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INFLAMMATION AND IDIOSYNCRATIC DRUG REACTIONS: INFLAMMATORY MECHANISMS AND INTERACTIONS IN A MURINE MODEL OF TROVAFLOXACIN HEPATOTOXICITY

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INFLAMMATION AND IDIOSYNCRATIC DRUG REACTIONS: INFLAMMATORY MECHANISMS AND INTERACTIONS IN A MURINE MODEL OF TROVAFLOXACIN HEPATOTOXICITY

By

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ABSTRACT

INFLAMMATION AND IDIOSYNCRATIC DRUG REACTIONS: INFLAMMATORY MECHANISMS AND INTERACTIONS IN A MURINE MODEL OF TROVAFLOXACIN HEPATOTOXICITY

By

Patrick Joseph Shaw

Drug-induced liver injury is the leading cause of acute liver failure in the United States and is a major concern for both public health and the pharmaceutical industry. Idiosyncratic adverse drug reactions (IADRs), a rare form of drug-induced liver injury, have been the reason for the majority of postmarket regulatory actions on drugs. The liver is often a target of IADRs. IADRs are characterized by the toxicity being unrelated to the pharmacology of the drug and do not demonstrate obvious dose or time dependence. The erratic occurrence and lack of mechanistic evidence makes IADRs very difficult to predict. Hepatotoxicity induced by the fluoroquinolone antibiotic trovafloxacin (TVX) exhibited these characteristics. The mechanism underlying TVX-induced idiosyncratic hepatotoxicity is unknown. We and others have hypothesized that an inflammatory stress, commonplace and erratic in people, could alter the threshold for toxicity of certain drugs precipitating an IADR.

This dissertation tested the hypothesis that an inflammatory stress could precipitate idiosyncrasy-like TVX hepatotoxicity in mice. Administration of a nonhepatotoxic dose of TVX 3 h before a nonhepatotoxic dose of either lipopolysaccharide (LPS) or peptidoglycan-lipoteichoic acid mixture caused

significant hepatocellular necrosis and apoptosis. Levofloxacin (LVX), a fluoroquinolone antibiotic without IADR liability in humans, did not interact with LPS to cause hepatotoxicity. The remaining studies focused on understanding the mechanisms underlying TVX/LPS-induced liver injury.

Gene expression analysis at a time before the onset of liver injury segregated mice to their respective treatment groups. Therefore, gene expression analysis was able to distinguish TVX/LPS-treated mice from all other treatment groups.

Furthermore, LPS-induced increases in TNFα, IFNγ, thrombin activation, PAI-1 and VEGF were enhanced by TVX. The progression of TVX/LPS-induced liver injury was dependent on PMN activation, TNFα, IFNγ, thrombin activation, PAI-1 and VEGF. Based on this finding, mice were killed at a time near the onset of liver injury to explore how these mediators of inflammation interact with one another and the cascade of events which leads to TVX/LPS-induced hepatotoxicity. TNFα, IFNγ, PAI-1 and VEGF potentially interacted to form several cycles of dysregulated inflammation. These potential vicious cycles of inflammation might be involved in TVX/LPS-induced liver injury.

In summary, novel proinflammatory properties and potential cycles of inflammation were identified which might be involved in various models of inflammatory tissue injury. Additionally, these studies support the possibility of predicting and identifying mechanisms underlying IADRs by utilization of a drug/LPS coexposure model.

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LIST OF ABBREVIATIONS

ALT	alanine aminotransferase
ANOVA	analysis of variance
ATP	adenine triphosphate
cAMP	cyclic adenosine monophosphate
CYP	cytochrome P450
DAG	sn-*1,2*-diacylglycerol
DILI	drug-induced liver injury
DNA	deoxyribonucleic acid
egr-1	early growth response-1
Erk	extracellular regulated kinase
FAK	focal adhesion kinase
FDA	U.S. Food and Drug Administration
Flk-1	VEGF receptor 2
Flt-1	VEGF receptor 1
HIF-1α	hypoxia-inducible factor α
HPC	hepatocyte
IADR	idiosyncratic adverse drug reaction
IFNγ	interferon γ
IFNGR1	interferon γ receptor 1
IFNGR2	interferon γ receptor 2
IKK-i	IκB kinase-i
IL-1	interleukin 1
IL-4	interleukin 4
IL-6	interleukin 6
IL-10	interleukin 10
IL-12	interleukin 12
IL-15	interleukin 15
IL-18	interleukin 18
IP-10	IFNγ-inducible protein 10
IP_3	inositol (1,4,5)-triphosphate
IRAK	IL-1-receptor-associated kinase
IRF3	interferon regulatory factor 3
Jak1	janus activated kinase 1
JNK	c-jun-N-terminal kinase
KC	keratinocyte chemoattractant
LBP	LPS-binding protein
LPS	lipopolysaccharide
LTA	lipoteichoic acid
LVX	levofloxacin
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein 1α
MIP-2	macrophage inflammatory protein 2

MPO	myeloperoxidase
MyD88	myeloid differentiation factor 88
NFκB	nuclear factor-κB
NK cells	natural killer cells
NKT cells	natural killer T cells
p55	TNF receptor 1
p75	TNF receptor 2
PAI-1	plasminogen activator inhibitor 1
PAR-1	protease activated receptor-1
PBMC	peripheral blood mononuclear cell
PGN	peptidoglycan
PI	pharmacologic interaction
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol (4,5)-biphosphate
PKC	protein kinase C
PLCγ	phospholipase C γ
PMN	neutrophil
PTX	pentoxifylline
RIP	receptor-interacting protein
ROS	reactive oxygen species
Sck	Shc-like protein
SOD2	superoxide dismutase 2
STAT1	signal transducer and activator of transcription 1
TAB-2	TAK1 binding protein
TACE	TNFα-converting enzyme
TAK1	transforming growth factor-b-associated kinase
TAT	thrombin:antithrombin
TBK1	TANK binding kinase 1
TCR	T cell receptor
TIRAP	Toll/IL-1R domain-containing adapters
TLR	toll-like receptor
TNFα	tumor necrosis factor α
t-PA	tissue plasminogen activator
TRAF6	tumor necrosis factor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-containing adapter molecule
TVX	trovafloxacin
u-PA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VRAP	VEGF receptor-associated protein

CHAPTER 1

General Introduction and Specific Aims

1.1 Idiosyncratic adverse drug reactions

1.1.1 Overview of idiosyncratic adverse drug reactions

Adverse drug reactions are a serious problem for not only the public health, but also for pharmaceutical companies and drug-regulatory agencies. In a study in the United Kingdom, adverse drug reactions accounted for more than 6% of hospital admissions. Of these admissions due to adverse drug reactions, the mortality rate was 2% (1). In addition to the risk to public health, adverse drug reactions are a major issue for drug development. A significant amount of time and money is expended in the effort to predict the risk of adverse reactions from drug candidates. Despite comprehensive preclinical drug testing and clinical trials, over 10% of drugs approved during 1975-2000 were either withdrawn from the market or have been highly restricted in use (2). In 1998, the pharmaceutical industry spent over 20 billion dollars on drug discovery and development, with screening assays and toxicity testing accounting for about 20% of the total amount spent (3). Despite such extensive efforts, in 1999 over 258,000 post-marketing adverse events were reported in the United States, suggesting that this is a persistent major issue (4).

Adverse drug reactions can occur in any number of tissues, but the liver is often the target organ. Of the 28 drugs removed from the US market between 1976 and 2005, 6 were withdrawn due to hepatotoxicity (5). Drug-induced liver injury (DILI) accounts for more than 50% of acute liver failure cases (6). It is associated with significant mortality; therefore, a number of drugs which have

been associated with DILI have been removed from the market. For example, bromfenac (7), troglitazone (8, 9) and tienilic acid (10) have been completely removed from the market due to hepatotoxicity. In addition, hepatotoxicity of other drugs such as trovafloxacin (TVX) (11, 12), nefazodone (13), and nevirapine (14, 15) has led to "black box" warnings limiting their use. DILI is the leading cause for the withdrawal of drugs from the market by either the U.S. Food and Drug Administration or pharmaceutical companies (16).

An important subset of adverse drug reactions which cause DILI are idiosyncratic adverse drug reactions (IADRs), which account for 13-17% of all cases of acute liver failure (6, 17). IADRs typically occur in a small fraction of people (generally < 1%) within the range of doses used clinically. The exact mechanisms underlying IADRs are unknown but typically do not involve the pharmacological properties of the drug. In addition, IADRs lack an obvious dose-dependence, meaning that a dose which causes toxicity in some patients does not in others. Another characteristic of IADRs is that the onset of toxicity relative to the duration of drug therapy is variable. Finally, there is a wide range in the severity of the reactions depending on the drug and individuals.

Despite extensive research, animal models do not exist which reproduce the hepatotoxicity caused by IADRs. The development of animal models is necessary to predict those drugs which cause IADRs and to decrease human suffering. A predictive animal model would be beneficial for several reasons. Prediction of drug candidates that could cause IADRs would prevent their development into marketed pharmaceuticals and thereby reduce risk to public

health. In addition, it would prevent pharmaceutical companies from sending such candidates to clinical trials or to market and would thereby save money spent on clinical trials, marketing and potential lawsuits from patients affected by IADRs.

Drugs which lead to IADRs are usually not identified in preclinical testing due to their typically rare occurrence and the use of relatively small numbers of animals in toxicity testing. The inability of animal tests to predict IADRs may be due, in part, to the reaction being idiosyncratic in animals as well as humans, and thus an extremely large number of animals would be needed to detect toxicity. It has been estimated that to predict an IADR confidently, toxicity testing would require 30,000 animals to be treated (18). In addition, the current animal testing paradigms might not include sufficient biological diversity to elucidate IADR toxicities. Since such large studies are not possible for drug candidates, it is critical that the modes of action of IADRs are better understood to develop predictive models.

1.1.2 Hypothesized mechanisms of idiosyncratic adverse drug reactions

Despite extensive research, the mechanisms underlying IADRs remain poorly understood and incompletely characterized. There exist several obstacles to understanding IADRs. A substantial challenge is that an animal model for the early detection of hepatic IADRs is currently unavailable. In addition, the tissue from afflicted individuals is often difficult to obtain for research purposes, although the DILI network is trying to address this obstacle. Even when tissue

from affected individuals is available, the tissue would have been harvested long after injury developed and is likely, therefore, to be of limited value for mechanistic studies. However, despite such limitations and difficulties, progress has been made in understanding IADRs. Such progress has led to the development of several diverse theories about IADR pathogenesis. To this point, none of the hypotheses to explain IADR pathogenesis have been proved or disproved. The prevalent hypotheses to explain IADR toxicity and supporting experimental evidence are described in more detail below.

Reactive Intermediate Hypothesis

One theory for the mechanism of IADRs is that a drug is metabolized into a reactive metabolite, which might bind with important cellular proteins, damage membrane integrity, alter calcium homeostasis or other intracellular signaling in ways which could lead to toxicity and that susceptible individuals have polymorphisms in the bioactivating enzyme(s) (19). Indeed, there are several cases in which a drug linked with IADRs has the ability to form a metabolite which is reactive (20). The reactive intermediate hypothesis can be closely associated with all of the hypotheses to be described, especially if a reactive metabolite and not the parent drug is the agent involved in the toxicity.

Troglitazone is an antidiabetic drug which was linked with serious idiosyncratic hepatotoxicity (9). Research conducted after troglitazone was removed from the market showed that it is metabolized in the rat to five intermediates with the ability to form glutathione conjugates that appear in bile

(21). In addition, metabolic activation by cytochrome P450 3A4 (CYP3A4) forms reactive metabolites which bind to proteins and nucleophiles (22). Whether these form protein adducts that play a role in toxicity is unknown. In addition, if the protein adducts are formed and involved in toxicity, the degree of protein adducts that constitute a threshold for troglitazone IADRs is unknown. Furthermore, several drugs which form reactive metabolites are not associated with an increased risk of IADRs (23). Moreover, one would expect an "intrinsic" (dose-related) toxicity picture in the absence of some metabolism-related sensitivity factor that renders a small fraction of patients susceptible to IADRs. Thus, although the reactive intermediate hypothesis is a reasonable one, a causal link between reactive metabolite generation and hepatotoxicity has not been established conclusively for drugs that cause IADRs.

Genetic Polymorphism Hypothesis

A related theory is that genetic polymorphisms among individuals can cause differences in the toxic responses of individuals to drugs. Many polymorphisms can lead to drug metabolism differences among individuals, leading to differences in pharmacokinetics and reactive intermediate formation (24). Human polymorphisms in genes encoding cytochrome P450 drug metabolizing enzymes have been identified and could lead to differences in drug metabolism and clearance that could render some individuals more susceptible to toxicity. In addition, it is possible that a polymorphism in drug metabolizing

enzymes might lead to the formation of a reactive intermediate not seen in the majority genotype.

Alternatively, a genetic polymorphism in a protective gene, such as an anti-inflammatory cytokine, might render individuals more susceptible to normally nontoxic doses of drugs, resulting in an IADR. For this hypothesis to explain IADRs, the genetic polymorphism of people on drug therapy would have to be as rare as the IADR itself or the IADR would have to be a result of a rare combination of several more common polymorphisms. Even if this explains the rarity of IADRs, the genetic polymorphism hypothesis does not explain other characteristics of IADRs such as the variability in the onset of toxicity.

An example often referenced by supporters of the importance of polymorphisms is toxicity caused by isoniazid, a first-line drug used in the prevention and treatment of tuberculosis. Isoniazid has been linked to several cases of liver injury (25). The susceptibility of individuals to isoniazid-induced liver injury has been linked to a polymorphism resulting in a rapid acetylator phenotype (25, 26). It was hypothesized that the rapid acetylators produce more of a reactive metabolite which causes hepatocellular necrosis. However, several epidemiological studies failed to find an association between the rapid acetylation polymorphism and liver injury (27). Another example of this hypothesis is evident from a study in which individuals were treated with the idiosyncratic drug, diclofenac. It was found that individuals who developed a toxic response had a greater rate of polymorphisms in the interleukin 10 (IL-10) and interleukin 4 (IL-4) genes than the group of individuals who did not develop a toxic response to

diclofenac (28). An association between IADRs and genetic polymorphisms does exist with some drugs; however, their roles remain uncertain, and it remains likely that other factors play a role in precipitating IADRs.

Hapten Hypothesis

A widely accepted theory to explain IADRs is that they result from an adaptive immune response. Some clinical characteristics of IADRs such as the delayed onset of toxicity, the lack of a simple dose-response relationship and eosinophilia have led some to postulate that IADRs are mediated by adaptive immunity (29). This has led to the formation of two related hypotheses. The hapten hypothesis states that a chemically reactive drug or a reactive metabolite binds to an endogenous protein. This protein adduct is then seen as a foreign antigen capable of initiating immunological recognition (30). According to this hypothesis, the drug-modified protein must be processed by antigen-presenting cells and presented to T cells. This results in sensitization of the T cells to the foreign antigen. The immune system develops memory to the foreign antigen, and upon subsequent exposure to the drug, robust immune system activation occurs, resulting in the formation of autoantibodies and/or the activation of cytotoxic T cells targeting self proteins (31). It is important to understand that both sensitizing and challenging exposures are required in this hypothetical mechanism.

In support of the hapten hypothesis, the presence of autoantibodies has been detected in patients with hepatic IADRs after exposure to several drugs,

including diclofenac, troglitazone, halothane and tienilic acid (28, 32). The study which found autoantibodies in the sera of patients who experienced diclofenac hepatotoxicity also reported the presence of autoantibodies in some patients treated with diclofenac who did not develop hepatotoxicity (28). Such a finding was also found in halothane-treated patients, in whom autoantibodies were found whether they developed toxicity or not (33). Thus, from these reports, a clear cause and effect relationship between autoantibodies and idiosyncratic hepatotoxicity is lacking. The clinical evidence supporting the role of the adaptive immune system may in some cases be explained by immune system activation occurring secondary to tissue damage. Efforts have been undertaken to show the involvement of the specific immune system in hepatotoxic IADRs; however, in all of the current animal models of drug immunogenicity, an adaptive immune response was detected in the absence of liver damage (34). Accordingly, experimental support for this hypothesis is incomplete, and an animal model of drug hepatotoxicity with an adaptive immune mechanism has not emerged so far.

The danger hypothesis

A theory closely related to the hapten hypothesis described above is the danger hypothesis, which proposes that a damaging immune system activation occurs only if the drug binds to a protein which causes some type a of a stress response, such as inflammation or cell death, resulting in a 'danger' signal (35). Thus, according to the danger hypothesis, the formation of a drug-protein adduct is insufficient to cause injury, a secondary signal during sensitization such as

mild cell death or cytokine release then results in adaptive immune system activation and pathogenesis (36). It has been postulated that reactive drug metabolites themselves could cause this danger signal, and this is what determines which reactive metabolites lead to IADRs (37, 38). However, the 'danger' signal could be from a number of independent factors including an infection causing an innate immune response, resulting in an inflammatory stress.

