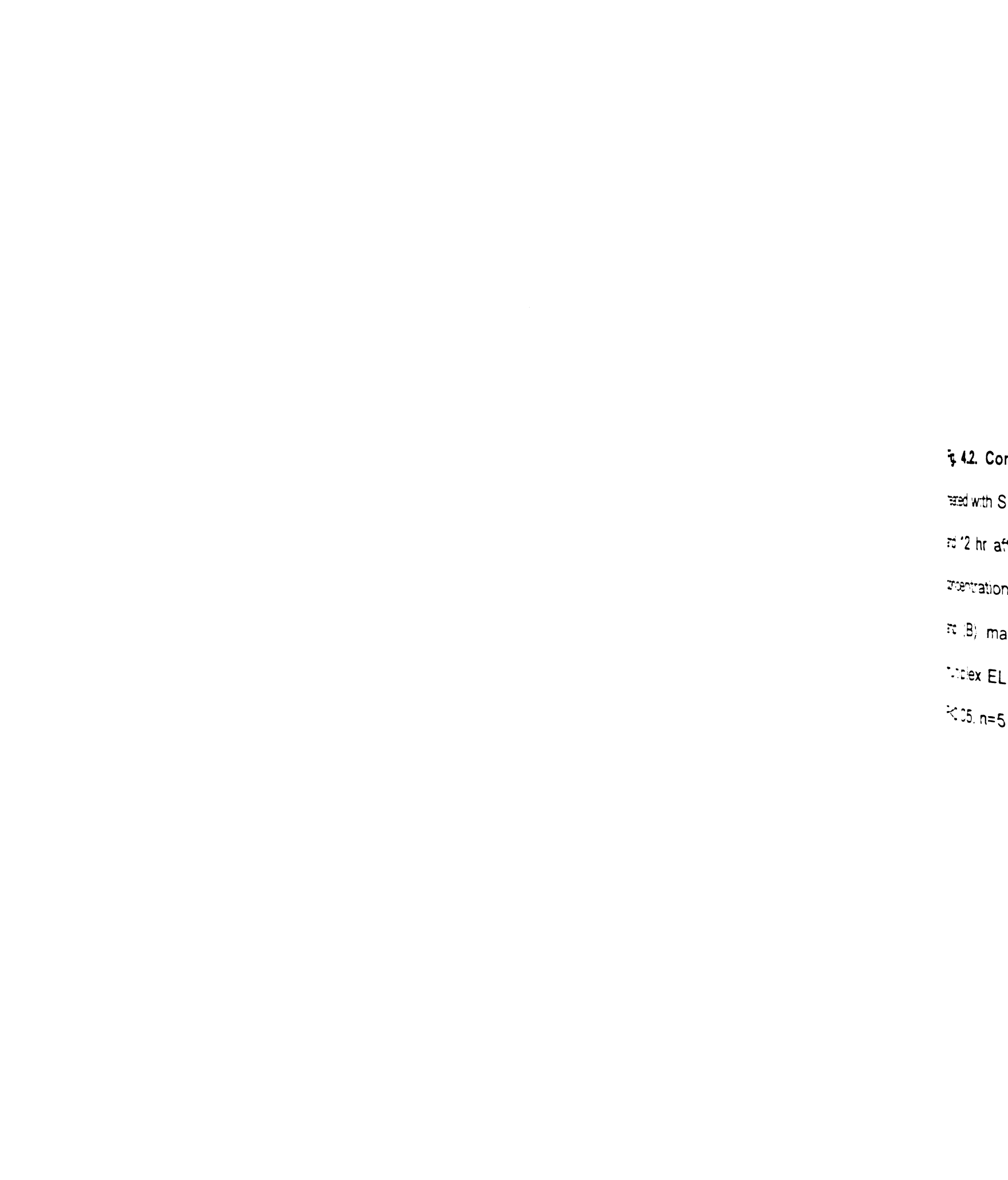

Fig. 4.2. Concentrations of PMN chemokines in rat plasma. Rats were treated with SLD and LPS or their vehicles (Veh) as described in Fig. 4.1. At 1, 4 and 12 hr after the second administration of SLD, plasma was collected and concentrations of (A) cytokine-induced neutrophil chemoattractant-1 (CINC-1) and (B) macrophage inflammatory protein-1α (MIP-1α) were evaluated by multiplex ELISA. *significantly different from Veh/Veh group at the same time. $P<0.05$, n=5.

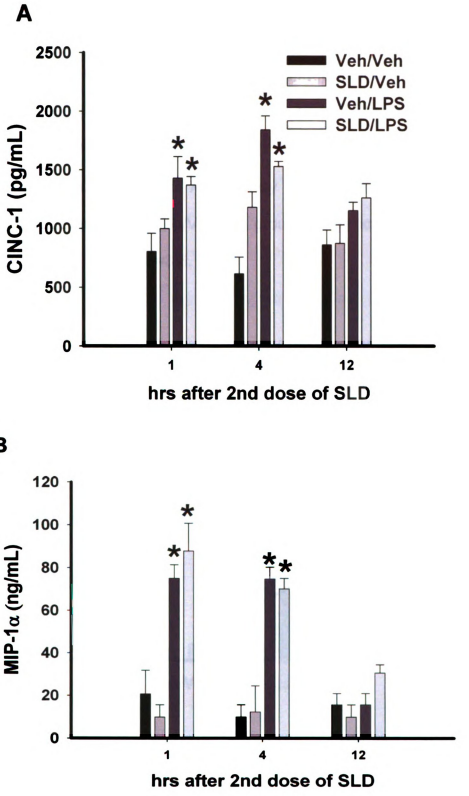
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Fig. 4.3. Effect of PMN depletion on SLD/LPS-induced liver injury. Rats were pretreated with either normal serum (NS) or rabbit anti-rat PMN serum (AS) half an hour before the first administration of SLD. Rats were euthanized 12 hr after the 2nd dose of SLD, and serum ALT activity was determined. Vehicle (Veh). *significantly different from Veh/Veh/NS group, #significantly different from SLD/LPS/NS group. P<0.05, n=3-6.

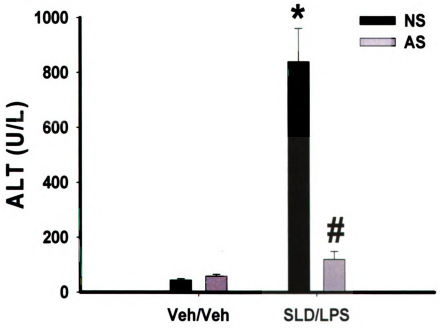
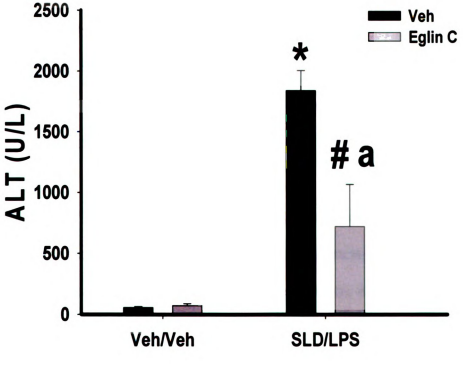


Fig.4.4. Effect of PMN protease inhibition on SLD/LPS-induced liver injury. Eglin C or its vehicle (Veh) was administered to rats 4, 6, and 8 hr after the 2nd administration of SLD. Rats were euthanized at 12 hr, and ALT activity in serum was determined. *significantly different from Veh/Veh/Veh. #significantly different from Veh/Veh/Eglin C group. [a]significantly different from SLD/LPS/Veh group. $P<0.05$, n=3-6.



4.4.4 Effect of TNF on PMN accumulation and activation

As noted above, cotreatment with SLD/LPS caused an increase in the number of PMNs in liver (Fig. 4.1A). Etanercept, which neutralizes TNF and inhibits its biological effects, did not affect PMN numbers in livers of rats treated with SLD/LPS (Fig. 4.5A). In contrast, etanercept prevented the elevation in SLD/LPS-induced formation of HOCl-protein adducts (Fig. 4.5B). These results suggest that TNF contributes to the release of cytotoxic factors from PMNs but not to PMN accumulation in the liver.

4.4.5 Role of PAI-1 in liver injury and accumulation and activation of PMNs

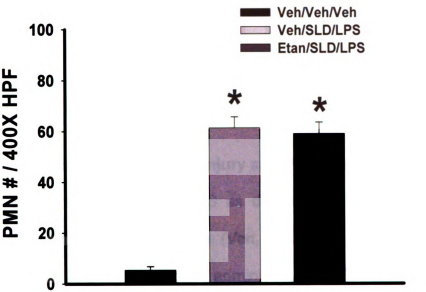
A previous study indicated that PAI-1 was selectively increased in the plasma of SLD/LPS- cotreated rats (Zou et al., 2009b); however, its role in liver injury has not been investigated. The PAI-1 inhibitor, PAI039, greatly attenuated liver injury, as followed by ALT measurements (Fig. 4.6a) induced by SLD/LPS cotreatment. PAI039 also reduced PMN activation (Fig. 4.6c) but not PMN accumulation at 8 hr (Fig. 4.6b).

4.4.6 Effect of TNF on plasma PAI-1 concentration

In previous studies, TNF was significantly increased as early as 1 hr by SLD/LPS cotreatment, and PAI-1 was increased by 8 hr (Zou et al., 2009b). To evaluate whether TNF regulates the production of PAI-1, plasma concentration of PAI-1 was evaluated in rats cotreated with etanercept. Etanercept given at a

Fig. 4.5. Effect of TNF inhibition on PMN accumulation and activation. Rats administered SLD/LPS were pretreated with etanercept or its vehicle (Veh) 1 hr before LPS. (A) PMN staining was performed on livers collected at 8 hr. The accumulation of PMNs in livers was evaluated by averaging PMN numbers in 10, randomly chosen, 400 X fields. (B) Quantification of HOCl-protein adducts in the livers of rats at 8 hr. *significantly different from Veh/Veh/Veh group. #significantly different from Veh/SLD/LPS group. $P<0.05$, n=3-6.

A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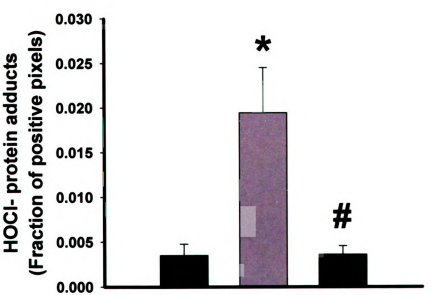
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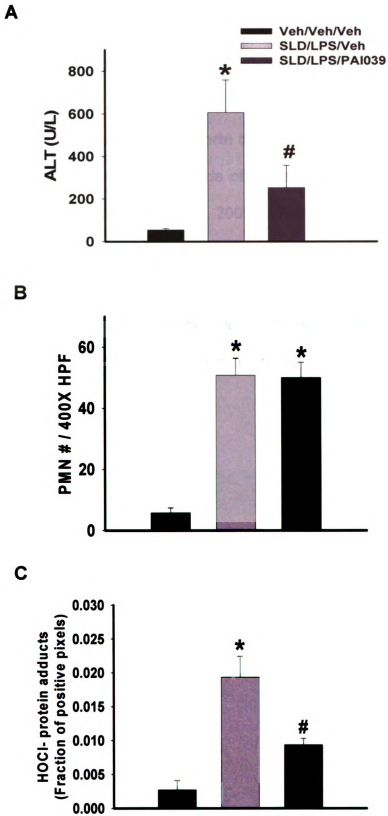
Fig. 4.6. Effect of PAI-1 inhibition on liver injury and PMN accumulation and activation. Rats were treated with SLD/LPS as described in Fig. 4.1. PAI-1 inhibitor, PAI039 (6 mg/kg, p.o.), or its vehicle (Veh, 0.5% methyl cellulose) was administered to rats at 1 hr after the second administration of SLD. Rats were euthanized at 12 hr to measure ALT activity (A) or at 8 hr to assess PMN accumulation (B) and activation (C). *significantly different from Veh/Veh/Veh. #significantly different from SLD/LPS/Veh group. $P<0.05$, n=4-16.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dose that protected against liver injury (Zou et al., 2009a) had no effect on the increase in plasma PAI-1 concentration caused by SLD/LPS (Fig. 4.7).

4.4.7 Effect of TNF and PAI-1 on fibrin deposition

Fibrin clots form in the sinusoids of livers of SLD/LPS-cotreated rats and result in hepatic hypoxia (Zou et al., 2009b). Both fibrin deposition and hypoxia were reduced by anticoagulant treatment, which protected against liver injury. Accordingly, we evaluated whether TNF or PAI-1 exerts its toxic effect by causing fibrin deposition in the liver. Etanercept had no effect on fibrin deposition caused by SLD/LPS cotreatment, whereas PAI039 reduced it (Fig. 4.8).

Fig. 4.7. Effect of TNF inhibition on plasma PAI-1 concentration. Rats were treated with etanercept (Etan), SLD and LPS or their vehicles (Veh) as described in the legend to Fig. 4.5. Plasma active PAI-1 concentration was determined at 8 hr. * significantly different from Veh/Veh/Veh. $P<0.05$, n=4.