The pharmacological interaction (PI) hypothesis

The PI hypothesis is closely related to the hapten and danger hypotheses, in that it suggests an active role for the adaptive immune system in the development of IADRs. The PI hypothesis proposes that drugs bind reversibly to the major histocompatibility complex (MHC) and T cell receptor (TCR) complex. It is hypothesized that the drug then acts like a superantigen to elicit an adaptive immune system response, precipitating an IADR (39). Much of the early work leading to the development of the PI hypothesis was done with sulfamethoxazole; which caused proliferation of T cells isolated from sulfamethoxazole IADR patients (40). However, there is no evidence that an IADR drug binding to the MHC-TCR complex is capable of eliciting an immune response. The role as a possible superantigen to the MHC:TCR complex has not not been shown with any other drugs linked with hepatotoxic IADRs. In addition, evidence is also lacking in support of a causal link between an adaptive immune response and the precipitation of a hepatotoxic IADR.

Mitochondrial dysfunction hypothesis

Another hypothesis for IADRs is that mitochondrial dysfunction and disturbances in mitochondrial integrity by oxidative stress are an underlying cause. Mitochondria play a critical role in providing the cell with energy, controlling the process of apoptosis and regulating intracellular oxidative stress. Mitochondrial dysfunction can encompass several changes such as decreased adenosine triphosphate (ATP) production, mitochondrial reactive oxygen species (ROS) production or depolarization of the mitochondrial membrane potential.

One way in which mitochondrial dysfunction can occur is through DNA alteration. Mitochondrial DNA alterations which could result in dysfunction are rare but are seen in humans. It was found in a epidemiological study that >12 in 100,00 people either had mitochondrial DNA disease or were at risk to develop it; these results reflect the minimum prevalence of mtDNA disease and pathogenic mtDNA mutations (41). It is hypothesized that either a mitochondrial disease or polymorphism could alter mitochondrial function and render cells sensitive to a drug, resulting in idiosyncratic toxicity (42). It is also postulated that genetic or acquired mitochondrial abnormalities can lead to silent and gradually accumulating mitochondrial injury which reaches a threshold and abruptly triggers liver injury (43).

There is extensive evidence linking IADR drugs with mitochondrial alterations. Troglitazone, tolcapone, diclofenac, valproic acid, and isoniazid are some of the drugs which cause IADRs and which have mitochondrial liability in hepatocytes (43-48). In addition, diclofenac and troglitazone are cytotoxic to

HepG2 cells through a mitochondrial mechanism (49, 50). In one study, superoxide dismutase 2 (SOD2) heterozygote mice, a model of silent mitochondrial abnormality, were chronically treated with troglitazone. This treatment had no effect on wild-type mice but resulted in hepatocellular necrosis in $SOD2^{+/-}$ mice (51). However, the hypothesis fails to explain the apparent lack of dose dependence that characterizes IADRs. In addition, there are several drugs that cause mitochondrial alterations *in vitro* but have not resulted in adverse drug reactions in people.

It is of importance to note that mitochondrial dysfunctions can be induced by a number of independent factors such as xenobiotics which might be taken concurrently, hypoxia or inflammation. Therefore, it is possible that alterations in mitochondrial function play a role in other hypothesized mechanisms of IADRs.

Failure to adapt hypothesis

Another hypothesis of IADRs is that a small fraction of people develop minor liver toxicity in response to a drug. Most of these individuals "adapt" and experience a resolution of liver injury even in the continued presence of the drug. However, it is proposed that a small fraction of these people fail to "adapt", and the injury progresses to overt toxicity (52). Reports of isoniazid hepatotoxicity seem to support this theory, inasmuch as 15% of patients taking isoniazid experience minor alanine aminotransferase (ALT) elevations, but less than 1% develop symptomatic hepatitis with continued treatment (53).

The mechanisms underlying the "adaptation" phenomenon are unknown. Adaptation may not be recognized in clinical trials because drug treatment is stopped when the serum ALT activity rises to greater than 3 times the upper limit of normal, making it impossible to distinguish between patients who would and would not adapt. In addition, there are currently few animal models in which adaptation can be studied. However, future studies made possible by the DILI Network will attempt to address these issues and determine possible reasons for increased susceptibility of certain individuals to IADRs. It is also of importance to note that the 'failure to adapt' hypothesis does not discount other hypotheses of IADRs, as toxicity may be due to any number of mechanisms to which certain individuals cannot adapt and therefore experience an IADR.

Multiple determinant hypothesis

The multiple determinant hypothesis proposes that idiosyncratic reactions are the result of multiple, discrete but necessary factors or processes all occurring simultaneously (54). Each factor has an independent probability of occurring, but all of them are required to precipitate an IADR, thus accounting for the rare occurrence rate. According to the hypothesis, an idiosyncratic reaction would only occur in an individual if all the critical steps occur within an appropriate time. An equation for the probability of an idiosyncratic reaction is proposed below:

$$P_{IADR} = P_{chem} \times P_{exp} \times P_{env} \times P_{gene}, \text{ where,}$$

P_{IADR} is the probability of an IADR, P_{chem} is the probability contributed by chemical properties, P_{exp} is the probability determined by the drug exposure to the critical organ(s), P_{env} represents probabilities determined by environmental factors (drug coexposure, inflammation, etc.) and P_{gene} is the probability related to genetic factors (54).

The multiple determinant hypothesis is a rather general and encompassing hypothesis which takes into account the other hypotheses mentioned above. However, it is important to understand in more detail the mechanistic aspects of IADRs to develop predictive animal models. Inasmuch as environmental and genetic factors might play a role in the probability of a specific drug causing an IADR, it is important to determine which factors are important to toxicity and why.

The hypothesis implies that an underlying factor has the potential to lower the toxicity threshold of a drug, rendering a normally therapeutic dose toxic. Several factors have the potential to affect the susceptibility of an individual to drug toxicity including age, gender, coexposure to other pharmacological agents, drug metabolism differences, and state of health.

Inflammatory stress hypothesis

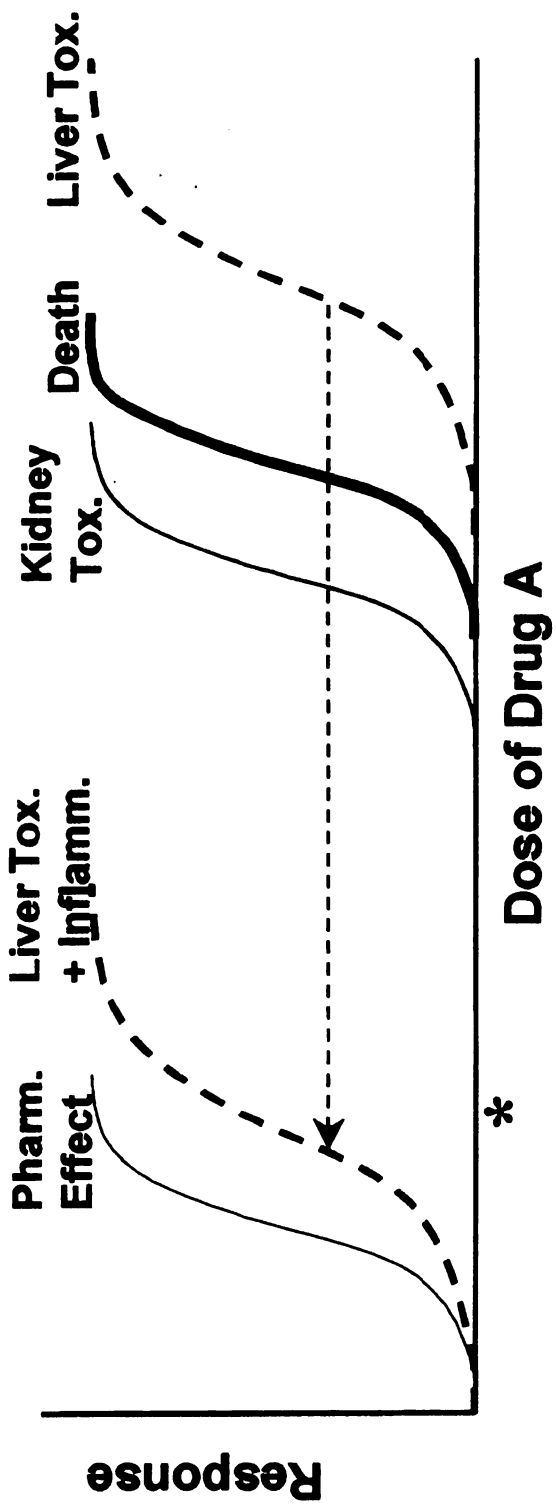
In the multiple determinant hypothesis, one environmental factor that might render an individual sensitive to a normally nontoxic drug dose is inflammatory stress. This idea has led to the inflammatory stress hypothesis,

which states that an episode of inflammation has the potential to interact with concurrent drug therapy to precipitate an IADR.

Inflammatory episodes are commonplace in people and occur erratically throughout life. Many are modest enough that they go unnoticed. A hypothetical relationship between inflammation and IADRs is illustrated in Fig. 1.1. For therapeutically useful drugs, the pharmacologic effect is seen at much smaller doses than signs of toxicity. Most drugs are developed so that the range between a therapeutic dose and the smallest toxic dose (ie., the therapeutic window) is as large as possible. As dose is increased, toxicity is seen (such as kidney toxicity in Fig. 1.1) and death ensues at large doses. Liver toxicity in this example is not observed because the toxicity threshold lies at doses higher than those that are lethal. The hypothesis is that a modest inflammatory stress can decrease the threshold for hepatic toxicity, thereby shrinking the therapeutic window and resulting in a toxic response at a normally safe and pharmacologically effective dose of the drug. In this case, an IADR would occur at a dose which is nontoxic to individuals not experiencing a concurrent inflammatory episode. The erratic nature of inflammatory episodes can explain the unpredictable nature of IADRs.

Lipopolysaccharide (LPS), a component of gram-negative bacterial cell walls, is one agent that can induce an inflammatory stress as described in more detail below. Experimental models have been developed in which nontoxic doses of IADR-causing drugs are rendered hepatotoxic upon coexposure to a nontoxic dose of LPS. For example, rats became susceptible to hepatotoxicity from several drugs known to cause IADRs when they were concurrently exposed to a

Fig. 1.1. Hypothetical relationship between inflammation and drug idiosyncrasy. Drug A is a relatively safe and efficacious drug. The asterisk indicates the usual therapeutic dose. The safety margin between pharmacological effect and kidney toxicity is quite large. A modest inflammatory response shifts the threshold for liver toxicity and precipitates and idiosyncratic response (55).


Response
Dose of Drug A
Pharm. Effect
Liver Tox. + Inflamm.
Kidney Tox.
Death
Liver Tox.
*

nontoxic dose of LPS. Drugs known to cause IADRs in humans such as trovafloxacin, ranitidine, sulindac, chlorpromazine and diclofenac were all rendered hepatotoxic to rats when coupled with a nontoxic dose of LPS (56-60) (Table 1.1). Drugs in the same pharmacologic class which were not associated with IADRs in humans were used when available. These drugs not associated with human IADRs did not interact with inflammatory stress to cause hepatotoxicity in animal models (58, 59).

Of the drugs tested, only the ones linked with IADRs in humans interacted with a concurrent inflammatory stress to cause hepatotoxicity in rats. This concordance suggested a potential role for inflammation in the mechanism of human IADRs. The results in animal models suggest that an inflammatory episode caused by LPS or other factors could render an individual susceptible to hepatotoxicity at normally nontoxic drug doses, thus causing an idiosyncratic reaction. A challenge still lies in understanding mechanisms of the hepatotoxicity observed with coexposure to LPS and an IADR-causing drug. The remainder of the Introduction and subsequent chapters of the thesis will explore inflammatory stress in greater detail and present work to develop and explore an inflammation/drug interaction model of TVX toxicity in mice.

Table 1.1. Concordance of LPS/drug coexposure model in rats for IADR-causing drugs in humans

Drug	Linked to hepatotoxicity in humans?	LPS/drug coexposure hepatotoxic to rats?
Trovafloxacin	Yes	Yes
Levofloxacin	No	No
Chlorpromazine	Yes	Yes
Ranitidine	Yes	Yes
Famotidine	No	No
Sulindac	Yes	Yes
Diclofenac	Yes	Yes

1.2 Inflammation

1.2.1 Overview of inflammatory stress

Inflammation is an innate immune system process critical for the host's defense against infection and foreign substances. The inflammatory response is a complex process encompassing the recruitment of cells, release of cytokines and other biologically active mediators, vasodilation, hemostatic system activation and complement activation. The magnitude of an inflammatory response depends on the cause and varies from one individual to the next. Modest inflammatory episodes occur sporadically and are commonplace in people. Inflammation occurs in response to a number of stimuli including tissue injury, microbial pathogens and other foreign substances.

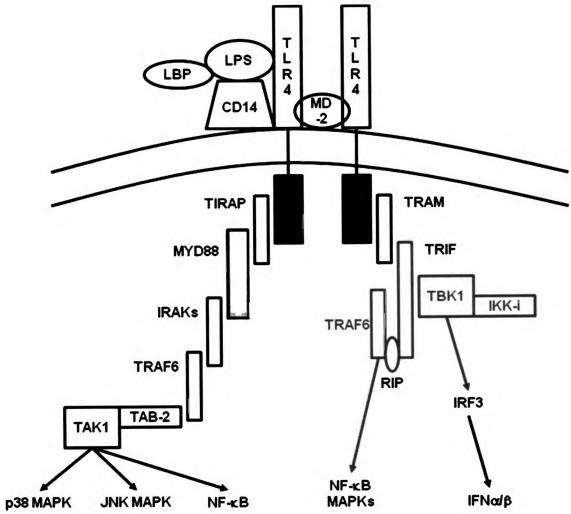
As mentioned above, recognition of microorganisms by various cell types within the body induces an inflammatory response. Components of gram-negative bacteria have been measured in the plasma of individuals and are increased by conditions such as gastrointestinal disturbances, alcohol consumption, surgery, alterations in diet, etc. (55, 61). In turn, a great deal of inflammation research has focused on host responses to gram-negative bacterial cell wall constituents. Endotoxin is a component of gram-negative bacterial cell walls and is released when bacteria undergo cell division or are damaged by antibiotics (62). A major, biologically active component of endotoxin is LPS. Chapter 3 presents some studies exploring the interaction between TVX and gram-positive bacterial cell wall components peptidoglycan and lipoteichoic acid,

which can also induce inflammation. However, the majority of the work will explore in detail the interaction between TVX and LPS. The mechanism by which LPS induces an inflammatory response will described below.

Toll-like receptors (TLRs) are conserved pattern recognition receptors that recognize bacterial components (63). The effects of LPS are elicited primarily through the activation of TLR4. LPS-binding protein and CD14 are required for presentation of LPS to TLR4; and the interaction of the co-receptor MD-2 with dimerized TLR4 is required to elicit activation of TLR4 by LPS (64, 65). After LPS activates TLR4, the resulting responses can be divided into those dependent on myeloid differentiation factor 88 (MyD88) and those independent of MyD88. The signaling pathways activated by TLR4 activation by LPS are described in more detail below and summarized in Fig. 1.2.

The activated TLR4 dimer recruits Toll/IL-1R domain-containing adapters (TIRAP), TIR-containing adapter molecule (TRIF) and TRIF-related adapter molecule (TRAM) (66). TIRAP recruitment and activation results in MyD88 recruitment. MyD88 is an adapter protein which activates inflammatory signaling pathways. The activation of MyD88 leads to the recruitment and phosphorylation of members of the IL-1-receptor-associated kinase (IRAK) family (67). Phosporylated IRAK then dissociates from MyD88 and interacts with tumor necrosis factor receptor-associated factor 6 (TRAF6) (68). Activated TRAF6 associates with TAK-1 binding protein-2 (TAB-2), causing activation of transforming growth factor-β-associated kinase 1 (TAK1). TAK1 is a mitogen-activated protein kinase (MAPK). At this point, TAK1 activates the p38 MAPK

Fig. 1.2. Toll-like receptor 4 signaling pathways. Schematic summary of TLR4 signaling following activation by LPS. LPS-binding protein (LBP) and soluble CD14 assist in the presentation of LPS to the TLR4 dimer. The TLR4 dimer is associated with MD-2 in the cell membrane. Upon LPS binding, Toll/IL-1R domain-containing adapter (TIRAP) and TRIF-related adapter molecule (TRAM) are recruited to the intracellular domain of TLR4. TIRAP recruitment and activation allows for myeloid differentiation factor 88 (MyD88) recruitment and activation. IL-1-receptor-associated kinase (IRAK) binds to the activated MyD88 and is in turn activated. IRAK activation allows for the binding and subsequent activation of tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 binds to a complex formed by TAK-1 binding protein-2 (TAB-2) and transforming growth factor-β-associated kinase 1 (TAK1). The binding of TRAF6 to the TAB-2/TAK1 complex results in TAK1 phosphorylation. TAK1 is a mitogen-activated protein kinase (MAPK) which in turn activates p38 and JNK MAPK pathways and NF-κB. The recruitment of TRAM also results in MyD88-independent signaling. Toll/IL-1R domain-containing-containing adapter molecule (TRIF) binds to TRAM and becomes activated. TRIF then serves as an adapter molecule for the activation of TRAF6, again leading to MAPK and NF-κB activation. TRIF binds to the TANK binding kinase1 (TBK1)/IκB kinase-I (IKK-i) complex which is believed to phosphorylate and activate TBK1. TBK1 phosphorylates and activates the transcription factor interferon regulatory factor 3 (IRF3), the activation of which results in interferon α and β (IFNα/β) expression.

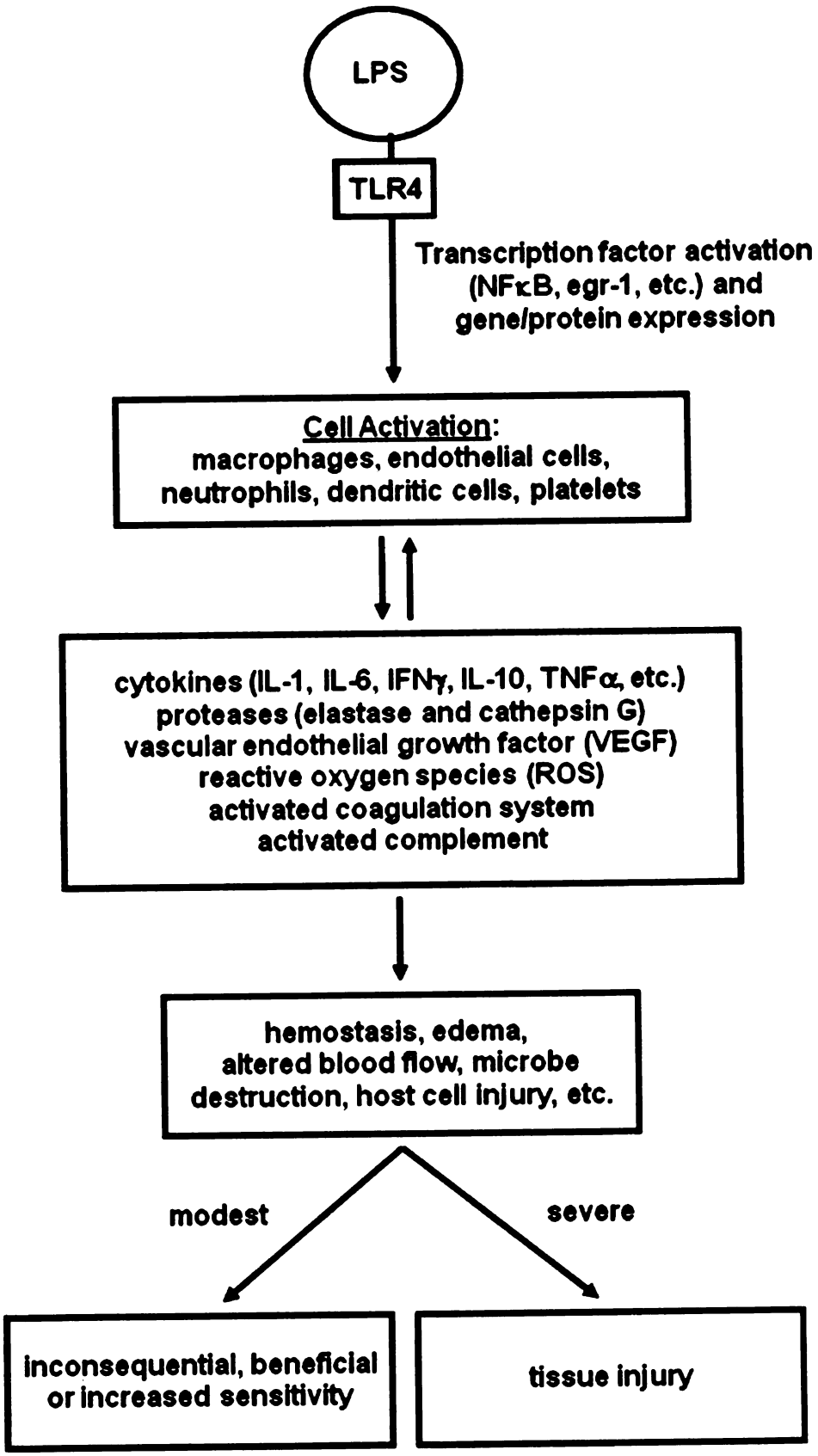

LBP
LPS
CD14
TLR4
MD-2
TLR4
TIRAP
TRAM
MYD88
TRIF
IRAKs
TBK1
IKK-i
TRAF6
TRAF6
RIP
IRF3
TAK1
TAB-2
p38 MAPK
JNK MAPK
NF-κB
NF-κB
MAPKs
IFNα/β

pathways, c-jun-N-terminal kinase (JNK) MAPK pathways, and the inflammatory transcription factor, nuclear factor-κB (NF-κB) (69). NF-κB, p38 and JNK activation leads to gene/protein expression of several mediators of inflammation and cell death (70).

In addition, LPS activation of TLR4 results in a MyD88-independent response via the recruitment of TRAM and TRIF (also known as TICAM1) (66). TRIF then can directly activate TRAF6 with the help of receptor-interacting protein (RIP), resulting in MAPK and NF-κB activation independent of MyD88. In addition, TRIF can bind to the TANK binding kinase 1 (TBK1)/IκB kinase-i (IKK-i) complex (71). TRIF binding of the TBK1/IKK-i complex is believed to phosphorylate and activate TBK1. Activated TBK1 directly activates interferon regulatory factor 3 (IRF3), a transcription factor the activation of which results in interferon α and β expression (72).

TLR4 is a conserved receptor expressed on a number of cell types including macrophages, endothelial cells, neutrophils, dendritic cells and platelets; therefore, these cell types are activated following LPS activation of the signaling pathways described above. TLR4 activation causes NFκB and MAPK pathway activation, which in turn activates other transcription factors such as early growth response-1 (egr-1). The cellular activation and transcription factor activation by LPS leads to the release of cytokines, neutrophil proteases, vascular endothelial growth factor, coagulation system activation and complement activation. This collection of factors induced by LPS can have several profound effects such as hemostasis, edema, altered blood flow, microbe

Fig, 1.3. A summary of the inflammatory process. LPS activates TLR4, leading to transcription factor activation as described in Fig. 1.2. The signal transduction following TLR4 activation causes the activation of several cell types, resulting in several inflammatory processes including the production and release of cytokines: interleukin 1 (IL-1), interleukin 6 (IL-6), interferon γ (IFNγ), IL-10 and tumor necrosis factor α (TNFα). In addition to the production of cytokines, cellular activation will result in neutrophil protease release, vascular endothelial growth factor (VEGF) release, ROS production, coagulation system activation and complement activation. These inflammatory processes can induce several several physiological changes such as hemostasis, edema, altered blood flow, microbe destruction or host cell injury. The degree of inflammatory episode then dictates the result. If modest, the inflammatory episode could be inconsequential, beneficial or could increase the sensitivity of the host to another insult. However, a severe inflammatory episode has the potential to cause direct tissue injury to host.


LPS
TLR4
Transcription factor activation
(NFκB, egr-1, etc.) and
gene/protein expression
Cell Activation:
macrophages, endothelial cells,
neutrophils, dendritic cells, platelets
cytokines (IL-1, IL-6, IFNγ, IL-10, TNFα, etc.)
proteases (elastase and cathepsin G)
vascular endothelial growth factor (VEGF)
reactive oxygen species (ROS)
activated coagulation system
activated complement
hemostasis, edema,
altered blood flow, microbe
destruction, host cell injury, etc.
modest
severe
inconsequential, beneficial
or increased sensitivity
tissue injury

destruction or host cell injury; but can also feedback to cause more cellular activation of macrophages, endothelial cells, neutrophils, dendritic cells, platelets and other cell types. Fig. 1.3 illustrates a simplified version of the inflammatory stress induced by LPS exposure.

The magnitude and location of the inflammatory episode dictate its ultimate effects. If the inflammatory episode is severe and uncontrolled, it can lead to host tissue injury. However, inflammation is essential for the defense of an organism against foreign pathogens. If the inflammatory episode is modest it can lead to one of several consequences: it might not have any effect, it might be beneficial by killing a foreign organism, or the inflammatory stress could sensitize the tissue to another insult potentially resulting in injury (55, 73). Based on this latter condition, we have hypothesized that an inflammatory stress induced by LPS could precipitate a toxic response to a therapeutic and normally nontoxic dose of a drug. The following sections will discuss the role of selected inflammatory factors in liver injury and their interactions with other inflammatory mediators.

1.2.2 Tumor necrosis factor α (TNFα)

TNFα is a pleiotropic cytokine that induces a number of cellular responses that include cell proliferation, production of inflammatory mediators, upregulation of adhesion molecules and programmed cell death. It is a key mediator of inflammatory responses, including both tissue damage and host defense mechanisms (74, 75). TNFα plays a critical role in several models of

hepatotoxicity including endotoxemia, viral hepatitis, acetaminophen hepatotoxicity and ischemia/reperfusion (76-79).

The main cellular sources of TNFα production are macrophages, but several other cell types produce TNFα including mast cells, endothelial cells, stellate cells, fibroblasts and neuronal cells (80, 81). TNFα is produced as a transmembrane protein, which is biologically active, but it may also be released as a soluble form via proteolytic cleavage by TNFα-converting enzyme (TACE) (80). Large amounts of TNFα are produced in response to TLR activation by microbial products, including LPS. Resident liver macrophages, ie, Kupffer cells, are a major source of TNFα production and release following TLR4 activation by LPS in liver (82).

The biological effects of TNFα are elicited via two high affinity cell surface receptors, p55 (TNF-R1) and p75 (TNF-R2) (83). The two TNF receptors are structurally similar but functionally very different. Expression of p55 is found in a wide variety of mammalian cell types, whereas the expression of p75 is typically found only on immune cells (80). Signaling through the p55 receptor is the key mode of TNFα signaling in most cell types. The cells of the lymphoid system are the exception, in which signaling through the p75 receptor plays a major role.

The intracellular domains of p55 and p75 are the main difference between the two receptors. The intracellular domain of the p55 receptor contains a death domain, which couples the receptor's activation to caspase activation and cell death (84). The p75 receptor lacks the death domain. However, both receptors recruit members of the TRAF family when activated. Activation of TRAF proteins

Table 1.2. The effects of TNFα on other mediators of liver injury

Mediator of liver injury	TNFα effects
Neutrophils (PMNs)	• Induces PMN proliferation or apoptosis depending on environment and concentration of TNFα (85) • Induces activation of respiratory burst by PMNs via both the p55 and p75 receptor or potentiates effects of PMN stimuli (86) • Increases PMN adhesion molecules on endothelial cells leading to increased "rolling" (87)
IFNγ	• Induces Th1 response leading to IFNγ production (88)
Hemostatic system	• Increases tissue factor expression on endothelial cells, promoting coagulation system activation (89) • Stimulates IL-6 production, which causes new platelet formation with increased prothrombotic activity (90)
VEGF	• Increases VEGF expression (91) • Synergistically enhances VEGF activation of egr-1 (92)

leads to MAPK activation and NF-κB activation, as described earlier. Ligand activation of the receptors is another functional difference; membrane bound TNFα has the ability to activate both p55 and p75 receptors (80, 93), whereas soluble TNFα only activates the p55 receptor and is the dominant signal for p55 activation (94).

The role of each receptor has been studied in several models of liver injury. The p55 receptor has been studied more extensively in hepatotoxicity, and evidence has emerged for critical roles in endotoxemia, acetaminophen and carbon tetrachloride (77, 95, 96). In contrast, roles for both receptors have been demonstrated only in a few models of hepatotoxicity, such as from concanavilin A, *Pseudomonas aeruginosa* exotoxin A and adenovirus (97-99).

As summarized in Table 1.2, TNFα can modulate several inflammatory factors that contribute to various models of liver injury. The role of TNFα and the effects of TNFα on these inflammatory factors will be explored in an inflammation/drug interaction model of TVX toxicity in mice in subsequent chapters.

1.2.3 Neutrophils

Neutrophils (PMNs) are a major component of the innate immune system. They are recruited to inflammatory sites and are capable of phagocytosing and enzymatically digesting microbes. They circulate in the blood and are recruited to the site of invading microorganisms or dead/dying cells. Proinflammatory signals of microorganisms or dead/dying cells cause the upregulation of selectin

adhesion molecules on both PMNs and endothelial cells. The selectins cause PMNs to bind to and "roll" along endothelial cells. In response to inflammatory cytokines and chemokines, PMNs then increase expression of integrins, i.e. CD11b/CD18, on their cell surface. The expression of integrins causes PMNs to extravasate from the blood and into the tissue. Chemokines released from homeostatically altered cells can contribute to this process. The extravasation of PMNs into the tissue is critical for their antimicrobial function and for cytotoxicity to host cells (100). Infiltrated PMNs can become activated by a number of mediators that cause release of their granules, which contain serine proteases (such as elastase and cathepsin G), myeloperoxidase (MPO), defensins and vascular endothelial growth factor (VEGF). Although PMNs are critical to host-defense against pathogens, the proteases and reactive oxygen species released from activated PMNs can cause tissue injury. Indeed, PMNs play a major role in several models of liver injury including endotoxemia, ischemia/reperfusion and LPS/ranitidine interaction (101-103).

1.2.4 Interferon γ (IFNγ)

IFNγ is a type II interferon and is integral to both the innate and adaptive immune responses. It plays a crucial role in innate immune host defense mechanisms by inducing neutrophil activation and activating macrophage functions such as phagocytosis, respiratory burst and cytokine secretion (104). In addition, IFNγ plays a critical role in the adaptive immune system host defense by promoting secretion of IgG2A antibodies by B cells, antigen presentation,

induction of Th1 cell differentiation and maturation of T cells (104, 105). The importance of IFNγ in host defense is demonstrated by the increased susceptibility of IFNγ$^{-/-}$ mice to a variety of infections (106). However, IFNγ is also an important mediator of inflammatory injury, such as septic shock (107).

The cellular sources of IFNγ are T cells, dendritic cells, natural killer (NK) cells, and natural killer T (NKT) cells. The control of IFNγ production in these cell types is mediated by a variety of factors. NK, NKT and dendritic cells can be stimulated to produce IFNγ by cytokines such as interleukins 12,15 and 18 (IL-12, IL-15, and IL-18) by activated macrophages and dendritic cells (108, 109). In addition, the production of IFNγ by T cells is induced by activation of the T cell receptor complex and interaction of CD28 with B7 proteins (109).

The biological effects of IFNγ are mediated through the IFNγ receptor, which is composed of two integral membrane proteins, IFNGR1 and IFNGR2 (110). The IFNγ receptor is expressed on nearly all cell types. IFNGR1 plays an important role in ligand binding, ligand trafficking through the cell and signal transduction; whereas IFNGR2 plays only a minor role in ligand binding but is required for signaling (110-112). Both subunits are required for eliciting the effects of IFNγ. The intracellular domains of IFNGR1 and IFNGR2 have binding sites for janus activated kinase 1 (Jak1) and Jak2, respectively. Jak1 and Jak2 bind to the intracellular domains in unstimulated cells. IFNγ binds to IFNGR1 thereby generating binding sites for IFNGR2 (113). This oligomerization of the subunits leads to transphosphorylation and activation of Jak1 and Jak2 (114).

Table 1.3. The effects of IFNγ on other mediators of inflammatory liver injury

Mediator of liver injury	IFNγ effects
Neutrophils (PMNs)	• Induces the expression of a wide variety of chemokines and adhesion molecules (115, 116) • Enhances the oxidative burst of activated neutrophils (117-119)
TNFα	• Leads to TNFα production and release from PMNs (120) • Induces TNFα production itself and enhances the LPS-induction of TNFα in Kupffer cells (121)
Hemostatic system	• Enhances LPS-induction of tissue factor (122) • Dampens the fibrinolytic response of endothelial cells to TNFα (123)
VEGF	• Induces VEGF release in monocytes and macrophages (124) • Induces initial VEGF production, but dampens VEGF production at later times (125)

The activated Jaks then phosphorylate an important tyrosine on IFNGR1, creating a docking site for signal transducer and activator of transcription 1 (STAT1) (126). STAT1 is then phosphorylated and in turn activated by the JAKs. Once phosphorylated, STAT1 dislocates from the receptor and translocates to the nucleus. STAT1 then binds specific promoter sites in IFNγ-inducible genes, resulting in increased expression (127). Thus, IFNγ stimulation leads to the expression of a number of antitumor, proapoptotic, and proinflammatory genes.