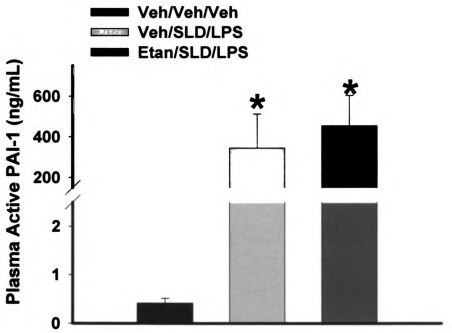
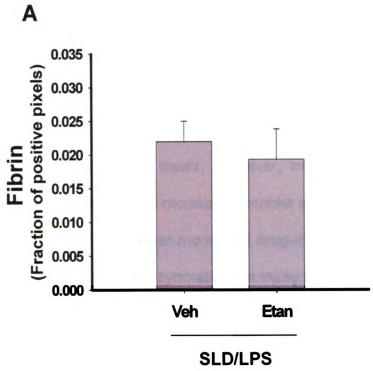


Fig. 4.8. Effect of TNF or PAI-1 inhibition on fibrin deposition in liver. Rats were treated with SLD/LPS and etanercept (Etan, A) or PAI-1 inhibitor (B) respectively. Fibrin deposition was evaluated at 8 hr. * significantly different from SLD/LPS/Vehicle (Veh). $P<0.05$, n=4-7.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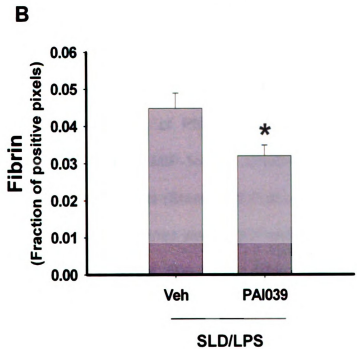
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4.5 Discussion

PMNs are a double-edged sword in the innate immune response to microbial infection and tissue trauma (Butterfield et al., 2006). Stimulated by inflammatory signals, PMNs attach to endothelial cells via adhesion molecules and transmigrate to the site of infection/trauma, where they become activated to release cytotoxic factors. PMNs can be beneficial by removing invading organisms and stimulating tissue repair. However, excessive PMN activation causes tissue injury in many animal models (Jaeschke et al., 1990; Hewett et al., 1992). PMNs are involved in several models of drug-induced liver injury and in drug-LPS interaction models of idiosyncratic liver injury (Deng et al., 2006; Deng et al., 2007b; Ramaiah and Jaeschke, 2007; Shaw et al., 2009d).

As has been reported, LPS administration causes PMNs to accumulate in liver. Although SLD mildly inhibited the adhesion of PMNs to nylon-wool columns in vitro (Venezio et al., 1985), it increased the LPS-induced PMN accumulation in liver before the onset of liver injury (Fig. 4.1A). Two PMN chemokines, MIP-1α and CINC-1, are potent inducers of PMN recruitment and extravasation. A neutralizing antibody to either MIP-1α or CINC-1 attenuated neutrophil sequestration in LPS-treated rodents (Standiford et al., 1995; Zhang et al., 1995). The concentrations of both chemokines were significantly increased in plasma by LPS, whereas SLD had no effect (Fig. 4.2). Thus, both chemokines might contribute to PMN accumulation in livers of LPS-treated rats; however, other factors must be involved after SLD/LPS cotreatment. The reason why SLD enhanced PMN accumulation is unknown, but some possibilities arise from

previous results. It is known that SLD/LPS cotreatment caused fibrin deposition in the liver (Zou et al., 2009b). It is possible that entrapment of PMNs in the meshwork of sinusoidal fibrin occurred. In addition, as a result of fibrin clots in sinusoids hypoxia occured in livers of cotreated rats (Zou et al., 2009b). Hypoxia can enhance the adherence of PMNs to human endothelial cells in vitro (Milhoan et al., 1992), and such an effect might further explain the SLD-induced increase in PMN accumulation.

Generally, PMNs that sequester in the liver are not injurious unless extravasation of them into the parenchyma and activation occur (Chosay et al., 1997). Although PMNs accumulated in livers in rats treated only with a small dose of LPS (Fig. 4.1), staining for HOCl-modified proteins, specific markers for neutrophil-induced oxidant stress, suggested no activation of PMNs. SLD/LPS cotreatment, however, increased HOCl-protein adducts, indicative that the MPO-hydrogen peroxide-chloride system of PMNs becomes activated between 4 and 8 hr, when the onset of liver injury occurred. Our observations parallel recent findings in patients with steatohepatitis in which liver chemokine expression was higher in patients with MPO-mediated oxidation products and correlated with hepatic neutrophil sequestration (Rensen et al., 2009). The role of PMNs in SLD/LPS-induced liver damage was further tested using anti-PMN serum, which markedly reduced PMNs in the circulation. The protection by anti-PMN serum shows that PMNs are critical to the development of SLD/LPS-induced liver injury (Fig. 4.3).

Activated PMNs release various lysosomal hydrolases including serine proteases, among which elastase and cathepsin G have been identified as primary mediators in hepatocyte killing by PMNs in vitro (Ho et al., 1996). Eglin C is an inhibitor of elastase and cathepsin G. It attenuated liver injury (Fig. 4.4), suggesting that PMN proteases also play a role in the pathogenesis. Compared to the complete protection by PMN depletion, eglin C incompletely reduced SLD/LPS-induced liver injury; thus, the proteases released from PMNs might not be the only PMN-derived mediators contributing to the pathogenesis. In numerous inflammatory liver injury models, antioxidants attenuated PMN-mediated liver injury in vivo (Liu et al., 1995; Jaeschke and Smith, 1997). Our results to date cannot rule out a role for reactive oxygen species other than HOCl, but this is a topic of current investigation.

TNF neutralization significantly attenuated liver injury induced by SLD/LPS in vivo (Zou et al., 2009a). In addition, TNF directly interacted with SLD sulfide to kill hepatocytes in vitro. TNF can also activate endothelial cells to promote PMN migration (Smart and Casale, 1994). In results presented here, the number of PMNs sequestered in the liver was not affected by TNF neutralization, but TNF neutralization did reduce PMN activation in the liver (Fig. 4.5). These results suggest that TNF contributes to PMN activation, but not to hepatic accumulation of these cells.

Like PMN depletion, anticoagulation using heparin abolished the hepatotoxicity induced by SLD/LPS cotreatment of rats (Zou et al., 2009b), which suggests that there is an interaction between PMNs and the hemostatic system

in the pathogenesis. Hemostatic factors including thrombin and PAI-1 were increased in plasma by SLD/LPS cotreatment (Zou et al., 2009b). Interestingly, hemostatic factors can bind to PMNs and influence their accumulation and activation (Gillis et al., 1997). For example, thrombin can rapidly trigger lysozyme release from human PMNs and promote PMN activation in perfused rat liver after LPS exposure (Baranes et al., 1986; Copple et al., 2003).

PAI-1 is an inhibitor of plasminogen activator and a key negative regulator of fibrinolysis. PAI039, a PAI-1 inhibitor, significantly attenuated SLD/LPS-induced liver injury, suggesting that PAI-1 is a mediator of pathogenesis (Fig. 4.6A). PAI039 decreased fibrin deposition in livers of SLD/LPS-treated rats (Fig. 4.8B), which suggests that PAI-1 contributes to fibrin deposition in the SLD/LPS model.

In addition to inhibiting fibrinolysis, PAI-1 can regulate PMN migration and potentiate LPS-induced PMN activation through a c-Jun N-terminal kinase-mediated pathway (Kwak et al., 2006; Roelofs et al., 2009). Consistent with these findings, PAI-1 inhibition reduced HOCl-protein adduct staining in livers of SLD/LPS-cotreated rats (Fig. 4.6C), suggesting that PAI-1 is involved in PMN activation. Therefore, PAI-1 contributed to both PMN activation and fibrin deposition. It can also play a proinflammatory role by stimulating the production of cytokines and chemokines. For example, in a murine model of trovafloxacin/LPS-induced liver injury, PAI-1 knockout markedly decreased the plasma concentrations of interleukin-1β, interleukin-10, keratinocyte chemoattractant and monocyte chemoattractant protein-1, respectively (Shaw et al., 2009c). Whether PAI-1 similarly regulates the production of chemokines in

SLD/LPS- treated rats and the role of these cytokines in PMN activation are topics for further investigation.

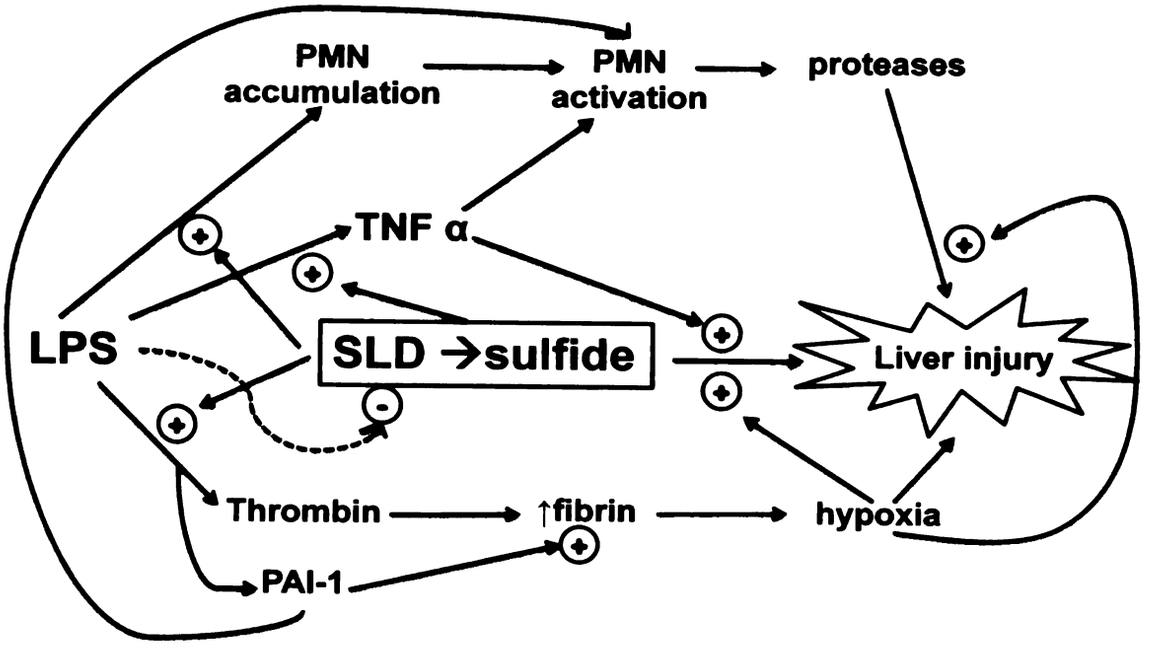
PMNs can exacerbate fibrin deposition by releasing proteases (Deng et al., 2007b). For example, proteases from PMNs can release PAI-1 from endothelial cells and platelets and thereby inhibit fibrinolysis (Pintucci et al., 1992). Eglin C treatment significantly decreased active PAI-1 concentration and fibrin deposition in a model of ranitidine/LPS-induced liver injury (Deng et al., 2007b). SLD/LPS cotreatment led to fibrin deposition at 4 hr, before the activation of PMNs at 8 hr. Therefore, PMNs do not contribute to the initial formation of fibrin, but proteases released by activated PMNs might prolong fibrin deposition.

The concentrations of PAI-1 and TNF in blood were both significantly greater in SLD/LPS-treated rats than in rats treated with either LPS or SLD alone. The peak of PAI-1 (4 hr) in plasma followed the peak of TNF production (i.e.,1 hr; Zou et al., 2009b). Although it has been reported that both TNF and LPS lead to PAI-1 release from endothelial cells in vitro (Riedo et al., 1990), inhibition of TNF did not decrease PAI-1 concentration in SLD/LPS-cotreated rats (Fig. 4.7). This indicates that PAI-1 production does not depend on TNF in this model. Consistent with this result, TNF did not affect fibrin deposition in liver (Fig. 4.8). Thus, the activation of hemostatic system is likely a direct effect of LPS but not mediated through TNF in this model. In contrast, TNF does mediate hemostatic system activation in ranitidine/LPS- or trovafloxacin/LPS-induced liver injury (Tukov et al., 2007a; Shaw et al., 2009e). Therefore, these results suggest that

TNF does not contribute to liver injury through the same mechanism in all drug/LPS interaction models.

From results of this and previous studies, we can summarize mechanisms of liver injury induced by SLD/LPS cotreatment (Fig. 4.9). Various mediators including TNF, hypoxia caused by hemostatic system activation and PMNs play critical roles in the pathogenesis of SLD/LPS-induced liver injury. SLD enhances TNF elevation induced by LPS. SLD/LPS cotreatment also leads to the production of hemostatic factors including thrombin and PAI-1, both of which contribute to fibrin clot formation in liver sinusoids (Zou et al., 2009b). As a result, the liver becomes hypoxic. Although the concentration of the toxic metabolite, SLD sulifide, is decreased by LPS in livers and plasma of rats, it synergistically kills hepatocytes in the presence of TNF and hypoxia (Zou et al., 2009a). PMNs are another critical player in SLD/LPS-induced liver injury (Fig. 4.3). PMN accumulation in the liver was primarily induced by LPS and this effect was enhanced by SLD (Fig. 4.1A). Activation of PMNs was observed in livers of rats treated with SLD/LPS (Fig. 4.1B). Both TNF and PAI-1 contribute to PMN activation independently (Fig. 4.5 to 4.7). When activated, PMNs release proteases which induce liver injury by interacting with hypoxia (Luyendyk et al., 2005). In summary, the studies presented here further our understanding of the roles of various mediators and their interaction in this SLD/LPS-induced idiosyncratic liver injury model.

Fig. 4.9. Mechanisms of SLD/LPS-induced liver injury. See text for details



CHAPTER 5

Wei Zou, Robert A. Roth, Husam S. Younis, Lyle D. Burgoon, and Patricia E. Ganey. Oxidative stress is an important player in the pathogenesis of liver injury induced by sulindac and lipopolysaccharide cotreatment

5.1 Abstract

Among all the nonsteroidal anti-inflammatory drugs, sulindac (SLD) is associated with the greatest incidence of idiosyncratic hepatotoxicity in humans. Previously, an animal model of SLD-induced idiosyncratic hepatotoxicity was developed by cotreating rats with a nonhepatotoxic dose of LPS. Tumor necrosis factor-alpha (TNF) was found to be critically important to the pathogenesis. In this study, we further explored the mechanism of liver injury induced by SLD/LPS cotreatment by analyzing gene expression in livers of rats before the onset of liver injury. The results suggested that oxidative stress might be a potential mediator. Moreover, protein carbonyls, products of oxidative stress, were elevated in liver mitochondria of SLD/LPS-cotreated rats. Antioxidant treatment with either ebselen or dimethyl sulfoxide attenuated SLD/LPS-induced liver injury. The role of oxidative stress was further investigated in vitro. SLD sulfide, the toxic metabolite of SLD, enhanced TNF-induced cytotoxicity and caspase 3/7 activity in HepG2 cells. SLD sulfide increased dichlorofluorescein fluorescence in HepG2 cells, suggesting generation of reactive oxygen species (ROS). Hydrogen peroxide and TNF cotreatment caused greater cytotoxicity than either treatment alone. Either antioxidant tempol or a pancaspase inhibitor Z-VAD-FMK decreased HepG2 cell death as well as caspase 3/7 activity induced by SLD sulfide/TNF coexposure. These results indicate that SLD/LPS treatment causes oxidative stress in livers of rats and that reactive oxygen species are important in the cytotoxic interaction of SLD and TNF by activating caspase 3/7.

5.2 Introduction

Reactive oxygen species (ROS) include oxygen free radicals and other nonradical but highly reactive molecules (e.g., hydrogen peroxide). Excessive generation of ROS tilts the balance between prooxidant and antioxidant influences in the cell and results in oxidative stress. ROS can directly oxidize proteins, DNA or membrane lipids in target cells, and such effects can result in cell death. One ROS-mediated pathway of cell death is through caspase-dependent intracellular apoptotic signaling initiated by oxidative stress (Jones et al., 2000). The oxidative stress in liver can be induced under various conditions that include consumption of ethanol or other drugs that cause inflammatory stress (Galati et al., 2002; Choi and Ou, 2006; Cederbaum et al., 2009).

In this study, gene expression was analyzed in livers from rats treated with sulindac and/or LPS. The results of Ingenuity pathway analysis of SLD/LPS-specific gene expression profiles pointed to the occurrence of oxidative stress. We tested the hypothesis that oxidative stress plays a role in the pathogenesis of liver injury induced by SLD/LPS in vivo. In hepatocytes, we evaluated the ability of SLD and its metabolites to prompt ROS generation and explored its role in cytotoxicity.

5.3 Materials and methods

5.3.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The LPS (Lot 075K4038) used in animal experiments was derived from *Escherichia coli* serotype O55:B5 and had an activity of 3.3×10^6 endotoxin units (EU)/mg. HepG2/C3A cells were obtained from American Type Culture Collection (Manassas, VA).

5.3.2 Animals

Male, Sprague-Dawley rats (Crl:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250 to 370 g were used in this study. They were allowed to acclimate for 1 week in a 12-hr light/dark cycle prior to use in experiments. Animals were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to spring water *ad libitum*. Experimental procedures complied with "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences).

5.3.3 Design of experiments in vivo

As described in a previous study (Zou et al., 2009b), rats were given the first administration of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose), and food was removed at this time. Sixteen hours later, SLD at the same dose or its vehicle was administered to rats. LPS (8.25×10^5 EU/kg, i.v.) or its vehicle

(saline) was administered via a tail vein half an hour before the second administration of SLD. As reported previously, this protocol results in liver injury in the SLD/LPS-cotreated rats (Zou et al., 2009b). At various times (4, 8 or 12 hr) after the second administration of SLD, rats were anesthetized with isoflurane, and blood was drawn from the vena cava. Serum was prepared from clotted blood. A portion of the right medial lobe of the liver was flash-frozen in liquid nitrogen for RNA extraction. Another portion was collected and cooled in ice-cold isolation buffer for mitochondrial preparation. In experiments designed to evaluate the effect of antioxidants on liver injury, ebselen (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) was given to rats 1.5 hr before the second administration of SLD; dimethyl sulfoxide (0.3 mL/g, i.p.) was given to rats at the same time as the administration of SLD.

5.3.4 Gene expression analysis

Total RNA was extracted from frozen liver tissue collected at 4 hr using a kit purchased from Qiagen Inc. (Valencia, CA) as described previously (Younis et al., 2006). Microarray analysis was performed using the standard protocol provided by Affymetrix, Inc. (Santa Clara, CA). Total RNA (10 μg) was reverse transcribed into cDNA in the presence of oligo dT primer using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). cDNA was purified, and biotin-labeled cDNA was synthesized using the Enzo RNA Transcript Labeling Kit (Affymetrix, Santa Clara, CA). After the labeled cDNA was purified and its quality was evaluated, cDNA was hybridized to a Rat Genome 230 2.0

Array (Affymetrix), which comprised more than 31,000 probe sets. The array was stained with streptavidin-phycoerythrin (Invitrogen, Carlsbad, CA) and scanned to generate signal intensity files.

Data normalization was performed using SAS version 9.1 on scanned image files (Eckel et al., 2005). Empirical Bayes analysis was used to calculate posteriori probabilities (P1(t) value) (Eckel et al., 2004). Ratios of gene expression were calculated by comparing SLD/Veh, Veh/LPS or SLD/LPS groups to the Veh/Veh group.

5.3.5 Evaluation of liver injury and protein carbonyls in mitochondria

Liver injury was assessed by measuring the activity of alanine aminotransferase (ALT) in serum using a diagnostic kit from Thermo Corp (Waltham, MA).

Mitochondria were isolated from livers of rats treated with SLD/LPS or vehicles at 8 hr using a mitochondrial isolation kit (Sigma, St. Louis, MO). Briefly, fresh livers (50 mg) were collected and homogenized in HEPES buffer containing 200 mM mannitol, 70 mM sucrose, and 1 mM EGTA. Liver homogenates were centrifuged at 600xg for 5 minutes. The supernatant fraction was transferred into a new tube and centrifuged at 11,000xg for 10 minutes. The pellets were washed and centrifuged at 11,000xg for 10 minutes again. The mitochondrial pellets were resuspended in HEPES for the evaluation of membrane potential or protein carbonyl concentration. Protein carbonyl concentration in liver mitochondria was

measured using a commercially available kit purchased from Cayman Chemical (Ann Arbor, MI).

5.3.6 Evaluation of mitochondrial membrane potential

The effect of SLD and its metabolites on mitochondrial membrane potential was evaluated using JC-1 mitochondrial membrane potential assay kit from Cayman Chemical (Ann Arbor, MI). Mitochondria isolated from normal rats (2 ug protein) were incubated with various concentrations of SLD or its metabolites at a final volume of 200 uL. After 30 min, the JC-1 dye solution (0.2 uL) was added. Fluorescence was read for JC-1 agglomerates and monomers respectively. The ratio of JC-1 agglomerates (excitation/emission=560/595 nm) to monomers (excitation/emission= 485/535 nm) in mitochondria was calculated, and the data were expressed as a percentage of vehicle control (0.5% dimethyl sulfoxide). The decrease in ratio of JC-1 agglomerates to monomers was associated with a decrease in membrane potential (Reers et al., 1995).

5.3.7 Evaluation of reactive oxygen species in HepG2 cells

ROS in HepG2 cells were assessed using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) purchased from Invitrogen, Inc. (Carlsbad, CA). HepG2 cells (4×10^5 cells/mL) in suspension were incubated with 10uM CM-H2DCFDA in Dulbecco's Modified Eagle's medium (DMEM) for 45 min. The cells were washed twice with DMEM with 10% fetal bovine serum. Cells were plated in 96-well plates and treated with

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250 uM SLD sulfide or its dimethyl sulfoxide (DMSO) vehicle in the presence or absence of recombinant human TNF (200 ng/ml) dissolved in DMEM vehicle. DCF fluorescence intensity was read at 0, 0.5, 1 and 3 hr after treatment.

5.3.8 Evaluation of cytotoxicity and capase 3 activity

HepG2 cells were plated in 96-well plates at a density of 4×10^5 cells/mL in DMEM with 10% fetal bovine serum. After overnight incubation, medium was renewed, and HepG2 cells were treated with tempol (0.2- 1 M) or with the pancaspase inhibitor, Z-VAD-FMK (Z-VAD,10uM). Half an hour later, SLD sulfide (200- 250 uM), hydrogen peroxide (0.2- 2 mM), TNF (200 ng/mL) or their vehicles were added depending on the purpose of the experiment. The percentage of LDH released was evaluated at 24 hr as described previously (Zou et al., 2009a). Caspase 3/7 activity was evaluated at 6 hr after treatment in HepG2 cells using a Caspase-Glo 3/7 assay kit (Promega, Madison, WI)

5.3.9 Statistical analyses

Results are expressed as means $\pm$ S.E.M. One-way or two-way analysis of variance was applied for data analysis as appropriate, and Student-Newman-Keuls test was used as a *post hoc* test to compare means. For all studies, the criterion for statistical significance was $P < 0.05$. For gene array analysis, empirical Bayes analysis was used and posterior probability (P1(t)- value) > 0.9 was set as the criterion for significance.

5.4 Results

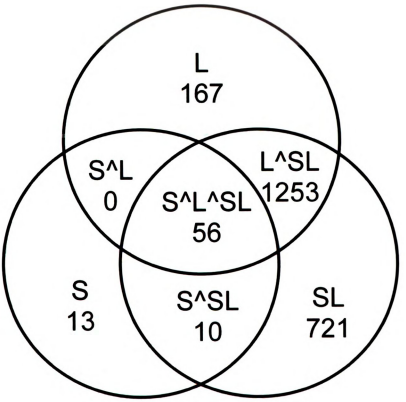
5.4.1 Gene expression changes regulated by treatment with SLD, LPS or SLD/LPS

In previous studies, neither LPS nor SLD was hepatotoxic when given alone; however, cotreatment with SLD/LPS caused severe liver injury in rats (Zou et al., 2009b). SLD/LPS-induced liver injury occurred between 4-8 hr after the second administration of SLD. Genes regulated by SLD/LPS at the onset of liver injury i.e., 4 hr, might be involved in the pathogenesis. Thus, livers were collected 4 hr after treatment with SLD and/or LPS or their vehicles, and gene expression was analyzed. To compare the number of gene expression changes (relative to Veh/Veh) caused by treatment with SLD/Veh, Veh/LPS or SLD/LPS, a Venn diagram was generated (Fig. 5.1). A large number of genes (1476) were changed by LPS administration. In contrast, SLD only caused a small number of genes expression changes (79). SLD/LPS cotreatment led to expression changes in 2040 genes. Not surprisingly, most of the gene expression changes caused by LPS (1309/1476) were represented in the genes regulated by SLD/LPS cotreatment. However, there were 721 genes regulated by SLD/LPS that were not affected by either SLD or LPS treatment alone (presented in appendix).

5.4.2 Gene expression changes specifically regulated by SLD/LPS point to oxidative stress

SLD/LPS was the only treatment that resulted in liver injury (Zou et al., 2009b). Accordingly, some of the 721 genes selectively regulated by SLD/LPS

Fig. 5.1. Venn diagram of probe sets regulated by SLD/Veh, Veh/LPS or SLD/LPS. Rats were treated with two administrations of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) with a 16 hr interval (n=5). Half an hour before the second administration of SLD, LPS (8.25X 10^5 EU/kg, i.v.) or its saline vehicle was administered via a tail vein. Livers were collected from rats euthanized at 4 hr after the second administration of SLD. RNA was isolated from liver, and gene expression was evaluated as described in Materials and Methods. The numbers of probe sets changed by SLD/Veh, Veh/LPS or SLD/LPS cotreatment was derived using Veh/Veh as baseline. S indicates SLD/Veh treatment, L indicates Veh/LPS treatment, and SL indicates SLD/LPS cotreatment. The intersection (^) of treatment groups represents the gene expression changed after both or all three of the indicated treatments.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are most likely to be involved in the pathogenesis of liver injury. These genes were further subjected to Ingenuity Pathway Analysis, which annotated 576 of the genes, including 83 that were upregulated and 493 that were downregulated by SLD/LPS. A list of toxicity pathways selectively affected by the 576 genes was determined (Table 5.1). Genes associated with pathway involved in fatty acid metabolism, LPS/IL-1-mediated inhibition of RXR function, NFkB signaling pathway and oxidative stress were highly impacted by SLD/LPS cotreatment.

5.4.3 Oxidative stress in SLD/LPS-cotreated rats

Protein carbonyl concentration was not affected in liver mitochondria of rats treated with SLD or LPS alone (Fig.5.2). In contrast, SLD/LPS cotreatment significantly increased protein carbonyls in mitochondria of livers collected at 8 hr, suggesting that hepatic oxidative stress was associated with SLD/LPS cotreatment.

Pretreatment of rats with either ebselen or DMSO reduced liver injury (Fig. 5.3). That is, ALT activity at 12 hr was significantly increased by SLD/LPS, and either ebselen or DMSO markedly decreased serum ALT activity.