IFNγ has a number of immunoregulatory functions, generally as a proinflammatory cytokine. IFNγ plays a special role in the liver, where there is an abundance of NK and NKT cells in addition to the presence of conventional T cells. NK and NKT cells represent about 25-40% of isolated liver leukocytes (128-130). It is thus not surprising that IFNγ plays a role in several models of liver injury, including endotoxemia-, acetaminophen- and concavalin A-induced hepatitis (115, 131, 132). In several models, IFNγ has profound effects on other mediators of liver injury such as PMNs, TNFα, the hemostatic system, and VEGF. Some of these proinflammatory effects of IFNγ are summarized in Table 1.3. A comprehensive analysis of the effects of IFNγ in a single model of inflammatory injury has not been done, and this would provide a better understanding of the role of IFNγ in liver injury.

1.2.5 The hemostatic system

The hemostatic system encompasses a complex interaction between platelets, blood vessels, procoagulant factors, coagulation inhibitors and

fibrinolytic factors. The two functional arms, coagulation and fibrinolysis, exist in a delicate balance. The maintenance of such balance is critical to prevent blood loss from tissue and control fibrin deposition to maintain appropriate blood flow to tissue. If the hemostatic system is not tightly regulated, blood flow to tissues is interrupted resulting in tissue ischemia. The significance of the balance of the two arms is illustrated by the high sensitivity of organs to ischemia/reperfusion insult (76, 133, 134).

Coagulation system activation can be initiated by both the extrinsic and intrinsic pathways. The activation of blood coagulation occurs predominantly through the extrinsic pathway, beginning with tissue factor expression. However, the result of both pathways is the cleavage of prothrombin to active thrombin, also called factor II. Thrombin activation is regulated by the endogenous inhibitor, antithrombin III, which binds to and inactivates it. Antithrombin III binding to thrombin forms thrombin:antithrombin (TAT) dimers which can be measured in the plasma as a marker of coagulation system activation.

Thrombin is a protease with a major role in the vascular homeostasis. It plays an important role in fibrin deposition in several ways. Most importantly, it cleaves fibrinogen to fibrin monomers, which polymerize to form fibrin clots (135). In addition, thrombin positively feeds back on the coagulation pathway by activating coagulation factors XI, VIII, and V, which in turn can generate more thrombin (136-138). Also, thrombin elicits diverse biological effects through cleavage and activation of protease activated receptor-1 (PAR-1) (139, 140). PAR-1 is highly expressed on platelets which become activated and aggregate

following thrombin cleavage of PAR-1 (141). Some of the effects of PAR-1 activation are independent of the hemostatic system; for example, PAR-1 activation can stimulate mast cells and monocytes to release proinflammatory cytokines such as IL-1, IL-6 and TNFα (142, 143).

The active dissolution of fibrin clots is critical to maintaining normal blood perfusion of organs; this is controlled by the fibrinolysis arm of the hemostatic system. Fibrin clots are cleaved and dissolved by the serine protease plasmin (135). Plasmin is synthesized in an inactive form, plasminogen, by the liver and secreted into the plasma. Tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA) convert plasminogen to plasmin. The enzymatic activity of t-PA and u-PA is inhibited by plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2) (144).

PAI-1 is the major endogenous downregulator of fibrinolysis. PAI-1 is mainly produced by endothelium, but can also be released by other cell types such as adipose tissue. In addition to its role as a plasminogen activator inhibitor, PAI-1 has several proinflammatory properties such as enhancing PMN activation, inducing TNFα production and increasing VEGF expression (145-147). Thus, an increase in active PAI-1 can increase inflammation and decrease the formation of plasmin, thereby impairing the lysis of fibrin clots.

If the balance of the two functional components of hemostasis is altered, a possible outcome is unregulated activation of the hemostatic system, which could lead to fibrin deposition and occlusive fibrin clots. These clots have the potential to impair local blood flow and result in tissue hypoxia (103). By these and

Table 1.4. The effects of the hemostatic system and hypoxia on mediators of inflammatory liver injury

Mediator of liver injury	Hemostatic system and hypoxia effects
Neutrophils (PMNs)	• Primary hepatocytes are sensitized to PMN elastase-mediated cell death in hypoxic conditions (103) • Hypoxia causes PMN chemokine production by hepatocytes (148) • PAI-1 potentiates LPS-induced neutrophil activation (146)
TNFα	• Hypoxemia causes a significant increase in macrophage release of TNFα (149)
IFNγ	• Hypoxia enhances mature dendritic cell and macrophage IFNγ production in response to LPS or IL-18 (150)
VEGF	• Hypoxia induces VEGF expression through hypoxia inducible factor α (HIF-1α) activation (151) • PAI-1 increases VEGF expression (145)

perhaps other mechanisms, the hemostatic system contributes to liver injury in endotoxemia, acetaminophen hepatotoxicity and ischemia/reperfusion as examples (103, 152-154). The hemostatic system and hypoxia could play a role in liver injury via interactions with various inflammatory mediators, as summarized in Table 1.4. However, interactions between hemostasis and inflammation are still not fully understood. In addition to its interactions with inflammation, hypoxia itself has the ability to cause cell injury directly (155). The role of the hemostatic system and its effects on these inflammatory factors will be explored in an inflammation/drug interaction model of TVX toxicity in mice in subsequent chapters.

One consequence of tissue hypoxia is the stabilization of hypoxia-inducible transcription factors, which translocate to the nucleus where they associate with other factors, bind to transcriptional regulatory elements of DNA and initiate the transcription of genes encoding proteins involved in adaptation to hypoxia and cell death. One of these proteins is vascular endothelial growth factor.

1.2.6 Vascular endothelial growth factor (VEGF)

The VEGF family describes splice variants VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor. The best studied VEGF variant is VEGF-A, therefore, VEGF will refer to VEGF-A. It is a cytokine best known for its function as a stimulator of developmental, adaptive and pathological angiogenesis (156-159). However, beyond its role in vessel development, VEGF also stimulates

differentiation, survival, migration, proliferation, tubulogenesis and vascular permeability in endothelial cells (157, 160, 161). The importance of VEGFs and VEGF receptors (VEGFRs) has been shown in gene targeting studies, where the removal of a single VEGF allele results in embryonic lethality (156, 162). In addition to its role in angiogenesis and development, VEGF is involved in the inflammatory response, which will be discussed in more detail below.

VEGF is produced by endothelial cells, macrophages, activated T cells, and variety of other cell types as a result of multiple stimuli. As mentioned above, one potent stimulus of VEGF production is a low oxygen environment, which results in hypoxia inducible factor α (HIF-1α) stabilization and expression of VEGF (163). The production of VEGF can also be stimulated by a number of inflammatory cytokines including IL-1β, IL-1α, IL-6, oncostatin M, TNFα and IL-8 (164-169).

Members of the VEGF family act on tyrosine kinase receptors. The members of the VEGF family show different affinities for each of these receptors. VEGF acts through specific binding to two receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1). Flt-1 is the only VEGF receptor expressed on monocytes and macrophages, whereas endothelial cells and hematopoietic stem cells express both Flt-1 and Flk-1 (170). Flk-1 is considered to be the major mediator of the effects of VEGF on endothelial cells. Ligand binding of VEGF to the extracellular domain of the receptors causes dimerization and autophosphorylation of intracellular tyrosine residues. Several proteins bind to the phosphorylated tyrosine residues and are activated, including VEGFR-associated protein (VRAP),

Shc-like protein (Sck) and phospholipase γ (PLCγ) (171-173). PLCγ activation leads to the hydrolysis of phosphatidylinositol (4,5)-biphosphate (PIP_2) creating the second messengers *sn*-1,2-diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3). IP_3 binds to a specific receptor on the endoplasmic reticulum to release stores of intracellular Ca^{2+}, whereas DAG is an activator of protein kinase C (PKC). Active PKC initiates a signaling cascade resulting in extracellular regulated kinase (Erk) activation and transcription of several genes. In addition, VEGFR activation results in the activation of several other signaling proteins including phosphoinositide 3-kinase (PI3K), focal adhesion kinase (FAK) and p38 MAPK (174, 175). The mechanisms by which VEGFR activates these proteins are still unknown.

Proinflammatory effects of VEGF have been the focus of recent research. A number of VEGF's effects are mediated through endothelial cells. Indeed, the discovery of VEGF arose from its ability to increase microvascular permeability of endothelial cells (176). In addition, VEGF activates endothelial cells to induce the production of several chemokines and adhesion molecules (177, 178). It has profound effects on several mediators of inflammation as summarized in Table 1.5. In accordance its proinflammatory effects, VEGF is involved in the development of liver injury in animal models of endotoxemia and ischemia/reperfusion (179, 180). The role of VEGF in rat models of drug/LPS-induced liver injury has not been examined, but VEGF and Flt-1 mRNA are selectively upregulated in LPS/ranitidine-treated rats (181). The selective increase in VEGF and Flt-1 was only seen in the group which developed liver

Table 1.5. The effects of VEGF on mediators of inflammatory liver injury

Mediator of liver injury	VEGF effects
Neutrophils (PMNs)	• Induces adhesion molecules on endothelial cells and modulates PMN trafficking (180)
TNFα	• VEGF blockade in a murine model of sepsis reduces plasma TNFα concentration (179)
IFNγ	• Enhances the induction of chemoattractant IFNγ-inducible protein 10 (IP-10) by IFNγ (182)
Hemostatic system	• Induces tissue factor expression which can activate the coagulation system (183) • Induces PAI-1 in endothelial cells (184)

injury, suggesting that VEGF signaling might play a role in LPS/ranitidine-induced hepatotoxicity. The mechanisms by which VEGF might participate in the development of hepatotoxicity remain unclear.

As summarized in Table 1.5, VEGF affects several inflammatory factors that play critical roles in various models of liver injury. The role of VEGF in the development of liver injury and the effects of VEGF on these inflammatory factors will be explored in an inflammation/drug interaction model of TVX toxicity in mice in subsequent chapters.

1.3 Trovafloxacin-induced idiosyncratic liver injury

1.3.1 Overview of trovafloxacin

TVX is an example of a drug linked to human idiosyncratic hepatotoxicity which was not predicted by preclinical testing or evident in clinical trials. The antibacterial mechanism of action of quinolones is through inhibition of type II topoisomerase, bacterial gyrase. This enzyme is critical for bacterial DNA replication, recombination and repair. Interference with bacterial gyrase results in the arrest of bacterial cell growth. TVX is a broad-spectrum fluoroquinolone antibiotic with excellent gram-negative activity and a greater spectrum of bactericidal activity against gram-positive pathogens compared to other fluoroquinolones. In addition to its increased bactericidal spectrum, TVX has high bioavailability after oral dosing and a relatively long elimination half-life, allowing for once-daily dosing. TVX has excellent penetration into various tissues, with highest concentration in the liver, spleen, kidney and lung and the lowest concentration in the brain (185). These qualities made it an extremely attractive drug.

The bactericidal activity of TVX is exerted by the parent compound, not by a metabolite. Approximately 50% of a dose of TVX in humans is recovered unchanged in the feces (43%) and urine (6%). For the portion of TVX that is metabolized, phase II conjugation, specifically glucuronidation, plays a major role (186). The metabolism and clearance of TVX is unique compared to other fluoroquinolones. Conjugative metabolism is common for fluoroquinolones, but

oxidative metabolism is the predominant biotransformation, which is not seen with TVX. Urinary excretion plays a major role in clearance of most fluoroquinolones (187), but not for TVX. The main route of TVX excretion is biliary; only a small amount undergoes renal excretion or oxidative metabolism (188).

Recent metabolic studies using a model cyclopropylamine-containing surrogate molecule for TVX suggested that TVX might be metabolized to a reactive αβ-unstaurated aldehyde (189). The reactive intermediate was only found in the presence of Cyp1A2 or MPO. MPO is released exclusively by activated neutrophils, suggesting that TVX might be metabolized differently in a pro-inflammatory environment to a reactive species that might contribute to TVX toxicity.

1.3.2 Trovafloxacin-induced hepatotoxicity in people

Trovafloxacin was released in February, 1998, as a potent new fluoroquinolone antibiotic. In 1999, reports surfaced linking TVX with hepatotoxicity, which led to severe restriction of TVX uses and prescription. Before restrictions were placed on TVX usage, approximately 2.5 million prescriptions were filled. A total of 140 severe hepatic reactions were reported, making the incidence of TVX-induced severe hepatic reactions approximately 1 in 18,000 prescriptions. Of these 140 severe hepatic reactions, 14 cases led to liver failure, ie, approximately 1 in 178,000 prescriptions (190). Examination of the case reports revealed that duration of TVX therapy in patients did not

correlate with a toxic response. The hepatotoxic events linked with TVX were equal in males and females and were seen in individuals ranging in age from 21-91 years old. This clinical profile of TVX hepatotoxicity classified the toxicity as an IADR.

TVX was the first fluoroquinolone to have its use severely restricted because of liver toxicity (191). In retrospect, the hepatotoxic potential of TVX was seen in dogs at doses exceeding therapeutic doses (190). Some dogs developed elevated liver enzyme signals and centrilobular necrosis. However, because the toxicity was species-specific, reversible and no serious hepatotoxicity was seen in clinical trials, the liver injury observed in dogs was not considered relevant for patients treated with therapeutic doses. Since several other fluoroquinolones have not been associated with human hepatotoxicity, TVX IADRs do not seem to be related to the desired pharmacologic properties of the drug (191).

The pathology of TVX hepatotoxicity was similar in several cases reported. Viral, metabolic and autoimmune causes of hepatitis were excluded in the cases reported (11, 192, 193). A liver biopsy revealed centrilobular hepatocellular necrosis with collapsed sinusoids around the central vein, whereas the portal tracts appeared normal. In addition, parenchyma-based lymphocytes, plasma cells and eosinophils were present, and these inflammatory cells were highly concentrated at the peripheral edges of the centrilobular necrosis (11, 192, 193).

Some signs of hepatic dysfunction have also been linked to other fluoroquinolones such as temafloxacin and ciprofloxacin (194, 195). Temafloxacin was removed from the market due to hemolytic anemia, but it was

also linked to hepatic dysfunction. Both temafloxacin and trovafloxacin share a unique 2,4-difluorophenyl moiety at position 1 of the molecule which is unseen in other fluoroquinolones (193). This moiety is not included in the surrogate molecule mentioned above that formed a reactive metabolite in the presence of Cyp1A2 or MPO (189). It is possible that this structural component is a requirement to idiosyncratic hepatotoxicity of fluoroquinolones.

1.3.3 Interaction between trovafloxacin and inflammation

Clinical reports of TVX hepatotoxicity revealed the presence of inflammatory cells in liver biopsies (11, 192, 193). Based on these findings, it was hypothesized that an inflammatory stress might interact with TVX to induce hepatotoxicity. Indeed, a modest inflammatory stress induced by LPS interacted with a nontoxic dose of TVX to precipitate liver injury in rats (58). This hepatotoxic interaction was not seen with coexposure to LPS and levofloxacin (LVX), a fluoroquinolone lacking the propensity to cause IADRs in humans. TVX given to rats 2 h after a nonhepatotoxic dose of LPS resulted in midzonal lesions of coagulative necrosis (58). The finding that TVX, but not LVX, caused liver injury when administered with LPS, suggested that the interaction between TVX and an inflammatory stress is independent of the desired pharmacology of the drug.

Hepatic gene expression analysis identified a unique profile induced by LPS/TVX-coexposure, including that TVX enhanced the LPS-induced expression of a number of chemokines. The increased chemokine expression suggested a

role for PMNs, which was confirmed by the result that PMN depletion attenuated LPS/TVX-induced hepatotoxicity (58). The selectivity of LPS coexposure to only interact with TVX, and not LVX, to cause liver injury, suggests that inflammatory stress might play a role in TVX hepatotoxicity in humans. However, further study is needed to determine if this interaction between inflammation and TVX is species-specific. In addition, understanding the mechanisms of TVX-inflammation interaction might provide a better understanding of TVX hepatotoxicity in humans.

1.4 Hypothesis and specific aims

The overall hypothesis is that a coexposure of mice to TVX and an inflammatory stress results in idiosyncrasy-like liver injury that is dependent on the following factors: TNFα, IFNγ, hemostatic system activation and VEGF. These factors were chosen based on their involvement in other models of inflammatory liver injury. In addition, these factors were chosen based on the interactions of these inflammatory mediators with one another outlined in Tables 2-5. I hypothesize that these factors create vicious proinflammatory cycles possibly involved in the pathogenesis of liver injury. The goal of the specific aims proposed is to determine which factors are critical for the development of TVX/LPS-induced liver injury and how each mediator fits into the cascade of events leading to hepatotoxicity. These general hypotheses will be addressed by specific hypotheses represented in five specific aims:

Aim 1 Hypothesis: TVX interacts with an inflammatory stress to cause idiosyncrasy-like liver injury in mice. (Chapters 2 and 3)

Aim 2 Hypothesis: TNFα is critical to the development of hepatotoxicity caused by TVX/LPS treatment via interactions with PMNs, IFNγ, VEGF and/or hemostasis. (Chapters 2 and 4)

Aim 3 Hypothesis: TVX/LPS-induced liver injury is dependent on IFNγ, which positively regulates PMNs, TNFα, hemostatic system activation and/or VEGF. (Chapter 5)

Aim 4 Hypothesis: Hemostatic system imbalance is involved in TVX/LPS-induced hepatotoxicity, and this affects PMNs, TNFα, IFNγ and/or VEGF. (Chapter 6)

Aim 5 Hypothesis: VEGF is important in the development of liver injury caused by TVX/LPS treatment via interactions with PMNs, TNFα, IFNγ and/or hemostatic system activation. (Chapter 7)

1.5 Overview and significance of dissertation

The studies proposed outline the development of a murine model of TVX idiosyncratic liver injury and the identification of several factors involved in the pathogenesis. A murine model of idiosyncratic liver injury is potentially of great importance. First, it proves that the LPS/IADR-drug interaction seen in rats is not species-specific. In addition, commonalities across species within the TVX/LPS coexposure model of liver injury might extrapolate to human TVX hepatotoxicity. The development of a murine model of idiosyncratic hepatotoxicity will also prove useful for mechanistic studies through the use of genetically modified mice. Finally, a predictive animal model of IADRs could be added to preclinical testing paradigms to eliminate drug candidates with the potential to cause IADRs in humans. The use of a predictive model might prevent the release of drugs which could be harmful to patients. In addition, the elimination of drug candidates with the propensity to cause IADRs could save pharmaceutical companies millions of dollars in the financial investment in developing drugs that ultimately must be withdrawn from the market.

Identification of the role of TNFα, IFNγ, the hemostatic system and VEGF in TVX/LPS-induced liver injury is important to understanding the mechanism of TVX hepatotoxicity in humans. The roles of IFNγ, the hemostatic system and VEGF in hepatotoxicity have not been extensively studied. In addition, a comprehensive examination as to how these factors affect one another and how they fit into the cascade of events resulting in liver injury has not been reported. Such interactions among inflammatory mediators are not only of importance to

this model of liver injury, but have the potential to be extrapolated to other models of inflammatory tissue injury.

CHAPTER 2

Shaw, P.J., Hopfensperger, M.J., Ganey, P.E., and Roth, R.A. (2007). Lipopolysaccharide and trovafloxacin coexposure in mice causes idiosyncrasy-like liver injury dependent on tumor necrosis factor-alpha. Toxicol Sci. 100(1): 259-266.

2.1 Abstract

IADRs occur in a small subset of patients, are unrelated to the pharmacological action of the drug, and occur without an obvious relationship to dose or duration of drug exposure. The liver is often the target of these reactions. Why they occur is unknown. One possibility is that episodic inflammatory stress interacts with the drug to precipitate a toxic response. We set out to determine if LPS renders mice sensitive to TVX, a fluoroquinolone antibiotic linked to idiosyncratic hepatotoxicity in humans, and if the cytokine TNFα is involved in the development of liver injury. Male mice were treated with a nontoxic dose of TVX followed 3 h later by a nonhepatotoxic dose of LPS. Coexposure to TVX and LPS led to a significant increase in liver injury as determined by plasma alanine aminotransferase activity and histopathological examination. In contrast, coexposure of mice to LPS and LVX, a fluoroquinolone without liability for causing IADRs in humans, was not hepatotoxic. Measurements of TNFα concentration in the plasma revealed a significant, selective increase in TVX/LPS-treated mice at times prior to and at the onset of liver injury. Treatment with either pentoxifylline to inhibit TNFα transcription or etanercept to inhibit TNFα activity significantly reduced TVX/LPS-induced liver injury. The results suggest that the model in mice is able to distinguish between drugs with and without the propensity to cause idiosyncratic liver injury and that the hepatotoxicity is dependent on TNFα.

2.2 Introduction

As described in Section 1.1, the ability of an inflammatory stress to potentiate the hepatotoxicity of numerous xenobiotic agents has led us to hypothesize that inflammatory stress could alter the toxicity threshold of certain drugs precipitating idiosyncratic toxicity (55, 73). In rats, a modest inflammatory episode induced by LPS administration potentiates the hepatotoxicity of several idiosyncratic drugs including ranitidine, chlorpromazine, diclofenac, sulindac and trovafloxacin (56-60). Such inflammation-drug interaction leading to hepatotoxicity has not been demonstrated in mice. The purpose of this study was to test the hypothesis that an inflammatory stress induced by LPS would potentiate TVX hepatotoxicity in mice. Furthermore, the study tested the hypothesis that LVX, a fluoroquinolone without idiosyncratic liability, would not interact with LPS to cause liver injury. TNFα is a mediator of inflammation critically involved in several models of liver injury, as described in more detail in Section 1.2.3. Finally, we tested the hypothesis that TNFα is critically involved in the development of TVX/LPS-induced liver injury in mice.

2.3 Materials and Methods

2.3.1 Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Lipopolysaccharide derived from *Eschericia coli* serotype O55:B5 was used for these studies. Lot 024K4067 with activity of 9.2 x 10^6 EU/mg was used for the experiments represented in Fig. 2.1-2.3. Lot 075K4038 with an activity of 3.3 x 10^6 EU/mg was used for the experiments represented in Fig. 2.4-2.9. The activity was determined using a colorimetric, kinetic *Limulus* amebocyte lysate assay purchased from Cambrex Corp. (Kit 50-650U; East Rutherford, NJ). TVX and LVX were kind gifts from Abbott Laboratories (Abbott Park, IL). Infinity ALT reagent was purchased from Thermo Electron Corp. (Louisville, CO).

2.3.2 Animals

Male, C57BL/6J mice (Jackson Laboratory, Bay Harbor, ME), 9-11 weeks old and weighing 21-26 g were used for the studies. Animals were given continual access to bottled spring water and were fed a standard chow (Rodent Chow/Tek8640, Harlan Teklad, Madison, WI) ad libitum. Mice were allowed to acclimate for 1 week in a 12 h light/dark cycle. They received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals,* and procedures were approved by the MSU Committee on Animal Use and Care.

2.3.3 Experimental protocol

Mice fasted for 12 h were given various doses of TVX, LVX or their saline vehicle by oral gavage. They were then given LPS at 67 x 10^6 EU/kg or 2.0 x 10^6 EU/kg (lots 024K4067 or 075K4038, respectively) by intraperitoneal injection 3 h after drug dosing. During the course of these studies we were forced to change lots of LPS. The dose of LPS of the initial lot was chosen based on preliminary dose-response studies for which the objective was to identify a nonhepatotoxic dose of LPS. For the lot of LPS that was used to complete these studies, a dose was chosen that was nonhepatotoxic when given alone and produced liver injury in TVX-cotreated mice that was similar in magnitude and timing to that produced by the first lot.

Food was returned immediately after LPS administration. Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at various times, and blood was drawn from the vena cava into a syringe containing sodium citrate (final concentration, 0.76%) and transferred to an Eppendorf tube for preparation of plasma. The left lateral liver lobe was fixed in 10% neutral buffered formalin and blocked in paraffin within 72 h. For some studies, mice were treated with pentoxifylline (200 mg/kg) or sterile saline by intraperitoneal injection 1 h before LPS injection. In other studies, mice were treated with etanercept (8 mg/kg) or sterile water by intraperitoneal injection either 1 h before LPS injection (Fig. 2.7 and 2.8) or 1.5 h after LPS dosing (Fig. 2.9). Etanercept (Enbrel, Amgen Pharmaceuticals) was purchased from the Michigan State University Pharmacy (East Lansing, MI).

2.3.4 Histopathology

Formalin-fixed left lateral liver lobes were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin & eosin and examined by light microscopy. The tissue sections presented in the figures were from mice with plasma ALT activity close to the average of the respective treatment group.

2.3.5 TNFα analysis

The plasma concentrations of TNFα were measured using a mouse inflammation kit (Cat. No. 552364) purchased from BD Biosciences (San Diego, CA). The BD cytometric bead array analysis was performed on a BD FACSCalibur flow cytometer (BD Biosciences).

2.3.6 Statistical analyses

Results are presented as mean ± S.E.M. A 1-,2-, or 3-way analysis of variance (ANOVA) was used as appropriate after data normalization. For the TVX/LPS timecourse (Fig. 2.1B), an ANOVA on Ranks was used. All pairwise comparisons were made using Dunn's method. The criterion for significance was $p < 0.05$ for all studies.

2.4 Results

2.4.1 Dose-response and timecourse of liver injury

Administration of LPS after TVX caused a significant increase in plasma ALT activity in a TVX dose-dependent manner (Fig. 2.1A): TVX doses of 80 mg/kg or greater caused hepatotoxicity in LPS-treated mice. TVX alone did not cause a significant increase in ALT activity up to 1000 mg/kg (data not shown). Administration of TVX doses greater than 200 mg/kg followed by LPS led to death within 15 h. A TVX dose of 150 mg/kg and LPS given 3 h later provided a maximal response with approximately 90% survival of mice; this protocol was chosen for all additional studies.

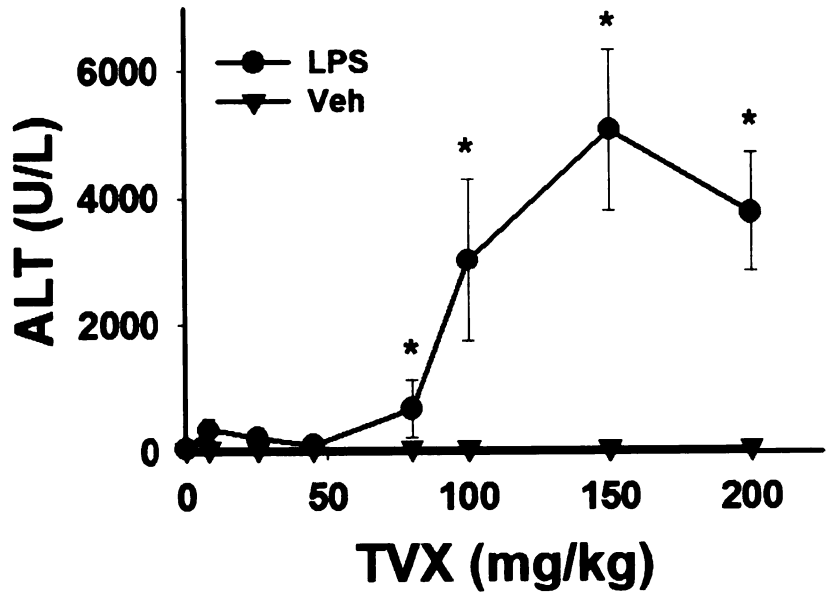
To evaluate the time-dependence of liver injury, TVX was administered 3 h before LPS dosing, and plasma ALT activity was measured at various times. TVX or LPS given alone did not significantly affect ALT activity compared to control mice at any time evaluated. Plasma ALT activity was significantly elevated by 9 h after TVX/LPS coexposure and peaked at 15-21 h after LPS (Fig. 2.1B).

2.4.2 Comparison of trovafloxacin and levofloxacin

Unlike TVX, LVX is not associated with human IADRs. We compared the hepatotoxic response to each of these in animals cotreated with LPS. The pharmacologically efficacious dose of TVX is similar in mice and humans (196, 197), and the same is true for LVX (198, 199). We chose a dose of

Fig. 2.1. Dose response and development of liver injury from TVX/LPS cotreatment in mice. A, Mice were given TVX at various doses (8, 25.3, 45, 80, 100, 150, and 200 mg/kg; p.o) and then 3 h later LPS (67 x 10^6 EU/kg; i.p.) or Veh (sterile saline). Hepatic parenchymal cell injury was estimated 15 h after LPS administration from increases in plasma ALT activity. *n* = 4-9 animals/group. * significantly different from Veh-treated group. B, Mice were treated with TVX (150 mg/kg; p.o.) or Veh and then 3 h later with LPS (67 x 10^6 EU/kg; i.p.) or Veh. Plasma ALT activity at various times after LPS dosing is depicted. *n* = 4-6 animals/group. * significantly different from 0 h group.

A


LPS
Veh
ALT (U/L)
6000
4000
2000
0
0
50
100
150
200
TVX (mg/kg)
*

B

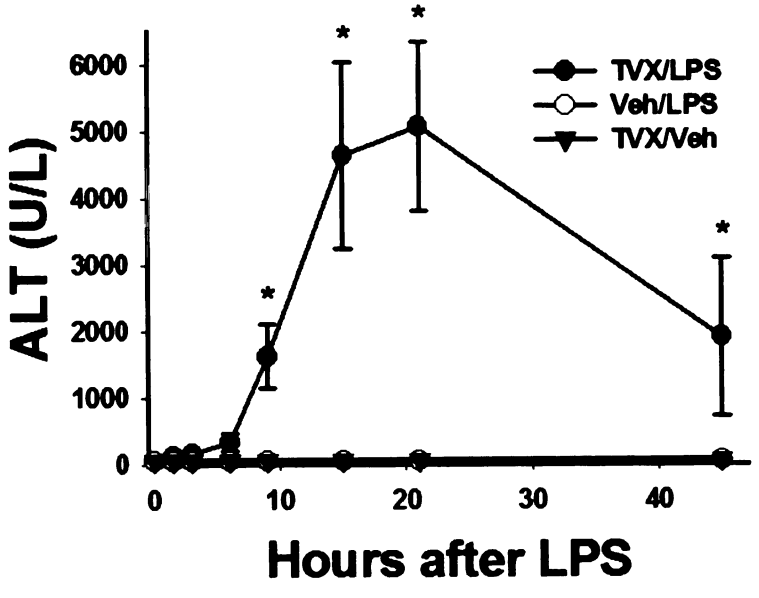
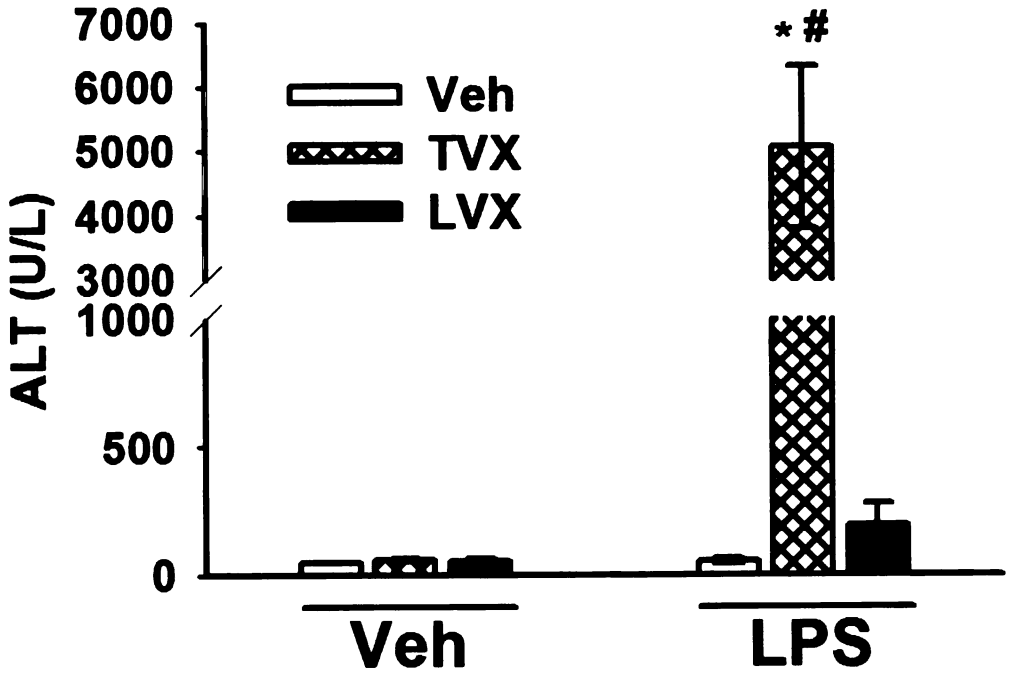

TVX/LPS
Veh/LPS
TVX/Veh
ALT (U/L)
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
Hours after LPS
*

Fig. 2.2. Comparison of TVX/LPS with LVX/LPS coadministration. TVX (150 mg/kg), LVX (375 mg/kg) or Veh (saline) was administered by oral gavage, and 3 h later LPS (67 x 10^6 EU/kg; i.p.) or Veh was administered. Mice were anesthetized and sacrificed 15 h after LPS administration. Hepatic parenchymal cell injury was estimated as increases in plasma ALT activity. *n* = 4-6 animals/group. * significantly different from Veh/Veh. # significantly different from Veh/LPS.