5.4.4 Effect of SLD sulfide on mitochondrial membrane potential

Mitochondria isolated from livers of untreated rats were incubated with SLD, SLD sulfide or SLD sulfone. Compared to vehicle treatment, SLD or SLD sulfone up to 1mM did not change the ratio of JC-1 aggregates and monomers, a marker of

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Table 5.1. Pathways associated with genes specifically changed by SLD/LPS cotreatment. Expression of 721 genes was changed specifically by SLD/LPS. These genes were imported into Ingenuity Pathway Analysis. A list of pathways was derived and ranked by p value, which indicates the deviation of observed number of genes for each pathway found in the imported list from the number expected to occur by chance. Ratio indicates the percentage of the number of genes affected in a particular pathway.

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LPS/I Inhibi Funct
Xenc Meta
Aryl Rece
Hepa
Mec Reg Perc Prol PPA
NFk Path
Cyto Par a Fa (Hu
Oxi
PP/ Act

Toxicity Lists	-Log(P)	Ratio (%)	Genes
Fatty Acid Metabolism	3.27	11.0	ALDH1A1,PECI,CYP4B1,AUH,ADH1C,CYP2D6, CYP2J2, ACAD9,ADHFE1, HSD17B4,ACSL1,GCDH
PXR/RXR Activation	2.95	12.7	PRKACB,GSTM2,ALDH1A1,AKT1,PRKACA,IL6,HNF4A,RXRA
LPS/IL-1 Mediated Inhibition of RXR Function	2.88	8.5	ALDH1L1,SLCO1A2,MAOB,GSTM2,ALDH1A1,MGST2, EG629219,XPO1,SULT1E1,LBP,RXRA,ACSL1,SULT1B1,MGST3,ALDH6A1
Xenobiotic Metabolism	2.55	9.1	GSTM2,MGST2,CYP4B1,ADH1C, ALDH1L1,CYP2D6,CYP2J2,ADHFE1, MGST3
Aryl Hydrocarbon Receptor Signaling	2.43	8.6	IL1A,GSTM2,ALDH1A1,MGST2,MAPK1,CDK4,ALDH1L1,NFIB,IL6,RXRA,ALDH6A1,MGST3
Hepatic Cholestasis	2.18	8.6	PRKACB,IL1A,NFKBIA,PRKACA,SLCO1A2,LBP,IL6,HNF4A,RXRA,CYP8B1,IRAK2
Mechanism of Gene Regulation by Peroxisome Proliferators via PPARα	1.66	8.60	PRKACB,IL1A,NFKBIA,MAPK1,PDGFA,ME1,RXRA,HSD17B4
NFkB Signaling Pathway	1.53	7.6	PRKACB,TLR2,IL1A,AKT1, GHR,NFKBIA,PRKACA,MAP3K8
Cytochrome P450 Panel - Substrate is a Fatty Acid (Human)	1.43	20.0	CYP4B1,CYP2J2
Oxidative Stress	1.28	8.93	GPX3,VCAM1,GSTM2,ME1,IL6
PPARα/RXR Activation	1.26	6.37	PRKACB,GHR,NFKBIA,MAPK1,PRKAA2,PRKACA,PLCG1,IL6,ADIPOR2,RXRA

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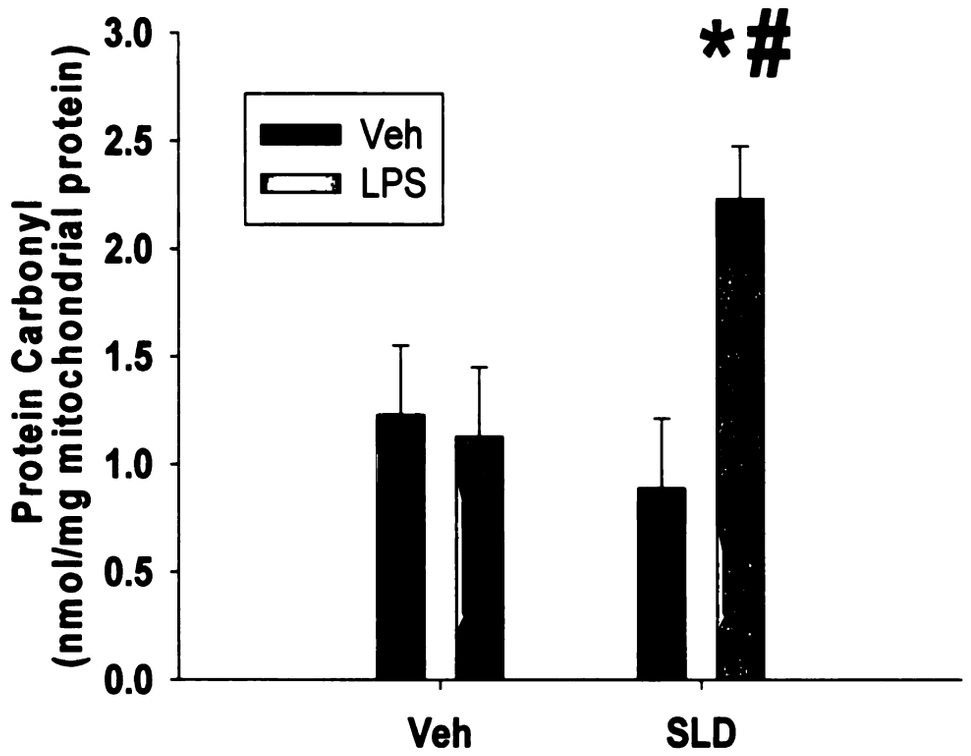
Fig. 5.2. Evaluation of protein carbonyl concentration in liver mitochondria. Rats were treated with SLD, LPS or their vehicles as described in Fig. 5.1. They were euthanized at 8 hr, and the livers were collected. Liver mitochondria were isolated, and protein carbonyl concentration was determined. *Significantly different from SLD/Veh group. #Significantly different from Veh/LPS group. $P<0.05$, n=4-8.



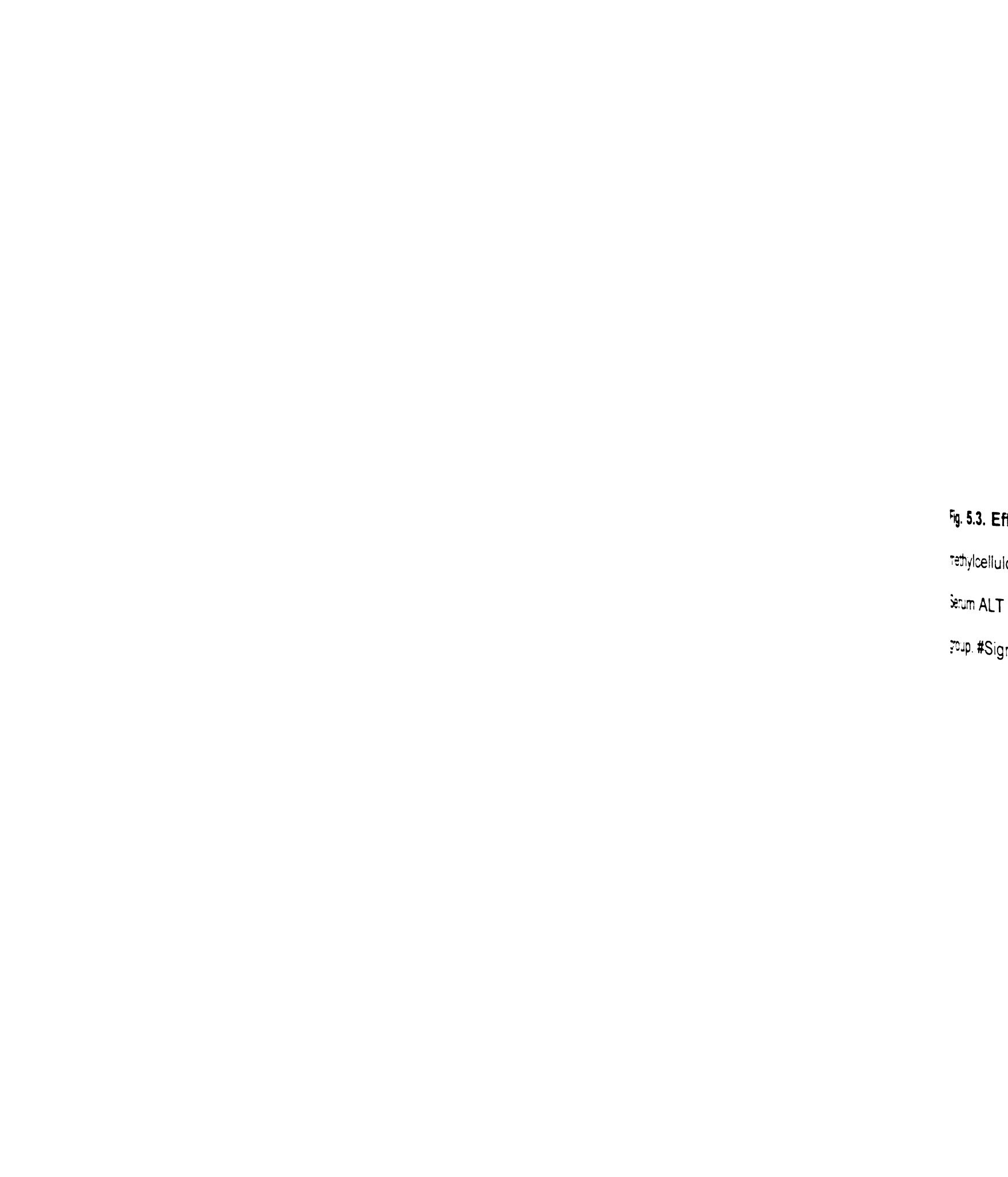


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Fig. 5.3. Effect of antioxidants on liver injury. (A) Ebselen, its vehcle (0.5% methylcellulose) or (B) DMSO was administered to rats treated with SLD/LPS. Serum ALT activity was evaluated at 12 hr. *Significantly different from Veh/Veh group. #Significantly different from SLD/LPS group. $P<0.05$, n=3-9.

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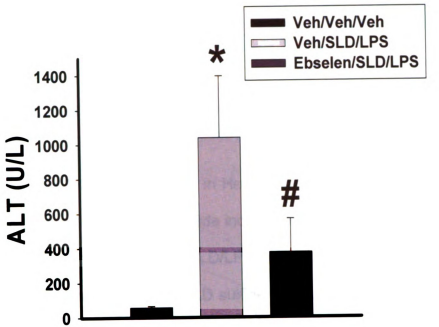
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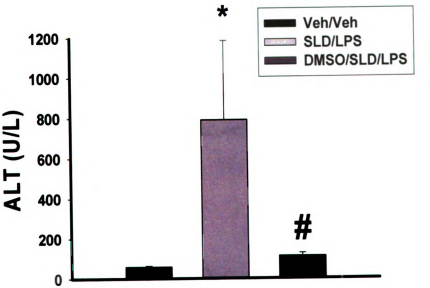
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membrane potential (Fig. 5.4). SLD sulfide caused a concentration-related decrease in mitochondrial membrane potential.

5.4.5 Effect of SLD sulfide on the production of reactive oxygen species in HepG2 cells

SLD sulfide increased DCF fluorescence in HepG2 cells at 0.5, 1 and 3 hr after addition (Fig.5.5), indicating that SLD sulfide induced ROS production. TNF plays a critical role in the pathogenesis of SLD/LPS-induced liver injury in part by synergistically killing hepatocytes with SLD sulfide (Zou et al., 2009a). However, TNF alone had no effect on DCF fluorescence, and the effect of SLD sulfide on ROS generation was not influenced by TNF.

5.4.6 Cytotoxicity of hydrogen peroxide and TNF to HepG2 cells

Neither TNF (200ng/mL) nor hydrogen peroxide (up to 1 mM) was toxic to HepG2 cells (Fig. 5.6). At 2 mM, hydrogen peroxide caused very modest cytotoxicity. When HepG2 cells were treated with TNF (200ng/mL) and hydrogen peroxide (1-2 mM) together, significant cytotoxicity occurred, as marked by a pronounced increase in LDH release. This indicated that TNF enhanced the cytotoxicity of hydrogen peroxide.

5.4.7 Effect of antioxidant treatment and caspase inhibition on cytotoxicity

SLD sulfide (250 uM) killed HepG2 cells (Fig. 5.7). Tempol, which is a superoxide dismutase mimetic, reduced the cell death caused by SLD sulfide. As shown

Fig. 5.4. Effect of SLD and its metabolites on mitochondrial membrane potential. Mitochondria were isolated from the livers of normal rats and incubated with SLD or its metabolites, SLD sulfone or SLD sulfide, for 30 min. Mitochondiral membrane potential was evaluated. *Significantly different from any other group at the same concentration. #Significantly different from vehicle group. $P<0.05$, n=3.

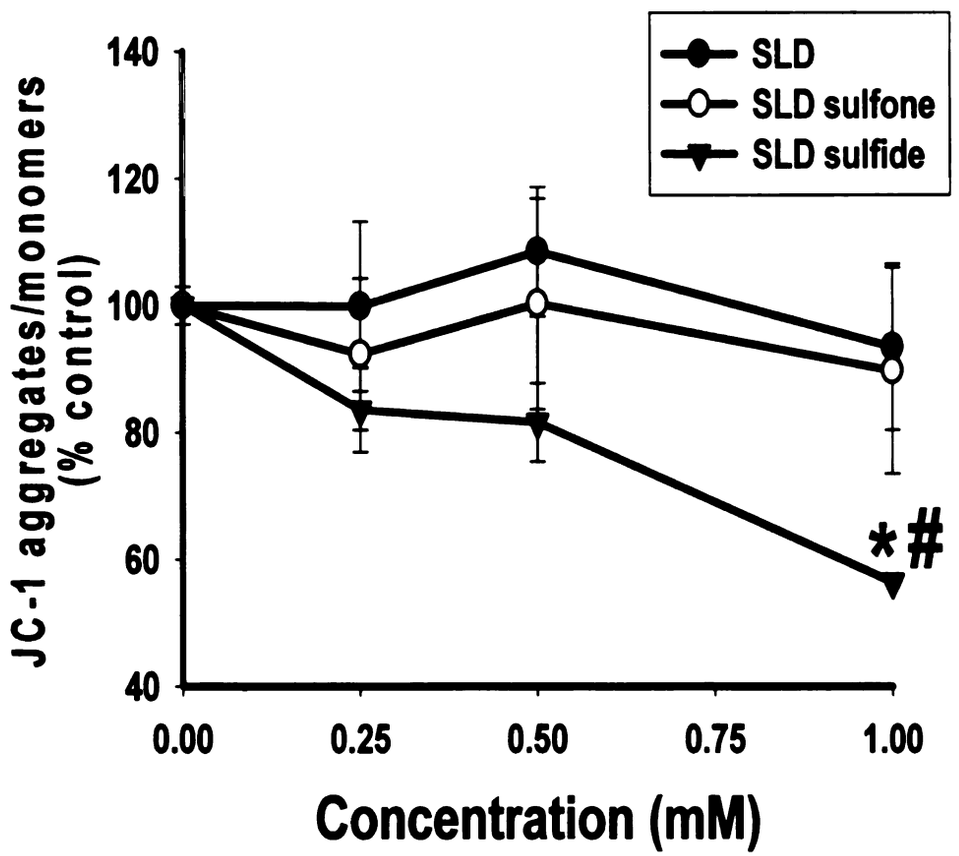

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Fig. 5.5. Effect of SLD sulfide and TNF on production of reactive oxygen species in HepG2 cells. SLD sulfide (250 uM) was administered to HepG2 cells in the presence of TNF (200ng/mL) or its medium vehicle. Reactive oxygen species generation at various times (0, 0.5, 1 and 3 hr) after treatment was evaluated using DCF fluorescence. *Significantly different from Veh/Veh group at the same time. $P<0.05$, $n=4$.

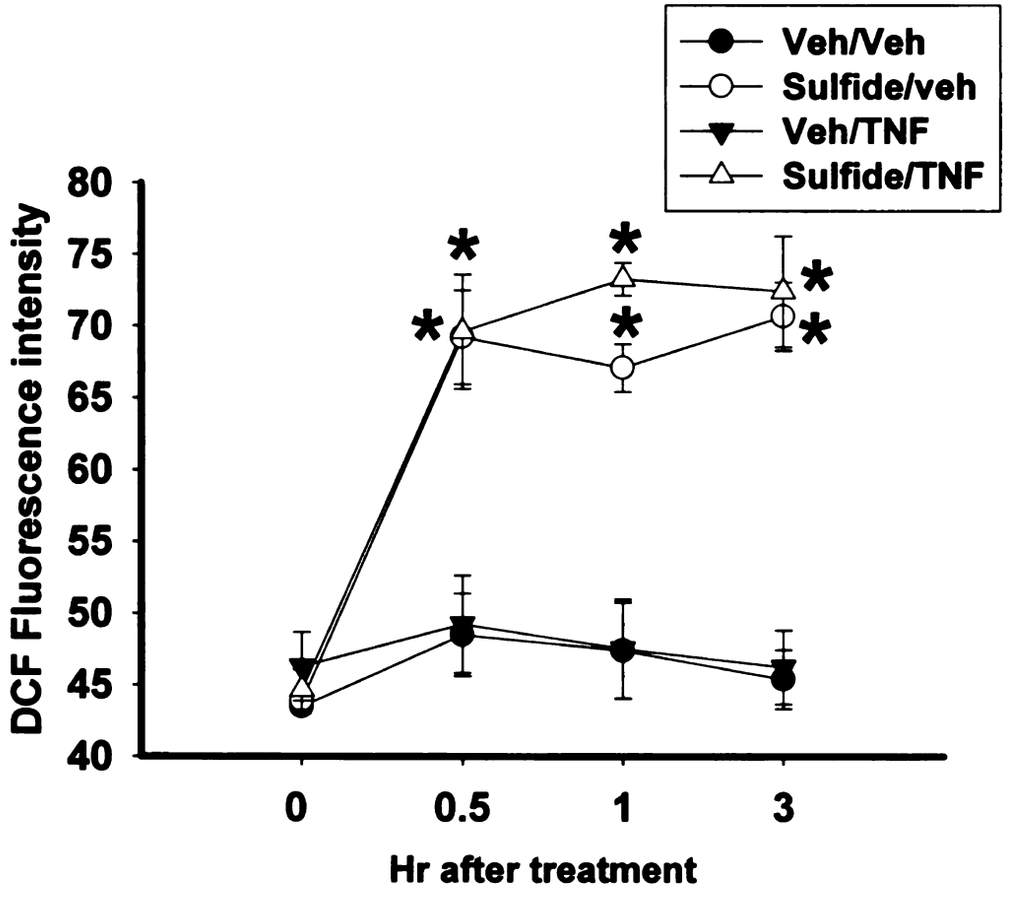

Fig. 5.6. Cytotoxicity induced by hydrogen peroxide and TNF. Hydrogen peroxide was administered to HepG2 cells in the presence of TNF (200ng/mL) or its medium vehicle. After 24 hr incubation, the percentage of LDH released from cells was evaluated as a marker of cell injury. *Significantly different from 0 concentration hydrogen peroxide group. #Significantly different from corresponding hydrogen peroxide/vehicle group. $P<0.05$, $n=4$.

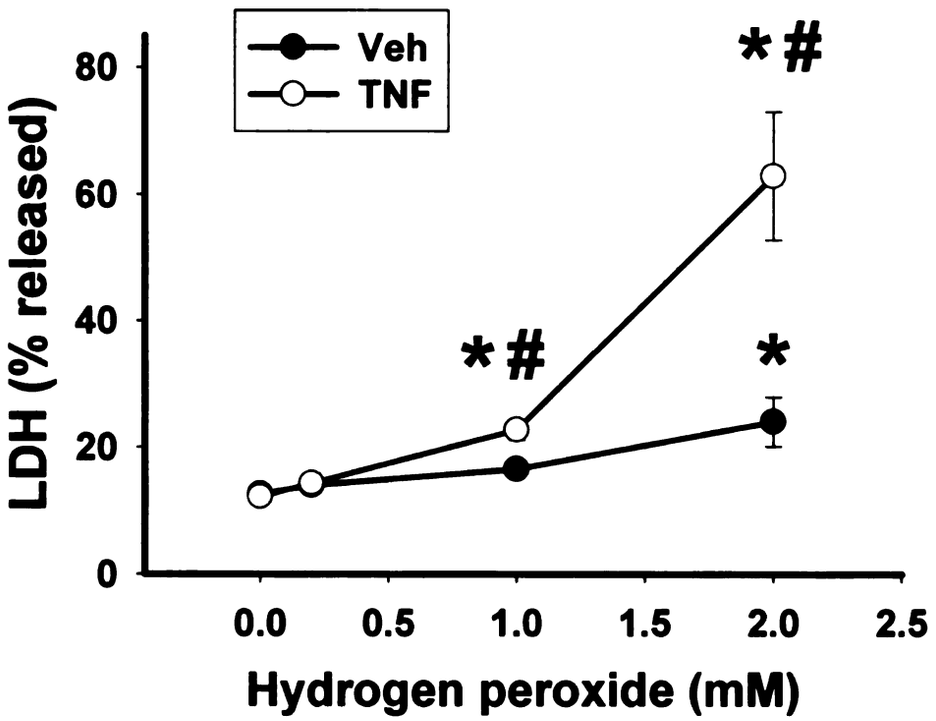

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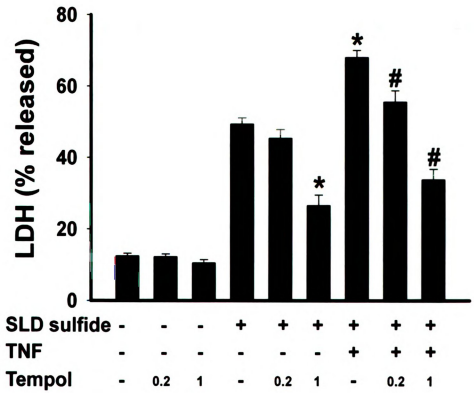
Fig. 5.7. Effect of antioxidant on cytotoxicity induced by SLD sulfide and TNF. Tempol was administered to HepG2 cells half an hour before the administration of SLD sulfide (250 uM), TNF (200ng/mL) or their vehicles. The percentage of LDH activity released was determined after 24 hr. *Significantly different from SLD sulfide group. #Significantly different from SLD sulfide/TNF group. $P<0.05$, n=5.



previously (Zou et al., 2009a), a nontoxic concentration of TNF (see Fig. 5.6) significantly enhanced the cytotoxicity of SLD sulfide. Tempol decreases the cytotoxicity due to the interaction of SLD sulfide and TNF.

5.4.8 Effect of antioxidant treatment on caspase 3/7 activity induced by SLD sulfide and TNF cotreatment

Either SLD sulfide or TNF alone at the concentrations used did not increase caspase 3/7 activity in HepG2 cells (Fig. 5.8). In contrast, coadministration of SLD sulfide and TNF activated caspase 3/7 activity at 6 hr. Pretreatment of HepG2 cells with antioxidant tempol or a pancaspase inhibitor Z-VAD abolished the increase in caspase 3/7 activity caused by SLD sulfide and TNF cotreatment. Z-VAD failed to protect HepG2 cells from the cytotoxic effect of SLD sulfide when it was given alone but reduced the cytotoxic effect of SLD sulfide/TNF coadministration (Fig. 5.9).

Fig. 5.8. Effect of antioxidant on caspase 3/7 activity. Tempol (200 uM), Z-VAD (10 uM) or their medium vehicle was administered to HepG2 cells. Half an hour later, SLD sulfide (250 uM) and/or TNF (200 ug/mL) was administered. Acitivity of caspase 3/7 in HepG2 cells was determined after 6 hr incubation. *Significantly different from any other group. $P<0.05$, n=4.

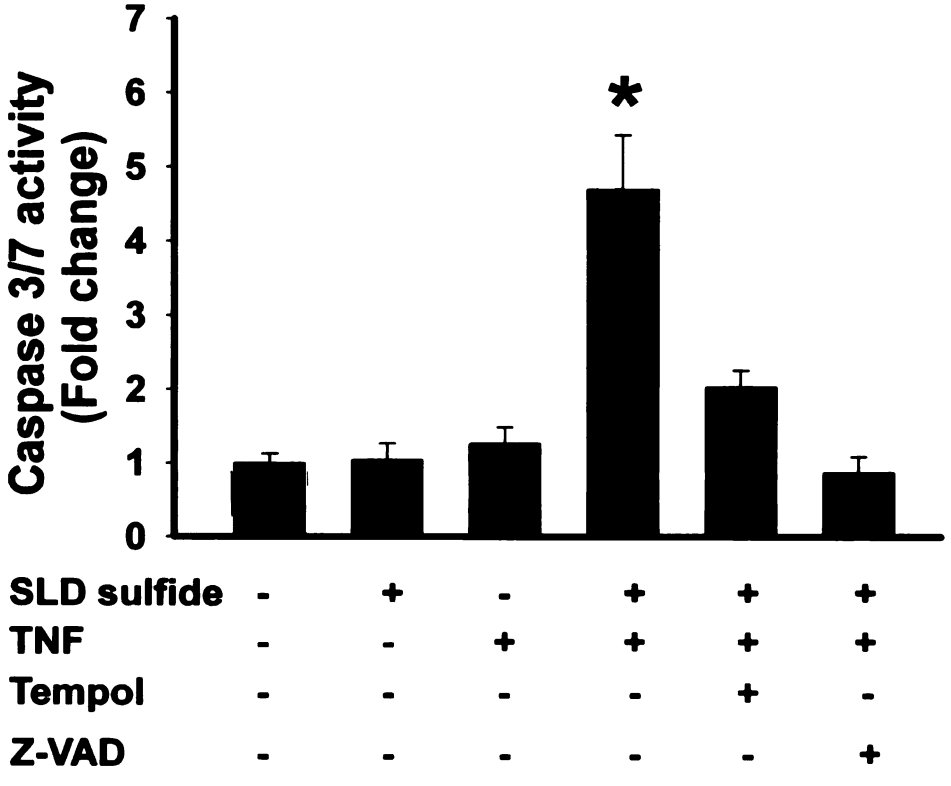
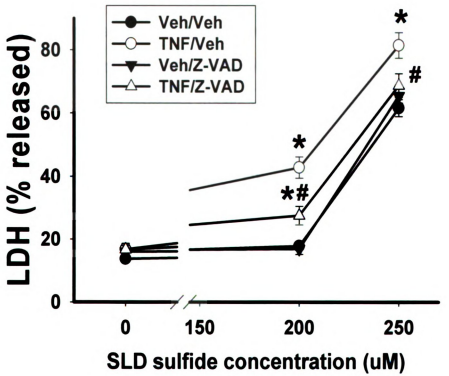


Fig. 5.9. Effect of pan-caspase inhibitor on cytotoxicity induced by SLD sulfide and TNF. Z-VAD (10 uM), SLD sulfide, TNF or their vehicles were administered to HepG2 cells as described in Fig.5.8. The percentage of LDH activity released from HepG2 cells was determined after 24 hr. *Significantly different from corresponding SLD sulfide-free group. #Significantly different from corresponding SLD sulfide/TNF group in the absence of Z-VAD. $P<0.05$, n=3.