7000
6000
5000
4000
3000
1000
500
0
ALT (U/L)
Veh
TVX
LVX
* #
Veh
LPS

LVX (375 mg/kg) to keep the dose ratio of TVX/LVX similar to the ratio of doses used clinically in humans (200). LVX, TVX or Veh was given 3 h prior to LPS or Veh, and then mice were sacrificed 15 h later to measure plasma ALT activity and for histologic examination of the livers. TVX, LVX or LPS were all nontoxic when administered alone (Fig. 2.2). TVX/LPS coexposure increased ALT activity in the plasma, suggesting hepatic parenchymal cell injury. ALT activity was not increased in LVX/LPS-treated mice.

There were no significant hepatocellular lesions in mice treated with Veh/Veh, TVX/Veh or LVX/Veh (Fig. 2.3A, 2.3B and 2.3C, respectively). Histopathological examination of livers from TVX/LPS-cotreated mice (Fig. 3E) revealed hepatocellular necrosis, which was not seen in Veh/LPS- (Fig. 2.3D) or LVX/LPS-treated mice (Fig. 2.3F). Inflammatory cell infiltration was seen in all LPS-treated groups. The coagulative necrosis seen in the TVX/LPS-treated group was located predominantly midzonally but could also be found in centrilobular regions. The appearance of these lesions in TVX/LPS-treated mice followed the same timecourse as was seen for ALT activity in the plasma (data not shown).

2.4.3 Timecourse of TNFα concentration in plasma

Mice were treated according to the protocol described above and were sacrificed at various times (0, 1.5, 3, 4.5 and 6 h) after LPS. These and subsequent studies were performed with a different lot of LPS than was used to generate data in Figures 2.1-2.3. The dose used for these studies was 2×10^6

Fig. 2.3. Liver histopathology in mice cotreated with LPS and either TVX or LVX. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg) or Veh (p.o) and then dosed with LPS (67 x 10^6 EU/kg; i.p.) or Veh. Liver sections are from mice treated with Veh/Veh (A), TVX/Veh (B), LVX/Veh (C), Veh/LPS (D), TVX/LPS (E), LVX/LPS (F) and killed at 15 h. The arrows indicate randomly distributed, variably sized foci of coagulative necrosis seen only in TVX/LPS-treated mice and which were observed predominantly in midzonal regions but also in some centrilobular regions.

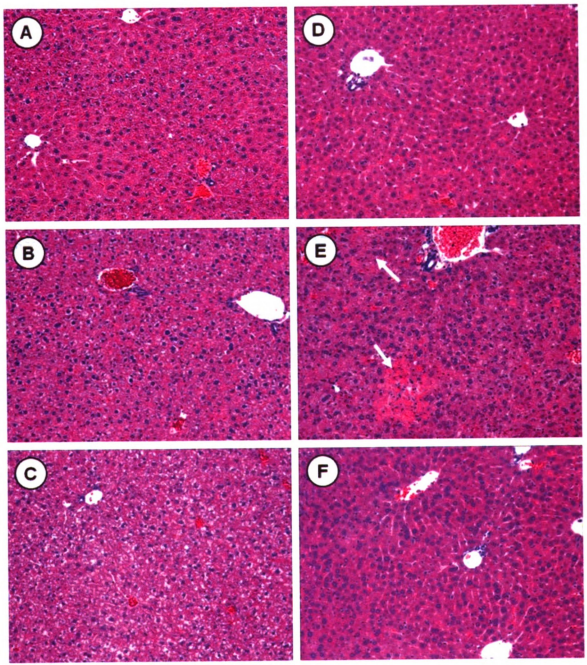
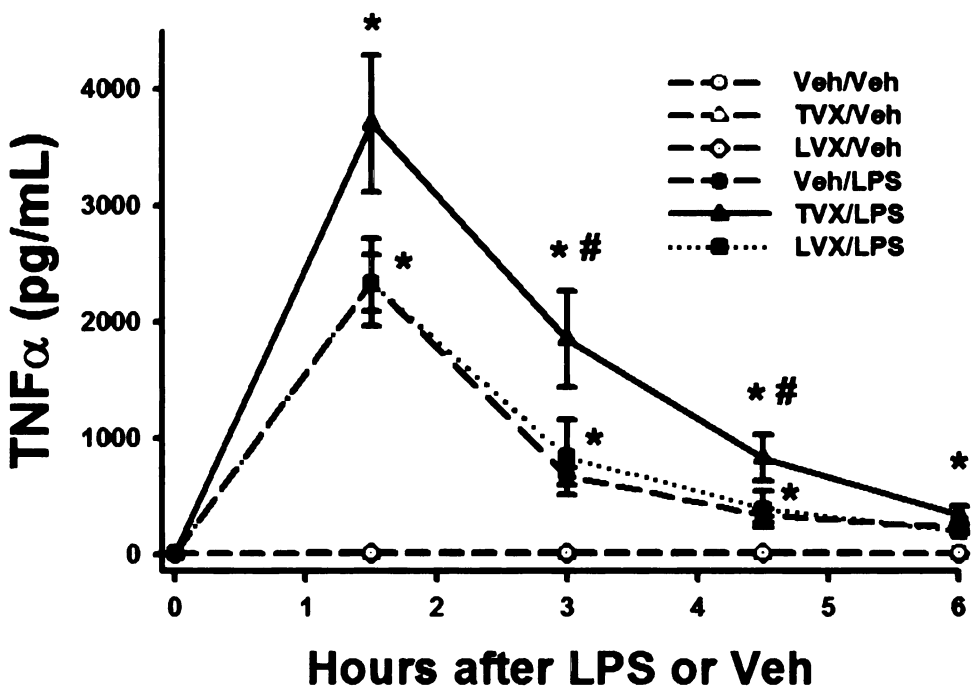

A
D
B
E
C
F

Fig. 2.4. Timecourse of TNFα concentration in the plasma of treated mice. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg) or Veh (p.o) and then dosed with LPS (2 x 10^6 EU/kg; i.p.) or Veh 3 h later. *n* = 3-10 animals/group. * significantly different from the same treatment group at 0 h. # significantly different from Veh/LPS-treated mice at the same time.


TNFα (pg/mL)
4000
3000
2000
1000
0
0
1
2
3
4
5
6
Hours after LPS or Veh
Veh/Veh
TVX/Veh
LVX/Veh
Veh/LPS
TVX/LPS
LVX/LPS
*
*
*
*
*
*
*#
*#

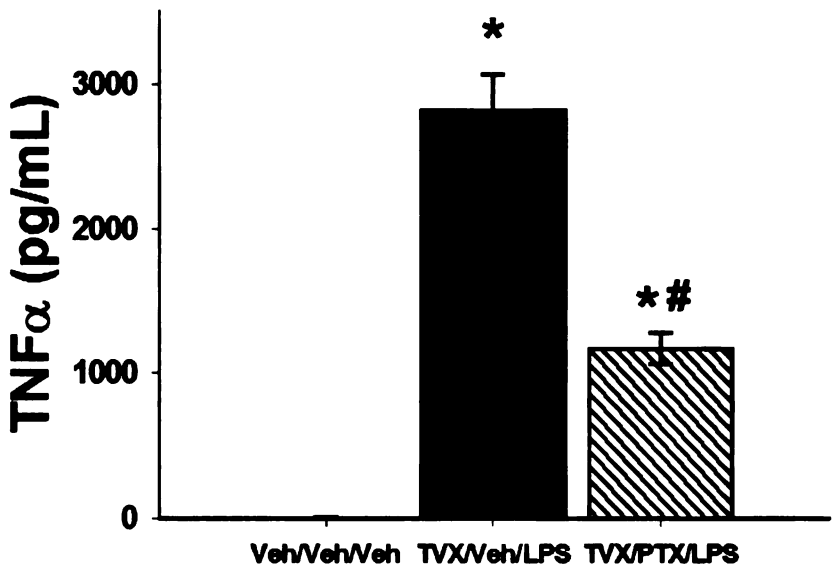
EU/kg, and despite the large difference in dose based on activity in the Limulus lysate assay, the results obtained with both lots were similar in terms of the magnitude and timing of liver injury. Plasma ALT activity was significantly and selectively increased in TVX/LPS-treated mice starting at 4.5 hrs after LPS (data not shown). LPS-treated groups showed a significant increase in plasma TNFα concentration at all times measured (Fig. 2.4). TVX administered prior to LPS caused greater elevation of TNFα concentration in the plasma compared to Veh/LPS-treated mice at 3 and 4.5 h after LPS. By contrast, LVX cotreatment had no effect on the LPS-induced change in plasma TNFα concentration.

2.4.4 Pentoxifylline study

As mentioned above, ALT activity was increased in TVX/LPS-cotreated mice 4.5 h after LPS administration, and plasma TNFα was selectively increased at this time. This result raised the possibility of a role for this cytokine in the development of hepatotoxicity in TVX/LPS-treated mice. Pentoxifylline (PTX) is a nonspecific phosphodiesterase inhibitor that inhibits LPS-induced TNFα production by increasing cAMP in monocytes/macrophages. The increase in cAMP inhibits the translocation and activation of NFκB, which controls TNFα expression (201). A dose of PTX 200 mg/kg (i.p.) given 1 h before LPS administration significantly decreased plasma TNFα concentration 1.5 h after LPS treatment (Fig. 2.5A). This dose of PTX significantly reduced TVX/LPS-induced liver injury, as estimated by plasma ALT activity 15 h after LPS dosing (Fig. 2.5B). TVX/Veh/LPS-treated livers had much less glycogen deposition and

Fig. 2.5. The effect of pentoxifylline (PTX) on LPS-induced TNFα expression and TVX/LPS-induced liver injury. A, Mice were treated with PTX (200 mg/kg; i.p.) or Veh (saline) 1 h prior to LPS. Mice were sacrificed 1.5 h after LPS treatment and plasma TNFα concentration was measured. B, TVX (150 mg/kg) or Veh was administered by oral gavage, followed by PTX or Veh 2 h later. LPS (2×10^6 EU/kg; i.p.) was given 1 h after PTX dosing. Mice were sacrificed 15 h after LPS treatment, and ALT activity was measured in the plasma. *n* = 5-10 animals/group. * significantly different from Veh/Veh/Veh control. # significantly different from TVX/Veh/LPS treatment group.

A


TNFα (pg/mL)
3000
2000
1000
0
*
*#
Veh/Veh/Veh
TVX/Veh/LPS
TVX/PTX/LPS

B

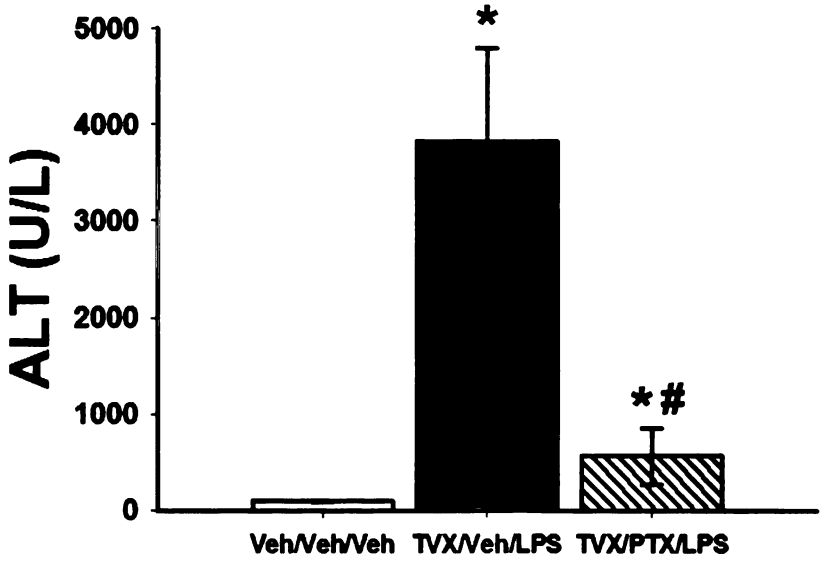
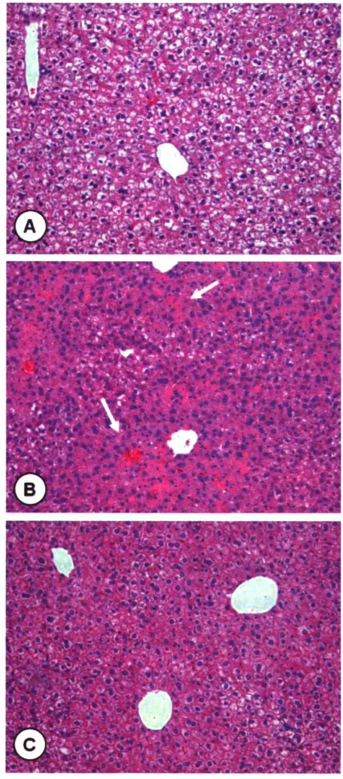

ALT (U/L)
5000
4000
3000
2000
1000
0
*
*#
Veh/Veh/Veh
TVX/Veh/LPS
TVX/PTX/LPS

Fig. 2.6. Effect of PTX on TVX/LPS-induced liver pathology. Mice were treated as described in Fig. 5 and sacrificed 15 h after LPS. Liver sections from mice treated with Veh/Veh/Veh (A), TVX/Veh/LPS (B), and TVX/PTX/LPS (C) were examined. The arrows indicate randomly distributed, variably sized foci of coagulative necrosis and hemorrhage seen only in TVX/Veh/LPS-treated mice. Lesions were not obvious in the TVX/PTX/LPS-treated group despite the slightly increased ALT activity in the plasma in this group (Fig. 5B).


A
B
C

had foci of midzonal hepatocellular necrosis compared to vehicle-treated control mice (Fig. 2.6B and 2.6A, respectively). PTX administration to TVX/LPS-treated mice reduced the midzonal hepatocellular necrosis and also reduced the glycogen depletion compared to the TVX/Veh/LPS group (Fig. 2.6C).

2.4.5 Etanercept inhibition of TNFα activity

Etanercept is a recombinant, human soluble TNFα receptor that inhibits TNFα activity. An etanercept dose of 8 mg/kg (i.p.) caused a significant decrease in plasma TNFα concentration in TVX/LPS-treated mice at 4.5 h after LPS administration (Fig. 2.7A). This dose of etanercept administered 1 h before LPS completely protected mice from the TVX/LPS-induced increase in plasma ALT activity (Fig. 2.7B) and from hepatocellular necrosis (Fig. 2.8). The TVX/LPS-treated mice consistently had midzonal and centrilobular foci of coagulative necrosis which were not observed when etanercept was administered (Fig. 2.8B and 2.8C, respectively).

In an attempt to determine if the prolongation of the LPS-induced plasma TNFα peak by TVX pretreatment (Fig. 2.4) was critical to TVX/LPS-induced liver injury, etanercept was administered at 1.5 h after LPS dosing (i.e., at the time plasma TNFα concentration had peaked). Etanercept administration at this time provided significant reduction in TVX/LPS-induced liver injury (Fig. 2.9).

Fig. 2.7. The effect of etanercept on TVX/LPS-induced TNFα expression and liver injury. A, TVX (150 mg/kg) or Veh was administered by oral gavage followed by etanercept (8 mg/kg; i.p.) or Veh 2 h later. LPS (2 x 10^6 EU/kg; i.p.) was given 1 h after etanercept dosing. Mice were sacrificed 4.5 h after LPS treatment, and plasma TNFα concentration was measured. B, Mice were treated as described above and sacrificed 15 h after LPS; ALT activity was measured in the plasma. *n* = 4-8 animals/group. * significantly different from Veh/Veh/Veh control group. # significantly different from TVX/Veh/LPS treatment group.

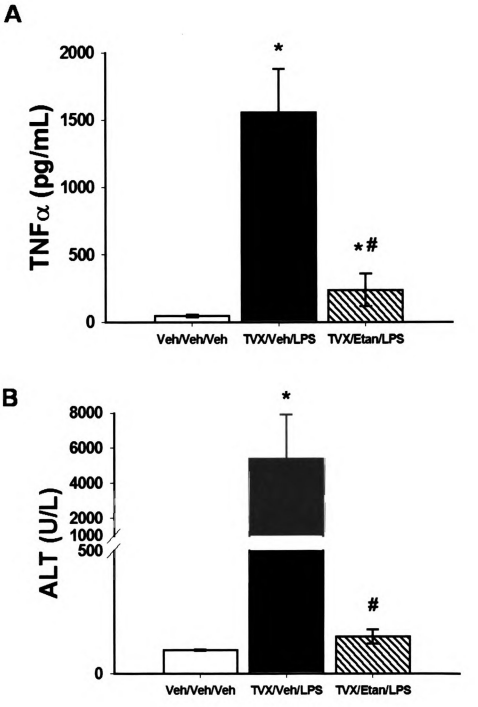

A
2000
1500
1000
500
0
TNFα (pg/mL)
*
*#
Veh/Veh/Veh
TVX/Veh/LPS
TVX/Etan/LPS
B
8000
6000
4000
2000
1000
500
0
ALT (U/L)
*
#
Veh/Veh/Veh
TVX/Veh/LPS
TVX/Etan/LPS

Fig. 2.8. Effect of etanercept on TVX/LPS-induced liver pathology. Mice were treated as described in Fig. 7 and sacrificed 15 h after LPS. Liver sections from mice treated with Veh/Veh/Veh (A), TVX/Veh/LPS (B), and TVX/etanercept/LPS (C) were examined. The arrows indicate randomly distributed, variably sized foci of coagulative necrosis and hemorrhage seen only in TVX/Veh/LPS-treated mice.