5.5 Discussion

SLD/LPS cotreatment induced liver injury in rats, whereas the doses of either SLD or LPS employed were not hepatotoxic when given alone. To investigate the interaction between SLD and LPS, gene array analysis was performed on livers collected at 4 hr, a time before the onset of liver injury. Gene expression changes caused by SLD, LPS and SLD/LPS were compared. The results suggested that SLD alone had only a modest effect on gene expression (Fig. 5.1). As expected, LPS treatment caused the changes in the expression of numerous genes. Interestingly, SLD interacted with LPS to cause a large number of genes to be expressed specifically after SLD/LPS cotreatment.

The pathway most impacted by SLD/LPS is fatty acid metabolism (Table 5.1). All the 12 genes in that category are downregulated, and half of them (ACSL1, AUH, ACAD9, ADHFE1, HSD17B4, GCDH) locate in the mitochondria, which might indicate an impaired function of mitochondria. Several LPS related pathways (LPS/IL-1-mediated inhibition of RXR function, NFkB signaling pathway) are highly impacted by SLD/LPS cotreatment. These results suggest SLD has an effect on the signaling pathway driven by LPS. Ingenuity pathway analysis indicated that genes related to oxidative stress were influenced by cotreatment. Two important genes involved in detoxification of reactive oxygen species (glutathione peroxidase 3 and glutathione S-transferase mu 2) were significantly downregulated by SLD/LPS. Glutathione peroxidase partially determines the susceptibility of cells to oxidative stress (Yang et al., 2006), and downregulation of glutathione S-transferase mu 2 has been associated with

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increased levels of superoxide anion (Zhou et al., 2008). Accordingly, this result suggests that the cellular defense system against oxidative stress was impaired before the onset of liver injury.

Oxidative stress has been associated with numerous models of liver injury. For example, ROS play a role in liver injury induced by alcohol and ischemia-reperfusion (Wiseman, 2006; Cederbaum et al., 2009). Hepatotoxic drugs such as acetaminophen can induce oxidative stress in mouse liver and in hepatocytes in vitro (Adamson and Harman, 1993; Lores Arnaiz et al., 1995). It has been suggested that ROS play a role in the idiosyncratic liver injury caused by NSAIDs (Boelsterli, 2002). SLD induces oxidative stress in cultured cell lines (Seo et al., 2007; Park et al., 2008). However, SLD has not been reported to cause oxidative stress in an animal model. In the model of SLD-LPS interaction, an increase in protein carbonyl concentration in isolated liver mitochondria was observed at 4 hr (Fig. 5.2), which suggests oxidative stress was induced in livers of rats cotreated with SLD/LPS before the onset of liver injury. Ebselen is a glutathione peroxidase mimic, and DMSO is a scavenger of ROS. Although DMSO can suppress conversion of the prodrug sulindac to its bioactive sulfide metabolite (Swanson et al., 1983), both of these agents decreased ALT activity in rats treated with SLD/LPS, suggesting that oxidative stress contributes to liver injury in this model.

Injured mitochondria can be a major source of ROS arising from leakage of electrons from the electron transport chain (Zorov et al., 2006; Orrenius, 2007). There are numerous reports that NSAIDs lead to mitochondrial dysfunction by

acting as mitochondrial uncouplers or causing mitochondrial membrane permeability transition pore opening (Moreno-Sanchez et al., 1999; Al-Nasser, 2000; Boelsterli, 2002). In isolated rat mitochondria, SLD sulfide decreased mitochondrial membrane potential, whereas SLD or SLD sulfone showed no effect (Fig. 5.4). Consistent with this result, a previous study using the JC-1 assay also suggested that SLD sulfide may lead to dissipation of mitochondrial membrane potential in HepG2 cells (Leite et al., 2006). Decreased ATP synthesis can be a direct consequence of decreased mitochondrial membrane potential, which might explain why SLD sulfide is more hepatotoxic compared to SLD or SLD sulfone (Zou et al., 2009a).

SLD sulfide also induced ROS generation in HepG2 cells (Fig. 5.5). However, exposure to SLD by itself failed to increase liver protein carbonyl concentration in rats; coexposure to LPS was necessary for this effect. Hypoxia, which occurs in the livers of rats cotreated with SLD/LPS as a result of hemostasis (Zou et al., 2009b), is a potential contributor of oxidative stress in vivo (Arteel et al., 1999). Moreover, PMNs accumulate and become activated in the livers of rats cotreated with SLD/LPS and contribute to liver injury at least in part by releasing cytotoxic proteases. During PMN activation that results in protease release, , NADPH oxidase assembles on the PMN plasma membrane and ROS are generated (Dahlgren and Karlsson, 1999). Thus, activated PMNs might contribute to ROS generation and consequent oxidative stress in this model.

A previous study revealed that TNF potentiated the cytotoxicity of SLD sulfide in HepG2 cells and primary hepatocytes in vitro (Zou et al., 2009a). However, TNF had no effect on ROS generation induced by SLD sulfide in HepG2 cells (Fig. 5.5), indicating that TNF does not contribute to cell death by enhancing oxidative stress. Interestingly, TNF enhanced the cytotoxicity of hydrogen peroxide in HepG2 cells (Fig. 5.6). This is consistent with previous observations that hydrogen peroxide and TNF synergistically kill primary mouse and rat hepatocytes in vitro (Imanishi et al., 1997; Han et al., 2006). These results might explain the synergistic killing by SLD sulfide and TNF; that is, although TNF does not enhance SLD sulfide-induced oxidative stress, it does render cells sensitive to ROS-mediated cell killing. This is supported by the observation that antioxidant tempol decreased the cytotoxicity of SLD sulfide and significantly reduced the cytotoxic interaction between TNF and SLD (Fig. 5.7). In vivo, it seems unlikely that SLD sulfide concentrations become great enough to cause ROS-mediated liver injury; rather, TNF production caused by LPS coadministration renders the liver more sensitive to otherwise noninjurious ROS generation. The gene expression results discussed above suggest that compromised antioxidant protective mechanisms could play a role in this heightened sensitivity.

JNK is a common target of TNF and ROS and regulates apoptosis (Kanda and Miura, 2004; Schwabe and Brenner, 2006). TNF-induced JNK activation leads to caspase activation, which in turn leads to liver injury (Wang et al., 2006). Activation of caspase 3/7 was only observed in HepG2 cells cotreated with SLD

sulfide and TNF (Fig. 5.8), whereas TNF or toxic concentration of SLD sulfide alone had no effect. This indicates that the cytotoxicity of SLD sulfide alone in vitro depended on ROS but not on caspase activation. However, both ROS and caspase activation were critical in the synergistic killing induced by TNF and SLD sulfide (Figs. 5.7 and 5.9). The observation that tempol reduced the activation of caspase 3/7 (Fig. 5.8) suggests ROS contribute to caspase activation induced by SLD sulfide/TNF interaction.

In summary, this study further revealed the mechanisms of SLD/LPS-induced liver injury in rats. According to the comparison of gene expression in the liver of rats treated with SLD and/or LPS or their vehicles, genes associated with oxidative stress were selectively regulated by SLD/LPS. SLD/LPS cotreatment led to an increase in protein carbonyl in the mitochondria of rat livers, and antioxidants protected against liver injury, which suggests oxidative stress is involved in SLD/LPS-induced liver injury. SLD sulfide exerts its cytotoxicity through decreasing mitochondrial membrane potential and increasing the production of ROS in vitro. The synergistical interaction of SLD sulfide and TNF to kill HepG2 cells is dependent on the oxidative stress induced by SLD sulfide. Under oxidative stress TNF leads to the activation of caspase 3/7, which contributes to the cytotoxicity of SLD sulfide/TNF interaction.

CHAPTER 6

Summary and conclusions

6.1 Summary and conclusions

The goal of this project was to test the hypothesis that LPS precipitates SLD-induced liver injury in rats. A model of SLD/LPS-induced liver injury was developed, and the mechanisms were further investigated. These studies provide additional evidence that supports the hypothesis that inflammation is a susceptibility factor for idiosyncratic drug hepatotoxicity. This project also enhances our understanding of the mechanisms of drug-LPS interaction.

First, to develop a liver injury model of SLD and LPS interaction, rats were treated with two administrations of SLD with a 16 hr interval. Two administrations of SLD were chosen because treatment with one administration of SLD with LPS was not sufficient to induce liver injury in rats. Half an hour before the second administration of SLD, a nonhepatotoxic dose of LPS was administered to rats via a tail vein. The ALT activity in rat serum increased at 8 hr and was significant at 12 hr (Fig. 2.3). Midzonal necrosis was also observed only in the livers of rats cotreated with SLD/LPS (Fig. 2.5). These results suggest that SLD interacts with the inflammatory stress induced by LPS, which leads to liver injury. Subsequently, several mediators potentially involved in the pathogenesis were investigated.

Hemostatic factors, including PAI-1 and thrombin, were elevated by LPS in rat plasma (Fig. 2.6) and were enhanced by SLD cotreatment. As a result, significant fibrin deposition was observed in liver sinusoids of rats cotreated with SLD/LPS compared to those treated with SLD or LPS alone (Fig. 2.9). Presumably resulting from the impaired blood flow, hypoxia occurred in the liver

of rats treated with SLD/LPS. Anticoagulant heparin, which attenuated fibrin deposition and hypoxia in the liver, protected against liver injury induced by SLD/LPS (Fig. 2.10 and 2.11). These results suggest that the hemostatic system and hypoxia resulting from fibrin deposition are critical to SLD/LPS-induced liver injury.

TNF is another mediator involved in the pathogenesis of liver injury induced by inflammation or inflammation-xenobiotic interaction. LPS led to an increase in TNF concentration in serum, which was enhanced by SLD cotreatment (Fig. 3.1). TNF neutralization using etanercept decreased serum ALT activity in rats cotreated with SLD/LPS, which shows that TNF plays an important role in the pathogenesis (Fig. 3.2).

The concentrations of SLD, SLD sulfone and SLD sulfide in plasma and livers of rats were decreased by LPS cotreatment (Fig. 3.3 and 3.4). In contrast, their concentrations in rat GI tract and feces were increased by LPS (Fig. 3.5). SLD is bioactivated to SLD sulfide, and this metabolite was much more cytotoxic to HepG2 cells and primary rat hepatocytes than SLD in vitro (Fig. 3.6). However, in rats the amount of SLD sulfide produced was insufficient to cause liver injury by itself. Presumably, it is this bioactivated metabolite that acts synergistically with LPS to precipitate liver injury in vivo. In vitro, SLD sulfide synergistically interacted with TNF to kill cells, whereas SLD or SLD sulfone had no interaction with TNF (Fig. 3.7 and 3.8).

PMNs are a critical contributor to liver injury in other drug/LPS interaction models. LPS led to PMN accumulation in the liver, and SLD enhanced the

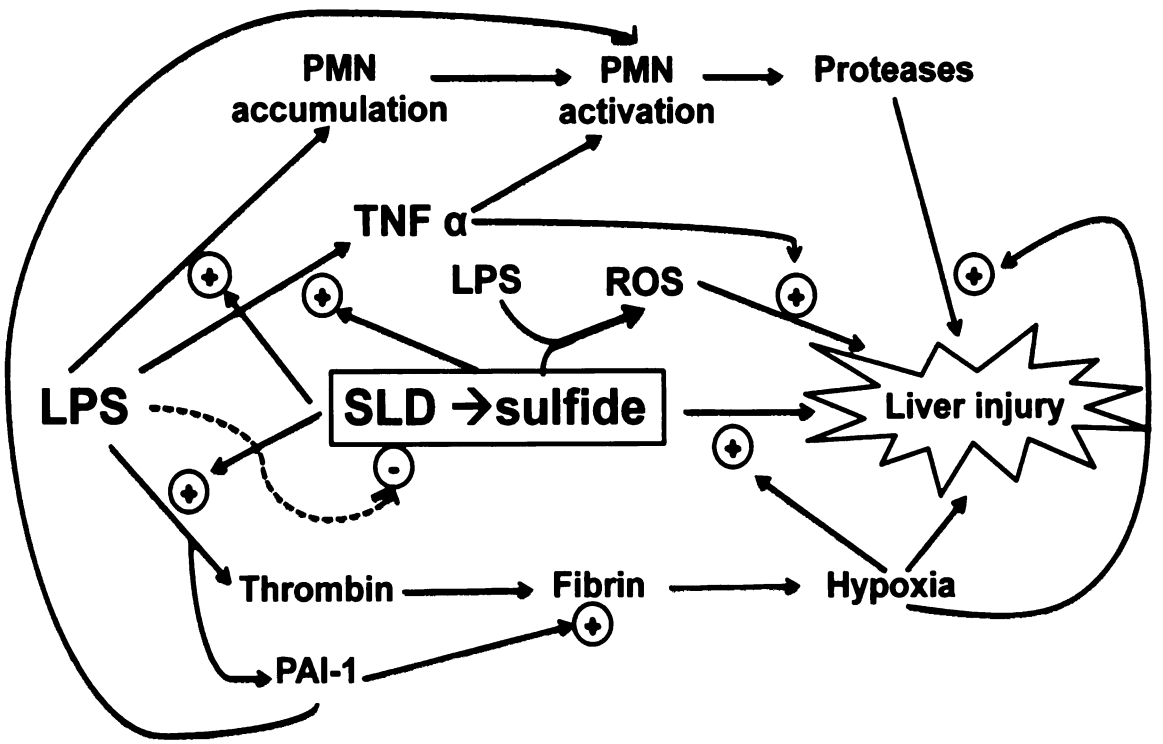
accumulation of PMNs induced by LPS (Fig. 4.1). However, HOCl-protein adducts were increased only in the livers of rats treated with SLD/LPS, suggesting that SLD/LPS cotreatment causes PMN activation in the liver. Either anti-PMN serum or the PMN protease inhibitor, eglin C, attenuated liver injury, which suggests that proteases released from activated PMNs participate in the pathogenesis (Fig. 4.3 and 4.4). Previous studies also showed that proteases synergistically interacted with hypoxia to kill hepatocytes (Luyendyk et al., 2005). This same interaction might contribute to SLD/LPS-induced liver injury. Several mediators are involved in PMN activation which is a prerequisite for protease release. Inhibition of TNF or PAI-1, both of which are important mediators in liver injury induced by SLD/LPS, decreased HOCl-protein adducts but had no effect on PMN numbers in the liver (Fig. 4.5 and 4.6). Thus, PMN activation but not accumulation is dependent on TNF and PAI-1. Neutralization of TNF had no effect on PAI-1 production, suggesting an independent relation between TNF and PAI-1 (Fig. 4.7).

SLD/LPS cotreatment produced a specific gene expression profile in the liver compared to treatments with SLD alone or LPS alone. Analysis of the genes specifically changed by SLD/LPS at 4 hr suggested that a pathway associated with oxidative stress is influenced before the onset of liver injury (Table 5.1). Antioxidants protected against liver injury induced by SLD/LPS in rats and reduced cytotoxicity caused by SLD sulfide and TNF interaction in vitro. These results suggest oxidative stress is also involved in the pathogenesis of SLD/LPS-induced liver injury. Moreover, ROS not only kills hepatocytes itself but also

interacts with other mediators. A study in vitro showed that TNF enhanced the cytotoxicity of hydrogen peroxide in HepG2 cells (Fig. 5.6).

Mechanisms of SLD/LPS-induced liver injury are summarized in Fig. 6.1. TNF, the hemostatic system, hypoxia, PMNs as well as reactive oxygen species play critical roles in the pathogenesis of SLD/LPS-induced liver injury. SLD enhances the activation of hemostatic factors, including thrombin and PAI-1, induced by LPS. An activated hemostatic system leads to fibrin clot formation in liver sinusoids. As a result, the liver becomes hypoxic which renders hepatocytes susceptible to injury. SLD also enhances the elevation in TNF induced by LPS. LPS decreased the bioavailability of SLD in rats, but the liver is nevertheless apparently able to produce enough SLD sulfide to precipitate a toxic interaction with LPS. SLD sulfide synergistically kills hepatocytes along with TNF and hypoxia in LPS-cotreated rats. PMNs are another critical player in SLD/LPS-induced liver injury. PMN accumulation in the liver was primarily induced by LPS, and this effect was enhanced by SLD. Activation of PMNs was only observed in livers of rats cotreated with SLD/LPS. TNF and PAI-1 contribute to PMN activation independently. Activated PMNs release proteases which induce liver injury by itself or by interacting with hypoxia.

Fig. 6.1. Proposed pathway in the pathogenesis of SLD/LPS-induced liver injury. See text for details.



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6.2 Commonality and differences among liver injury models of drug-inflammation interaction

So far, the evidence in animal models supporting the inflammatory stress hypothesis has been accumulating. In animals, inflammation precipitates liver injury induced by several drugs associated with idiosyncratic liver injury in humans. These drugs include ranitidine (RAN), diclofenac (DCLF), trovafloxacin (TVX), sulindac (SLD), halothane (HAL) and chlorpromazine (CPZ). The characteristics and mechanisms involved in the pathogenesis have been studied to various extents, and the results are summarized in table 6.1.

Previous studies suggest that inflammation induced by different kinds of inflammagens precipitates drug-induced liver injury. The inflammagens that have been evaluated so far in drug interaction models include the cell wall components of gram-negative bacteria (LPS), Gram-positive stimuli (a peptidoglycan-lipoteichoic acid (PGN-LTA) mixture) and the viral RNA mimetic (polyI:C). The routes of drug administration are different among the models, which suggest that liver injury is not dependent on a specific route of drug administration. SLD is the only model in which the drug was administered twice; one dose of SLD failed to induce a significant liver injury in rats. Times between drug and inflammagen administration needed to elicit a toxic response vary among different models.

RAN, TVX and SLD models are three that have been relatively well investigated using LPS as an inflammatory stimulus. Enhanced TNF production, activation of the hemostatic system and PMN accumulation in liver have been observed in these models. Neutralization or inhibition studies showed that TNF,

Table 6.1. Characteristics and underlying mechanisms of idiosyncratic liver injury models of drug-inflammation interaction. Y means the phenomenon or mechanism is observed in the model. N means the listed item is not true in the model. A blank entry means it has not been tested or reported. *indicates the inflammagen used in the mechanism studies. (See text for references)

	RAN	DCLF	TVX	SLD	CPZ	HAL
Inflammagen	LPS	LPS	LPS* or PGN+LTA	LPS	LPS	Polyl:C* or LPS
Administration route of the drug	i.v.	p.o.	i.p.	p.o.	i.p.	i.p.
Number of drug administrations	1	1	1	2	1	1
Time (hr) drug was given relative to LPS	2	2	-3	-15.5 and 0.5	2	-6
Hr after the drug administration when liver injury was observed	6	6	12	12 after 2nd SLD	24	15
Enhanced TNF production	Y		Y	Y		Y
TNF involved in pathogenesis	Y		Y	Y		Y
Hemostatic system activated	Y		Y	Y		
Fibrin deposition and hypoxia involved	Y		Y	Y		
PMN accumulation	Y	Y	Y	Y	Y	
PMN activation	Y			Y		
PMNs involved in the pathogenesis	Y	Y	Y	Y		
IFN / IL-18 dependent			Y			
ROS involved				Y		
Hemostasis affected by TNF	Y		Y	N		
PMN accumulation increased by TNF	N		N	N		
PMN accumulation affected by hemostasis	N					
PAI-1 increased by TNF	Y		Y	N		
PMNs activated by PAI-1 and TNF	Y			Y		
Kupffer cells and NK cells involved						Y

hypoxia and PMNs are critical mediators in the pathogenesis of liver injury induced by the interaction with LPS for all three of these drugs. Their roles in DCLF, HAL and CPZ models need to be tested. These results suggest that TNF, hypoxia and PMNs are important players shared by many liver injury models of drug-inflammation interaction. These mediators are not independent but interact with each other. However, their interactions differ among models.

Some mediators have been found to be critical in the pathogenesis in a single model but have not been tested in other models. For example, IFNγ and IL-18 are both involved in TVX/LPS-induced liver injury. Oxidative stress proved to play a role in SLD/LPS-induced liver injury. The roles of these mediators in other models need to be investigated.

In summary, evidence is accumulating to support the hypothesis that inflammation precipitates or enhances drug-induced liver injury. TNF, the hemostatic system and PMNs are involved in the pathogenesis of all of the liver injury models of drug-inflammation interaction in which their roles have been tested. The roles of oxidative stress or proinflammatory cytokines (IFNγ and IL-18) and other potential mediators remain to be determined in other models.

6.3 Potential future studies

This project generally clarified several important mediators involved in the liver injury induced by SLD/LPS coexposure. However, the mechanisms of SLD/LPS interaction remain incompletely understood. Potential directions for future studies of this idiosyncratic liver injury model are discussed below.

Although it was observed that SLD enhances the appearance of TNF and hemostatic factors, the mechanisms have not been clarified. SLD has been reported to be toxic to the GI tract and to cause ulcers. A possible explanation for the effect of SLD on TNF, thrombin and PAI-1 is that SLD causes bacterial translocation to the liver resulting from gastrointestinal toxicity of SLD. In concert with this hypothesis, diclofenac at large dose exerts hepatotoxiticy to rats by causing bacterial translocation from the GI tract. Another possible mechanism is that SLD directly acts on the TLR4 signaling pathway. For example, ranitidine specifically enhanced the activation of p38, which led to enhanced TACE activation and TNF production. Therefore, the activity of signaling proteins in TLR4 pathway is worth evaluation. Activation of TACE is a potential target of SLD.

The cellular sources of several mediators have not been identified. Kupffer cells are a major source of TNF in many inflammatory models. However, it is not known if they are the only source of TNF in the SLD/LPS model; hepatocytes and endothelial cells can also contribute to TNF production (Zhaowei et al., 2007). Kupffer cell depletion is a feasible approach to investigate the role of these cells in this model. The mechanisms by which the ROS are produced have not been

identified either. PMNs, Kupffer cells, hypoxia and TNF might all contribute to generation of ROS in the SLD/LPS model. Their roles can be investigated through their inhibition or depletion.

The relation and interaction among these critical mediators are not fully understood. According to results in the ranitidine model, PMN proteases exacerbate fibrin deposition by upregulating PAI-1. ROS might contribute to the activation of PMNs (Jaeschke, 2006). In the TVX/LPS model, PAI-1 contributes to the production of proinflammatory cytokines. These interactions could be investigated in the SLD/LPS model.

Despite of the discovery of various mediators including TNF, hypoxia, PMNs and ROS, there could be some participants yet to be discovered. IFNγ, which was upregulated in plasma by SLD/LPS and plays a critical role in the TVX/LPS model, is a potential candidate.

APPENDICES

Genes selectively regulated by SLD/LPS cotretment. The number of gene expression changes (relative to Veh/Veh) caused by treatment with SLD/Veh, Veh/LPS or SLD/LPS were depicted using Venn diagram (Fig. 5.1). According to the diagram, there were 721 probe sets regulated by SLD/LPS that were not affected by either SLD or LPS treatment alone. The genes in this group were listed as below.

ID	Log2 ratio	Symbol	ID	Log2 ratio	Symbol
1367589_at	-2.674	ACO2	1376009_at	-2.121	CTAGE5
1367614_at	3.064	ANXA1	1376094_at	-1.901	HINT3
1367636_at	-1.696	IGF2R	1376105_at	-1.540	COL14A1
1367672_at	-1.542	HSD17B4	1376321_at	1.883	FAM38A
1367673_at	-3.673	SELENBP1	1376337_at	-1.667	SMARCA2
1367695_at	-1.708	QDPR	1376569_at	1.774	KLF2
1367721_at	1.563	SDC4	1376692_at	-1.514	HIPK2
1367729_at	-1.814	OAT	1376706_at	-2.293	TMEM47
1367750_at	-2.102	PRPSAP1	1376715_at	-1.671	CBARA1
1367771_at	-2.808	TSC22D3	1376727_at	-1.663	YIPF4
1367807_at	-2.164	PLOD1	1376758_at	-2.106	ING1
1367815_at	-1.682	SLC5A6	1376771_at	-1.797	PPM1L
1367817_at	-1.884	HDGF	1376796_at	-1.664	RAB14
1367818_at	-1.716	COQ3	1376862_at	-2.386	UBE4B
1367857_at	-4.297	FADS1	1376930_at	-1.614	MRPL51
1367869_at	-2.537	OXR1	1377049_at	-1.555	PNPLA7
1367874_at	3.210	RHOQ	1377060_at	-1.862	MCCC2

1367889_at	-1.820	CAMK1	1377166_at	-1.522	ALS2
1367896_at	-2.700	CA3	1377209_at	-1.619	KLHL25
1367933_at	-1.658	AMD1	1377307_at	2.444	FAM89A
1367940_at	2.018	CXCR7	1377603_at	-2.121	SNX24
1367998_at	2.628	SLPI	1377654_at	-1.605	FAM3A
1368021_at	-1.676	ADH1C	1377657_at	-1.663	IBTK
1368057_at	-2.056	ABCD3	1377745_at	-1.751	LRRC40
1368067_at	-1.776	ZNF148	1377758_at	-2.274	HSD17B13
1368071_at	-1.775	MOSC2	1377810_at	-1.593	RALGPS2
1368085_at	-1.589	GCHFR	1377995_at	-1.860	ITFG3
1368091_at	-3.031	OPLAH	1378027_at	-2.013	PVRL3
1368096_at	-1.693	RAB7L1	1378146_at	-1.741	TBC1D24
1368115_at	-2.670	CLDN3	1378185_at	-1.556	NXT2
1368117_at	-1.685	GPHN	1378394_at	-1.819	MPPE1
1368122_at	-2.453	RNF103	1378842_at	-2.071	GABARAPL1
1368133_at	-1.598	MPDZ	1379044_at	-1.894	LARP2
1368144_at	2.208	RGS2	1379101_at	-1.549	DHX36
1368183_at	-1.787	PLCG1	1379315_at	-1.664	RASSF7
1368215_at	-2.300	TPP1	1379353_at	-1.737	AASDHPPT
1368265_at	-1.802	CYP2T4	1379375_at	1.601	PDGFA
1368272_at	-3.178	GOT1	1379441_at	-2.343	RNF160
1368277_at	-2.073	PPP3CA	1379456_at	-2.233	MCART2
1368378_at	-1.699	ALDH1L1	1379499_at	1.965	LTB
1368387_at	-1.990	BDH1	1379525_at	-1.780	CRLS1
1368427_at	-1.577	AKAP11	1379578_at	-1.807	ZBTB20
1368435_at	-4.049	CYP8B1	1379606_at	-3.277	RAB30
1368437_at	2.305	CA4	1379645_at	-1.859	PBRM1