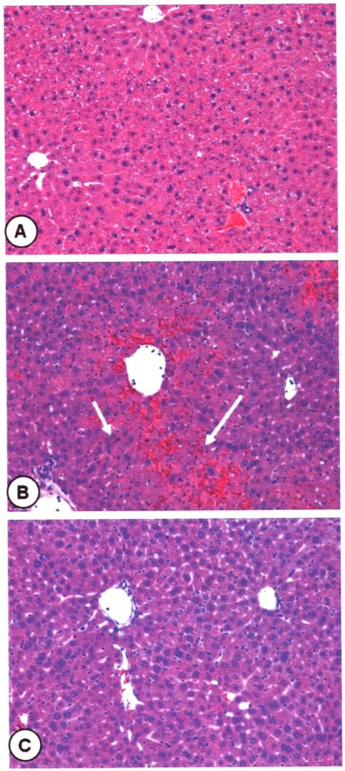
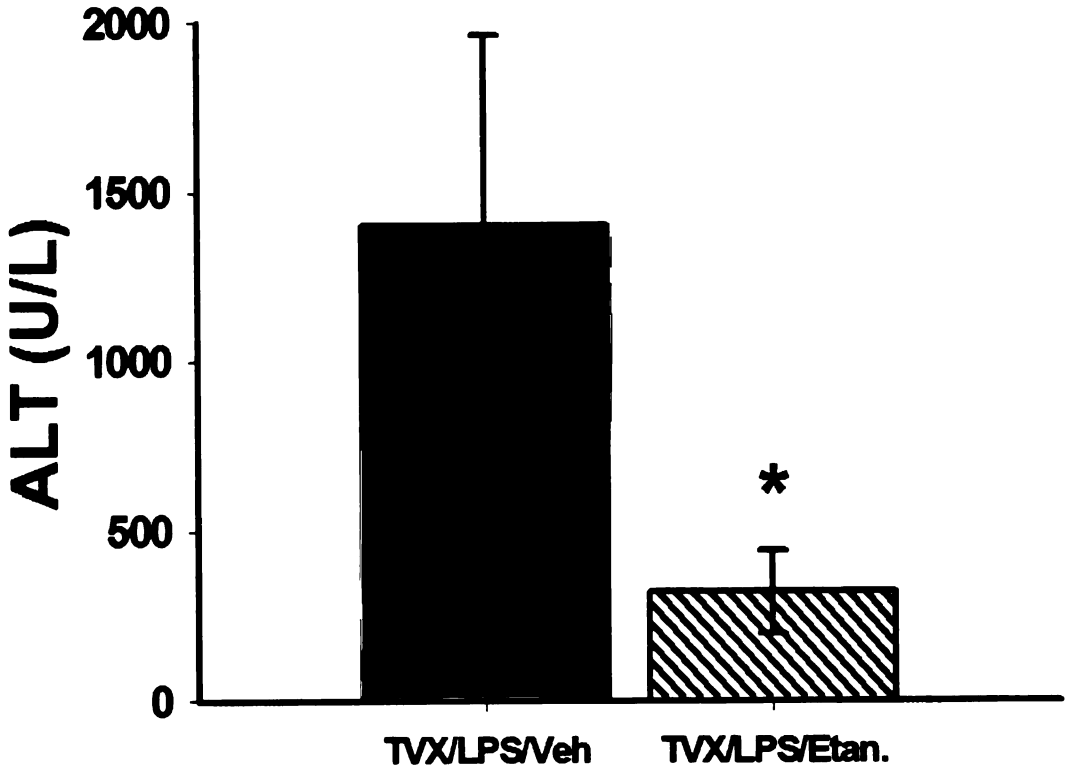

A
B
C

Fig. 2.9. Effect of etanercept treatment given at the peak of plasma TNFα on TVX/LPS-induced liver injury. Mice were treated with TVX (150 mg/kg; p.o.) 3 h before LPS (2 x 10^6 EU/kg; i.p.). Mice were then treated with etanercept (8 mg/kg; i.p.) 1.5 h after LPS dosing and sacrificed 15 h after LPS; ALT activity was measured in the plasma. *n* = 5 animals/group. * significantly different from TVX/LPS/Veh-treated group.


2000
1500
1000
500
0
ALT (U/L)
*
TVX/LPS/Veh
TVX/LPS/Etan.

2.5 Discussion

The underlying mechanisms behind hepatic IADRs in humans are unknown. One of the most widely accepted hypotheses is that they involve immune-mediated hypersensitivity reactions (202). However, for the majority of drugs there is limited evidence to support this theory. Another widely accepted hypothesis is that mitochondrial dysfunction from oxidative stress leads to hepatotoxic IADRs. There is evidence from human hepatocytes that TVX can cause oxidative stress and mitochondrial damage (203). In contrast, changes in markers of oxidative stress were not observed in rats treated with TVX, suggesting either that the effect may be species-specific or that the observation in vitro does not pertain in vivo (58). Thus, mechanisms of TVX-mediated liver injury remain unknown.

In rats, the TVX/LPS interaction was found to precipitate a hepatotoxic response (58). The timing of dosing in the rat model was different from the mouse model presented here in that the TVX was given 2 h after LPS administration. The rats were treated with TVX by i.v. rather than oral administration, which might explain the differences in protocols that caused maximally toxic responses. That is, greater time might be needed after oral dosing to reach effective plasma TVX concentration compared to i.v. injection. Additionally, the half-life of TVX in mice is much longer than in rats, and this might contribute to the differences in the dosing protocol needed to induce maximal liver injury (204, 205). The development of hepatotoxic TVX-inflammation interaction in both mice and rats demonstrates that the

phenomenon is not species-specific and might have common mechanisms which could be extrapolated to TVX IADRs in humans.

The degree of TVX/LPS-induced liver injury was much greater in mice (Fig. 2.1) compared to rats (58). This assessment is based on histopathology and on the fold increase in plasma ALT activity. In mice, the peak plasma ALT activity was about 30 fold greater than in the rat model, and the liver lesions were more pronounced. Both moderate and severe hepatotoxic responses have been reported in people who took TVX (206). The robustness of the murine model of liver injury resembles the severe hepatotoxicity caused by TVX in humans more so than the rat model. This might be due to the greater similarity in TVX pharmacokinetics in mice and humans (204, 205, 207). TVX binding to serum proteins is greater in rats compared to humans, 92 vs. 70%, respectively (205, 207). The degree of serum protein binding in mice is unavailable, but the more extensive serum protein binding in rats might contribute to the less robust liver injury.

Coexposure to LVX and LPS did not produce hepatotoxicity in mice, as indicated by both plasma ALT activity (Fig. 2.2) and histopathological examination (Fig. 2.3). Thus, for this class of drugs, the animal model is selective for a drug that produced IADRs in humans. The difference in response was probably not due to pharmacokinetic differences, as LVX and TVX have very similar elimination half-lives (208). Another possible explanation for the selective hepatotoxicity with TVX/LPS coexposure is that TVX is more potent against GI bacteria, causing release of LPS into the bloodstream, which, when paired with

LPS administration, precipitates a toxic response. This possibility, however, can be ruled out. If TVX or LVX caused LPS release from the GI tract, then it should have been reflected in increased plasma TNFα; however, neither of the drugs alone caused such an increase. Moreover, LVX failed to enhance the increase in plasma concentration of TNFα or to cause liver injury when coadministered with LPS. Thus, this model is selective for the IADR-causing TVX in both the development of liver injury and in the enhancement of LPS-induced increase in plasma TNFα concentration.

TVX pretreatment selectively prolonged the LPS-induced plasma TNFα peak before and during the onset of liver injury (Fig. 2.4). TNFα is critically involved in several models of liver injury including ischemia/reperfusion, and endotoxemia (209, 210). To explore the role of TNFα, both PTX and etanercept were used to inhibit TNFα activity. PTX pretreatment provided significant protection from TVX/LPS-induced liver injury (Fig. 2.5). In addition to inhibiting TNFα, PTX has several other effects including preventing platelet aggregation, decreasing other proinflammatory cytokines, and inhibiting hepatic fibrogenesis (211). Accordingly, a more selective inhibitor was also used.

Etanercept is a recombinant human soluble TNFα receptor which specifically neutralizes the activity of TNFα. Pretreatment with etanercept completely protected mice from TVX/LPS-induced liver injury (Fig. 2.7). Additionally, etanercept administration at a later time to eliminate the prolongation by TVX of the LPS-induced plasma TNFα peak also provided protection (Fig. 2.9). Thus, the prolonged TNFα presence caused by TVX

pretreatment seems to be critically involved in the TVX/LPS-induced liver injury. However, this finding does not rule out a critical role for the initial peak of TNFα (0-1.5 h after LPS). Whether TNFα directly causes hepatotoxicity or acts indirectly through other mediators will require further investigation.

The cellular source(s) of TNFα in this model have not been explored. In the liver, Kupffer cells can be stimulated by LPS to release TNFα and other cytokines. These cells are an important source of TNFα in several models of liver injury (212, 213) and seem likely to be involved in TVX/LPS-induced liver injury as well. Similarly, neutrophils were found to be critically involved in the TVX/LPS model in rats. It seems likely that they play a similar role in the mouse model, but additional studies are required to confirm this.

The hepatotoxic interaction between TVX and LPS presented here contrasts to a previous report showing that TVX reduces LPS-induced death in mice (214). The difference in the effect of TVX could be due to different timing of TVX administration. The protective effect of TVX was seen when it was administered at 47, 17 and 1 h before LPS. In our hands, the timing of TVX administration in relation to LPS was critical. For example, administration of TVX after LPS dosing did not lead to significant liver injury in mice (data not shown), suggesting that TVX had to be present in the body during LPS administration to precipitate liver injury. An alternative explanation for the contrasting response is that after a lethal dose of LPS (214), TVX might play a different role to reduce mortality, for example by killing bacteria translocated from the GI tract into the circulation. In addition, the previous study referenced used Swiss Webster mice

whereas C57/BL6 mice were used for these studies, so that strain differences might contribute to the disparate results.

It has been reported that TVX significantly reduces TNFα concentrations induced by LPS in mice (214, 215). These results contrast with data presented in Fig. 2.4. The difference in results could be due to different strains of mice, doses of LPS (lethal vs. nonhepatotoxic) or different treatment protocols, in which TVX was given 1 h (214) or 3 h (data presented here) before LPS. In that study, TVX increased plasma TNFα concentration, an effect not observed in our study. The plasma concentration of TNFα in control mice was reported to be 1.4 ± 0.5 ng/mL (214), a value that is extremely high for normal mice and might reflect an ongoing inflammatory response in their controls.

It has also been reported that alatrofloxacin, a prodrug of TVX, decreased LPS-stimulated expression of TNFα mRNA and protein *in vitro* in human peripheral blood mononuclear cells (PBMCs) (215), a result that contrasts with our findings. Interestingly, in rats cotreated with TVX and LPS, mRNA for TNFα in liver was not increased, but mRNA for TNF-induced protein was elevated (58), suggesting a post-transcriptional mechanism for increasing TNFα protein. It is also possible that Kupffer cells, a major source of TNFα in the liver, respond differently to the interaction of TVX with LPS than human PBMCs with respect to TNFα production. Another possibility is that a hepatic metabolite is involved in the TVX effect on LPS stimulation, and this metabolite might not be produced by isolated PBMCs. Additionally, our studies would have allowed more time for such a metabolite to form. Thus, although a previous study provided evidence for an

anti-inflammatory property of TVX, the treatment protocols, mouse strains, and doses contrast with those employed in this study.

In summary, a modest inflammatory stress induced by LPS rendered TVX, but not LVX, hepatotoxic in mice. TVX pretreatment prolonged the LPS-induced increase in TNFα in the plasma. The increase in TNFα plays a critical role in the development of TVX/LPS-induced liver injury. The demonstration of TVX/LPS toxicity in both mice and rats indicates that the interaction is not species-specific. The results suggest the possibility that inflammatory stress underlies the development of TVX-induced idiosyncratic liver injury and support the potential of animal models of drug-inflammation interaction as preclinical predictors of IADRs in humans.

CHAPTER 3

Trovafloxacin enhances the inflammatory response to a gram-negative or a gram-postitive bacterial stimulus, resulting in CD18-dependent liver injury in mice.

3.1 Abstract

Trovafloxacin, a fluoroquinolone antibiotic, was strongly linked with several cases of idiosyncratic hepatotoxicity leading to the severe restriction of TVX usage. Previous studies have shown that a modest inflammatory stress induced by LPS renders nontoxic doses of TVX hepatotoxic in mice. The liver injury is dependent on TNFα, suggesting TVX might enhance the response to an inflammatory stress. The purpose of this study was to examine the interaction of TVX with a subsequent inflammatory stress induced by either a gram-negative or a gram-positive bacterial stimulus. Mice were given TVX 3 h before LPS (gram-negative) or a peptidoglycan/lipoteichoic acid (PGN-LTA) mixture isolated from *S. aureus* (gram-positive). Administration of TVX, LPS or PGN-LTA was nonhepatotoxic. In contrast, TVX administration prior to LPS or PGN-LTA resulted in liver injury starting by 4.5 h which was maximal at 15 h. Histopathology revealed that TVX/LPS-coexposure resulted in primarily midzonal hepatocellular cell death, whereas TVX/PGN-LTA-induced necrosis was primarily centrilobular. LPS or PGN-LTA alone increased plasma concentrations of IL-18, IL-1β, IL-6, IL-10, keratinocyte chemoattractant (KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), VEGF, IFNγ, TNFα and macrophage inflammatory protein 2 (MIP-2) at 4.5 h. TVX administration enhanced the LPS induction of all of these cytokines/chemokines. In contrast, TVX enhanced the PGN-LTA-induced increase of all except TNFα and IFNγ. TVX/LPS- and TVX/PGN-LTA-induced liver injury was significantly attenuated by CD18 antiserum treatment. In summary, TVX significantly

enhanced the inflammatory response of mice to either a gram-negative or gram-positive stimulus and caused hepatotoxicity which was dependent on CD18.

3.2 Introduction

A nontoxic inflammatory stress induced by LPS, a cell wall component of Gram-negative bacteria, can interact with a nontoxic dose of TVX to cause TNFα-dependent hepatotoxicity in both rats and mice (58, 216). Toll-like receptors recognize pathogens and bacteria as foreign as described in Section 1.2.1. Whereas LPS activates cells through binding to TLR4, TLR2 serves as the primary receptor for Gram-positive bacteria and their cell wall components, peptidoglycan (PGN) and lipoteichoic acid (LTA) (217, 218). Activation of TLR2 by PGN and LTA leads to the activation of the transcription factor NF-κB, which increases the expression of inflammatory cytokines and chemokines (217). This study determined if an inflammatory stress induced by gram-positive microbial products interacts with TVX to cause liver injury.

Additionally, TVX enhanced the LPS-induced increase of TNFα (216). One purpose of this study was to determine if TVX pretreatment enhanced the LPS- or PGN-LTA-induced increase of cytokines. The profile of cytokines altered by TVX coexposure with either inflammatory stimuli were compared. Finally, we tested that hypothesis that CD18, involved in PMN activation, plays a critical role in the development of hepatotoxicity resulting from TVX/inflammatory stress coexposure.

3.3 Materials and Methods

3.3.1 Materials

Peptidoglycan and lipoteichoic acid isolated from *S. aureus* were purchased from Sigma (St. Louis, MO). Rabbit anti-murine CD18 antiserum was designed against amino acids 89-100 and purchased from New England Peptide (Gardner, MA). Please refer for Section 2.3.1 for additional information on this topic.

3.3.2 Animals

Please refer to Section 2.3.2 for information on this topic.