1368446_at	-2.661	SPINK1	1379784_at	-1.796	PEX7
1368453_at	-2.931	FADS2	1379794_at	2.159	GZMB
1368474_at	1.989	VCAM1	1379803_at	1.675	LMO4
1368482_at	1.829	BCL2A1	1379850_at	-2.093	PSMC6
1368509_at	-1.541	BBS2	1379901_at	-1.582	TBC1D17
1368514_at	-1.993	MAOB	1379909_at	-1.968	GKAP1
1368519_at	4.112	SERPINE1	1379935_at	2.280	CCL7
1368545_at	1.614	CFLAR	1380063_at	3.086	CH25H
1368592_at	1.688	IL1A	1380229_at	1.790	MAFF
1368657_at	3.211	MMP3	1381012_at	-1.547	SERPINF1
1368702_at	1.585	PAWR	1381193_at	-1.936	LPGAT1
1368711_at	-2.039	FOXA2	1381768_at	-1.523	MTHFS
1368733_at	-1.784	SULT1E1	1381973_at	-1.569	SLC25A30
1368814_at	-1.592	ALDH6A1	1382024_at	1.906	DNAJB6
1368860_at	3.143	PHLDA1	1382101_at	-1.722	HS2ST1
1368862_at	-1.755	AKT1	1382150_at	-1.581	SLC25A46
1368869_at	3.134	AKAP12	1382200_at	-2.381	CENPV
1368914_at	2.918	RUNX1	1382216_at	-1.574	GEMIN6
1368924_at	-3.846	GHR	1382274_at	1.703	RARRES1
1368960_at	-1.855	LGALS8	1382285_at	-1.656	NAGA
1369063_at	-2.462	ANP32A	1382325_at	-1.750	GCAT
1369069_at	-2.219	AKAP1	1382332_at	-1.736	STAG2
1369070_at	-1.549	PEX12	1382371_at	-1.538	DRAM2
1369078_at	-1.734	MAPK1	1382402_at	-1.961	ULK1
1369150_at	-2.259	PDK4	1382496_at	-1.624	HNF4A
1369169_at	-1.577	SLC23A1	1382602_at	-1.696	UBR3
1369191_at	2.780	IL6	1382843_at	-1.907	SGPL1

1369268_at	3.449	ATF3	1382935_at	-2.046	KIAA0141
1369278_at	-1.660	GNA12	1383004_at	-1.550	AHCYL1
1369393_at	2.703	MAP3K8	1383037_at	-1.693	POLDIP2
1369453_at	-1.737	EPN1	1383050_at	-2.286	CENPV
1369492_at	-1.816	AADAC	1383118_at	-1.901	TMEM209
1369654_at	-2.490	PRKAA2	1383155_at	-1.610	FAM117B
1369785_at	-1.900	PPAT	1383159_at	-1.936	TOM1L2
1369837_at	-1.685	GULO	1383282_at	-1.689	THAP11
1369922_at	-1.733	PLBD2	1383358_at	-2.712	AKAP1
1369926_at	-1.507	GPX3	1383359_at	-1.628	LNX2
1369931_at	1.577	PKM2	1383395_at	-1.878	AGMAT
1369936_at	1.687	CALM1	1383462_at	-1.678	RNF160
1369942_at	-1.539	ACTN4	1383463_at	-1.565	ZFP91
1369950_at	-2.125	CDK4	1383474_at	2.182	IRAK2
1369956_at	-1.764	IFNGR1	1383732_at	-3.490	BC021614
1369960_at	-1.593	FXYD1	1383863_at	1.529	LMO2
1369982_at	-1.524	AP2A2	1383933_at	-1.607	KIAA0564
1369989_at	-1.978	PNPO	1383960_at	-1.712	PEX16
1369997_at	-1.577	DVL1	1384029_at	-1.646	XPA
1370029_at	-1.643	CTBP1	1384131_at	-2.195	ATL2
1370036_at	-2.348	SUOX	1384205_at	-2.590	NGLY1
1370047_at	-1.679	ENPP1	1384254_at	2.056	OTUD1
1370067_at	-2.173	ME1	1384293_at	-1.962	C20ORF191
1370112_at	-1.865	PTEN	1384383_at	-1.700	AGPAT6
1370121_at	-1.711	ADD1	1384628_at	-1.722	IYD
1370174_at	2.146	PPP1R15A	1384903_at	-2.443	GPT2
1370177_at	3.345	PVR	1385160_at	-1.621	STAB2

1370190_at	1.529	H3F3C	1385266_at	-1.590	NLK
1370200_at	-1.557	GLUD1	1385566_at	1.767	AKAP2
1370236_at	-1.525	PPT1	1385690_at	-1.948	MUT
1370249_at	2.047	TSPO	1385845_at	-1.753	D730039F16RIK
1370285_at	-1.690	CALCOCO1	1385889_at	-1.927	C2ORF64
1370319_at	-2.026	PPIF	1386280_at	-1.574	METTL7B
1370322_at	-1.896	STK16	1386764_at	1.946	AKAP2
1370329_at	-1.957	CYP2D6	1386895_at	-2.147	MAGED1
1370334_at	-1.877	PLEKHB1	1386977_at	-3.225	CA3
1370359_at	-1.809	AMY2A	1387006_at	-2.315	EG629219
1370360_at	-1.738	C3ORF34	1387018_at	-1.566	SORBS2
1370375_at	-2.340	GLS2	1387022_at	-1.921	ALDH1A1
1370399_at	-2.607	CYP4B1	1387023_at	-3.142	GSTM2
1370501_at	-1.843	UBE2G1	1387093_at	-1.683	SLCO1A2
1370516_at	1.679	SLC15A3	1387094_at	-2.694	SLCO1A2
1370548_at	-1.807	SLC16A10	1387186_at	-1.732	RAB9A
1370808_at	-2.317	CYB5R3	1387188_at	-1.866	SLC17A1
1370814_at	-1.501	DHRS4	1387190_at	-1.542	DGKA
1370818_at	-1.892	DECR2	1387209_at	-2.259	SEC16B
1370848_at	1.688	SLC2A1	1387214_at	-1.997	ES22
1370875_at	2.092	EZR	1387219_at	1.927	ADM
1370881_at	-1.504	TST	1387244_at	-2.280	CGRRF1
1370891_at	1.770	CD48	1387296_at	-2.141	CYP2J2
1370905_at	-1.728	DOCK9	1387314_at	-1.763	SULT1B1
1370939_at	-1.571	ACSL1	1387375_at	-2.454	KHK
1371034_at	-2.912	ONECUT1	1387567_at	-1.863	SLCO1A1
1371070_at	1.819	ZBP1	1387652_at	-1.638	IDE

1371317_at	-1.606	LDB1	1387725_at	-2.041	GULO
1371322_at	1.956	LAMC1	1387782_at	-1.676	DYNLL2
1371362_at	-1.700	KDELR3	1387786_at	-1.610	MTPN
1371385_at	-1.651	PSMG1	1387790_at	-2.156	PAICS
1371388_at	-1.576	PDHB	1387821_at	-1.570	RAB3IP
1371400_at	2.932	THRSP	1387852_at	2.101	THRSP
1371404_at	-1.935	EIF4B	1387857_at	-1.707	STX7
1371405_at	-1.871	C2ORF64	1387861_at	-1.693	AES
1371432_at	-1.544	VAT1	1387864_at	-1.936	KIDINS220
1371443_at	-1.626	C1ORF174	1387865_at	-1.830	DUT
1371445_at	1.935	LRRC59	1387868_at	1.863	LBP
1371447_at	2.064	PLAC8	1387900_at	-1.928	CDIPT
1371460_at	-1.648	C12ORF62	1387901_at	-1.746	PTPRS
1371461_at	-1.802	FAM54B	1387920_at	-1.505	MAN2C1
1371464_at	-1.542	ZFAND6	1387921_at	-1.524	ZC3H14
1371471_at	-1.628	GLTSCR2	1387948_at	-1.521	ICK
1371483_at	-1.689	NNT	1387959_at	-1.989	ASPG
1371493_at	-2.337	AP2A2	1388136_at	-1.522	TIMM9
1371525_at	-1.886	SLC12A7	1388150_at	-1.707	XPO1
1371527_at	1.788	EMP1	1388155_at	1.510	KRT18
1371531_at	-1.584	LOC678880	1388167_at	-1.991	NFIB
1371553_at	-1.955	MRPL36	1388300_at	-2.351	MGST3
1371560_at	-1.856	IRF3	1388324_at	-2.044	NIT1
1371576_at	-2.016	MRPS36	1388338_at	-2.086	PPP2R4
1371578_at	-1.993	PRKACA	1388364_at	-1.578	NDUFS3
1371583_at	3.100	RBM3	1388382_at	-1.605	C1ORF43
1371611_at	-1.713	EXT2	1388404_at	-1.873	GM12751

1371620_at	-1.728	PRELID1	1388410_at	-1.733	UGP2
1371634_at	-2.053	TMEM126A	1388430_at	-1.915	PTOV1
1371637_at	-1.660	HP1BP3	1388441_at	-1.920	LOC689574
1371668_at	-3.060	RXRA	1388471_at	-1.574	TCP11L2
1371697_at	-1.641	PNPLA2	1388474_at	-1.589	UBE2I
1371716_at	-1.507	SMARCC2	1388486_at	-1.596	DPP8
1371717_at	-1.751	MFN1	1388497_at	-1.599	ACOT13
1371747_at	-2.748	PPDPF	1388503_at	-2.238	EID1
1371799_at	-1.909	GAA	1388507_at	1.727	EIF6
1371835_at	-2.283	PRKACB	1388518_at	-1.902	FBXW5
1371853_at	-1.631	MRPL42	1388533_at	-2.024	CTDSP1
1371920_at	-1.604	POLDIP2	1388540_at	-1.744	MAZ
1371948_at	-1.972	PIGP	1388549_at	-1.891	NCOA4
1371956_at	-1.968	LOC683077	1388567_at	-2.072	THUMPD1
1371963_at	-1.780	PCCA	1388617_at	-1.766	BPHL
1371964_at	-2.291	GRSF1	1388636_at	-1.554	RNF167
1371982_at	-2.173	DPY30	1388658_at	-1.970	SURF2
1371983_at	-1.763	JOSD1	1388666_at	1.796	ENC1
1371993_at	-1.516	CPNE3	1388672_at	-2.232	ZCCHC24
1372071_at	-1.533	CD320	1388680_at	-1.563	C1GALT1C1
1372073_at	-1.625	GATAD2A	1388685_at	-1.905	DGCR2
1372074_at	-1.752	NUDT3	1388725_at	-1.822	LEPROT
1372085_at	-1.959	ATL2	1388732_at	-1.549	SLC35F5
1372099_at	-1.863	FAM21A	1388755_at	-1.968	SEC23A
1372102_at	-1.687	C20ORF191	1388783_at	-1.596	HMGB1
1372124_at	-2.491	EIF4B	1388809_at	-1.757	SMPDL3A
1372149_at	-1.684	AUH	1388815_at	-1.933	SAPS1

1372158_at	-1.531	MACROD1	1388817_at	-1.578	FAM63A
1372214_at	-1.645	MRPS33	1388823_at	-2.642	RAB5B
1372217_at	-1.727	TMEM199	1388831_at	-1.735	SLC9A3R2
1372281_at	-1.768	LYPLAL1	1388833_at	-1.548	POLE3
1372284_at	-2.080	TRAPPC3	1388877_at	-1.718	MRPS5
1372286_at	-1.558	TSPAN6	1388908_at	-1.755	PECI
1372295_at	-1.635	NARF	1388913_at	-1.978	PPAP2C
1372306_at	-1.987	ETHE1	1388965_at	-2.086	PPP2R5E
1372310_at	-1.667	ISOC1	1388976_at	-1.507	BOLA3
1372372_at	-1.894	CMBL	1388995_at	-2.779	RNF14
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1372394_at	-2.382	HECTD1	1389128_at	-2.226	WDFY3
1372395_at	-1.632	MARCH6	1389139_at	-2.060	TTC15
1372408_at	-1.731	GGA2	1389146_at	-1.547	FAM107B
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1372562_at	-1.682	C8ORF82	1389351_at	1.854	LRRFIP1
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1372597_at	-1.671	MRPL14	1389363_at	-1.829	ADI1
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1372663_at	-1.616	PTDSS2	1389567_at	-2.071	SCAP
1372720_at	-1.530	BTBD1	1389676_at	-1.612	CCDC101
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1372946_at	1.654	CXORF40A	1390591_at	-1.582	SLC17A3
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1373469_at	-2.332	RGD1565496	1392543_at	-1.903	RBBP4
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1373523_at	2.919	FCGR3A	1392916_at	-1.827	MAP7
1373547_at	-1.985	C7ORF25	1392929_at	-1.642	C20ORF194
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1373686_at	-1.864	SERPINA6	1393005_at	-1.500	SFT2D2
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1373874_at	-1.871	SGPP1	1393171_at	-1.707	TMEM47
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1374303_at	-1.505	ALKBH2	1395565_at	-1.691	COPS4
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1374396_at	-1.733	ATP6V1C1	1396112_at	-1.676	MTMR10
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1375170_at	2.108	GM5068	1398282_at	-3.201	KYNU
1375173_at	-1.523	PLBD2	1398286_at	-1.814	CSAD
1375298_at	-1.664	PPP2R5C	1398295_at	-1.525	SLC29A1
1375357_at	-1.570	TOR1A	1398341_at	-1.625	CISD3
1375429_at	-1.864	ZH16	1398350_at	2.334	BASP1
1375431_at	-1.752	C2ORF69	1398472_at	-1.651	C10ORF2
1375524_at	-1.949	ARID1A	1398514_at	-2.151	HGD
1375536_at	-1.919	NUMB	1398591_at	2.643	CCRL2
1375634_at	-2.387	CCDC53	1398642_at	-2.039	MTRR
1375638_at	-1.547	SDPR	1398808_at	-1.999	IMPA1
1375869_at	-2.210	ULK1	1398891_at	-2.331	MRPL15
1375934_at	-1.967	RNF128	1398902_at	-1.737	KIAA0664
1375951_at	1.799	THBD	1398976_at	-1.956	C20ORF191

1375977_at	-1.805	CETN2	1398981_at	-2.146	TRIAP1

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