3.3.3 Experimental protocols

Mice fasted for 12 h were given TVX (150 mg/kg) or Veh (saline) by oral gavage. They were then given LPS (2.0×10^6 EU/kg), PGN-LTA (30 mg/kg each) or Veh (saline) by intraperitoneal injection 3 h later. Food was returned immediately after this dosing. Mice were anesthetized with sodium pentobarbital (50 mg/kg; i.p.) and killed at either 4.5 or 15 h after the administration of LPS, PGN-LTA or Veh for various measurements. Blood was drawn from the vena cava into a syringe containing sodium citrate, resulting in a final concentration of 0.76%. The left lateral lobe was fixed in 10% neutral buffered formalin and paraffin blocked.

For some studies, mice were treated with CD18 antiserum. CD18 antiserum or rabbit control serum (0.25 mL; i.p.) was administered when food was removed and then again 2 h after LPS, PGN-LTA or Veh administration.

3.3.4 Histopathology

Formalin-fixed, left lateral liver lobes were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin & eosin and examined by light microscopy. The tissue sections presented in the figures were from mice with plasma ALT activity close to the average of the respective treatment group.

3.3.5 Cytokine measurements

The plasma concentrations of IL-1β, TNFα, IL-10, IL-6, IL-18, IFN γ, VEGF, MCP-1, KC, MIP-2 and MIP-1α were measured using custom Bio-plex cytokine assays purchased from Bio-Rad Laboratories (Hercules, CA) using the Bio-Plex 200 System (Bio-Rad Laboratories).

3.3.6 Neutrophil staining

Paraffin-embedded, left lateral liver lobes were stained for neutrophils (PMNs) using a rabbit anti-PMN Ig isolated from the serum of rabbits immunized with rat PMNs (101). Immunohistochemical staining of PMNs was done using the protocol as described previously (219). Neutrophil accumulation was then quantified by counting PMNs per high power field. The slides were coded, randomized and then visualized using a light microscope.

3.3.7 Statistical analyses

Results are presented as mean ± S.E.M. A student's t-test or a 2-way analysis of variance (ANOVA) was used as appropriate after data normalization. All pairwise comparisons were made using a Tukey test with the criterion for significance at $p < 0.05$.

3.4 Results

3.4.1 TVX coexposure with either LPS or PGN-LTA causes hepatotoxicity

TVX (150 mg/kg) administration 3 h before either LPS or PGN-LTA mixture caused a significant increase in plasma ALT activity as early as 4.5 h after the inflammatory stimulus (Fig. 3.1). It increased to a maximum at 15 h after either inflammatory stimulus. Administration of either LPS or PGN-LTA without TVX did not cause an increase in plasma ALT activity (data not shown).

The histopathology of the respective treatment groups corroborated the plasma ALT activity results. TVX/PGN-LTA coexposure resulted in hepatocellular oncotic necrosis and apoptosis primarily in the centrilobular regions (Fig. 3.2). In contrast, TVX/LPS coexposure caused hepatocellular death primarily in midzonal regions (Fig. 3.2).

3.4.2 TVX enhances cytokine induction by either inflammatory stimulus

Administration of either LPS or PGN-LTA caused a significant increase at 4.5 h of plasma concentrations of the following cytokines: IL-1β, TNFα, IL-10, IL-6, IL-18, IFNγ, vascular endothelial growth factor (VEGF), and MCP-1 (Fig. 3.3). TVX pre-treatment enhanced the LPS-induction of all cytokines listed above (Fig. 3.3). In contrast, TVX treatment enhanced the PGN-LTA-induced increase in all of the cytokines listed except for TNFα or IFNγ at 4.5 h (Fig. 3.3). In addition, TVX enhanced the induction of chemokines by both LPS and PGN-LTA (Fig. 3.4).

Fig. 3.1. Development of liver injury after TVX/LPS or TVX/PGN-LTA coexposure. Mice were treated with TVX (150 mg/kg; p.o.) 3 h before LPS (2 x 10^6 EU/kg; i.p.) or PGN-LTA (30 mg/kg each, i.p.). Mice were killed at various times and plasma ALT activity was measured. *n* = 5 animals/group. * significantly different from same treatment group at 0 h.

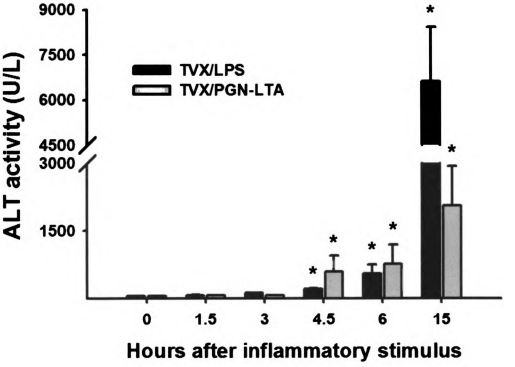

9000
7500
6000
4500
3000
1500
ALT activity (U/L)
TVX/LPS
TVX/PGN-LTA
*
*
*
*
*
*
0
1.5
3
4.5
6
15
Hours after inflammatory stimulus

Fig. 3.2. Histopathology of livers from mice treated with TVX/LPS or TVX/PGN-LTA. Mice were treated with TVX (150 mg/kg; p.o.) 3 h before either LPS (2 x 10^6 EU/kg; i.p.) or PGN-LTA (30 mg/kg each, i.p.). Mice were killed at 15h and representative photomicrographs were taken.

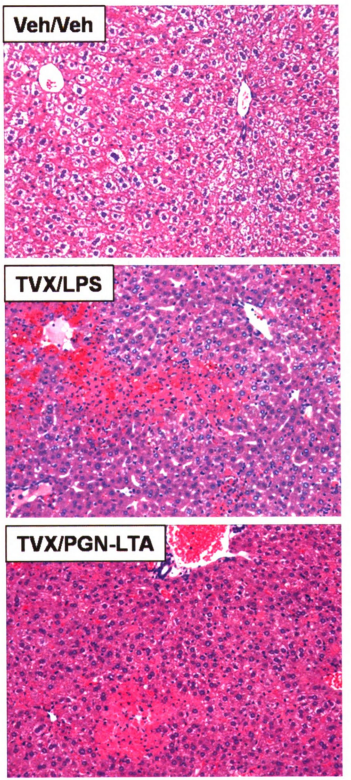

Veh/Veh
TVX/LPS
TVX/PGN-LTA

Fig. 3.3. Effect of TVX pretreatment on LPS- and PGN-LTA-induced increases in cytokines. Mice were treated with TVX or Veh and then LPS, PGN-LTA or Veh as described in Section 3.3.3. Mice were killed at 4.5 h, and plasma concentrations of IL-1β, TNFα, IL-6, IL-18, VEGF, MCP-1, IL-10 and IFNγ were measured. *n* = 4-6 animals/group. * significantly different from Veh/Veh-treated group. # significantly different from TVX/Veh-treated mice. $^{\Phi}$ significantly different from Veh-treated mice within the same treatment group.

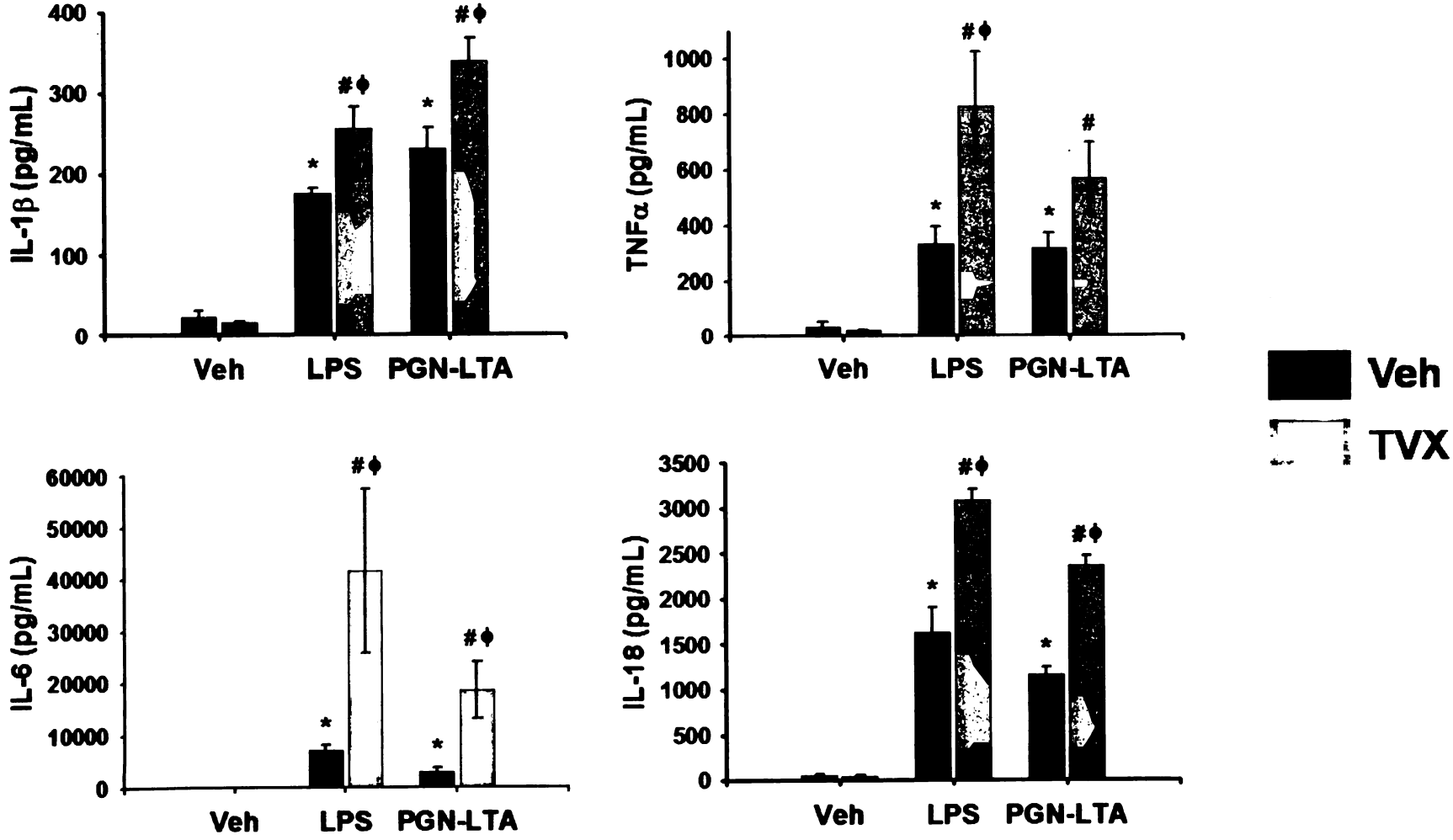

IL-1β (pg/mL)
400
300
200
100
0
Veh
LPS
PGN-LTA
TNFα (pg/mL)
1000
800
600
400
200
0
IL-6 (pg/mL)
60000
50000
40000
30000
20000
10000
0
IL-18 (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
Veh
TVX

Fig. 3.3 (cont'd).

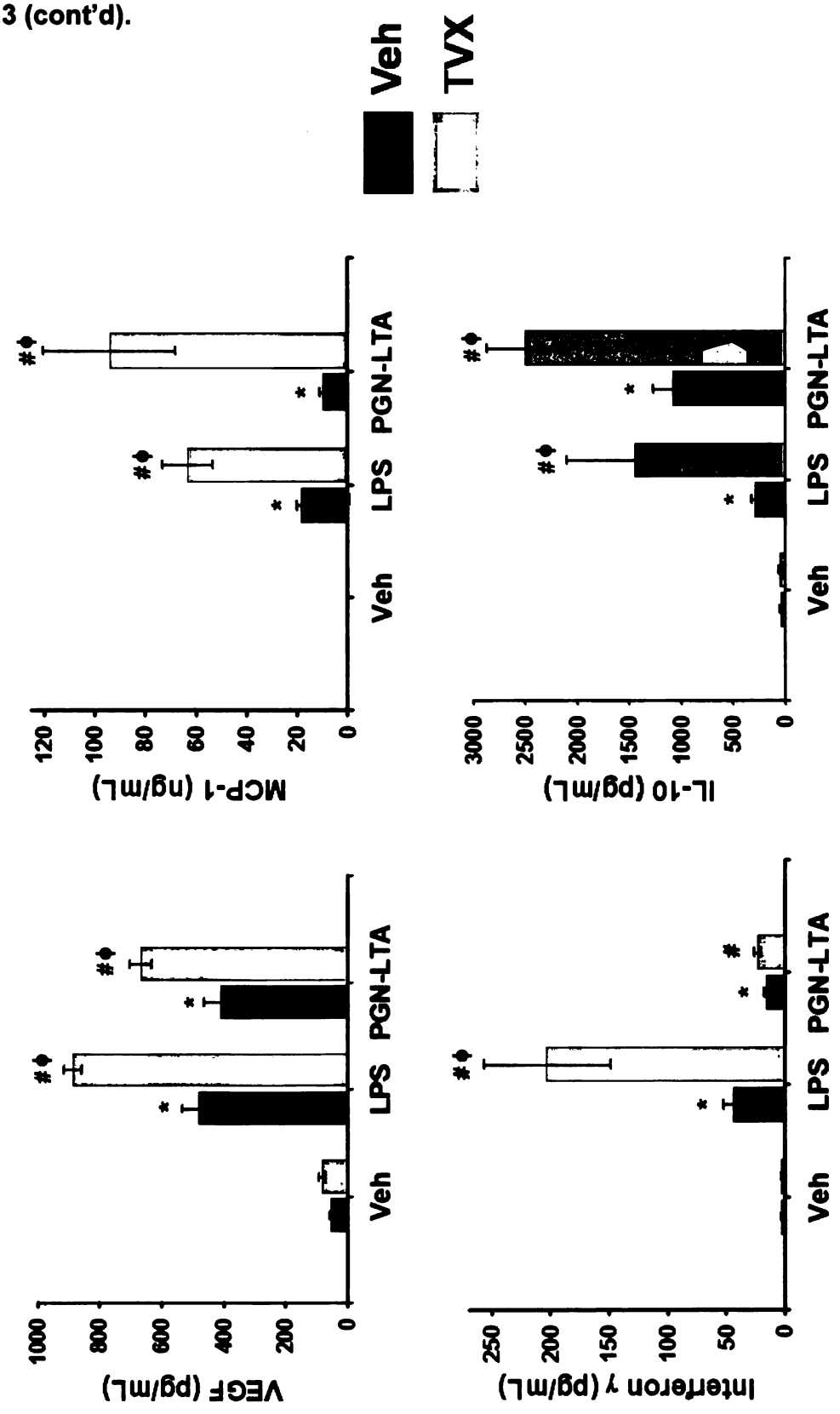
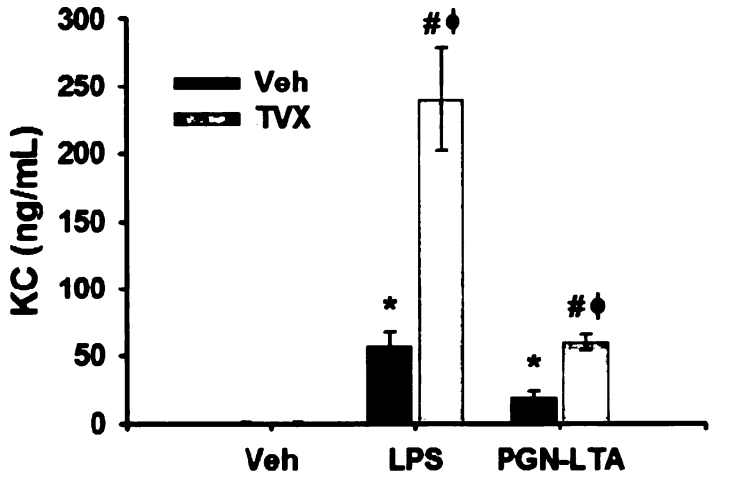
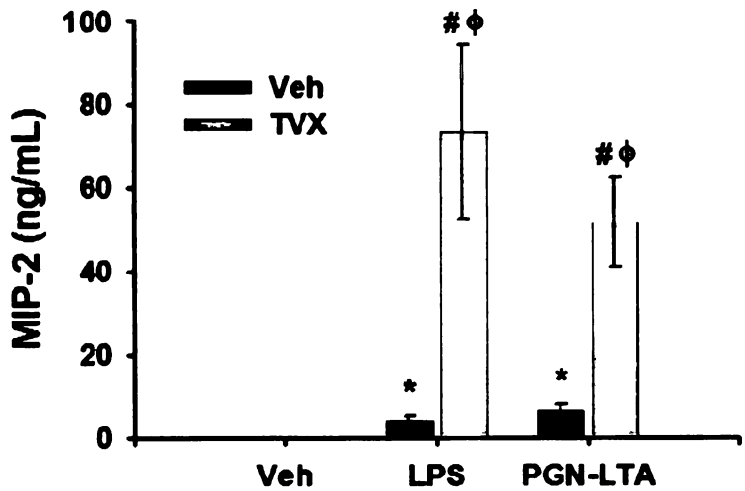
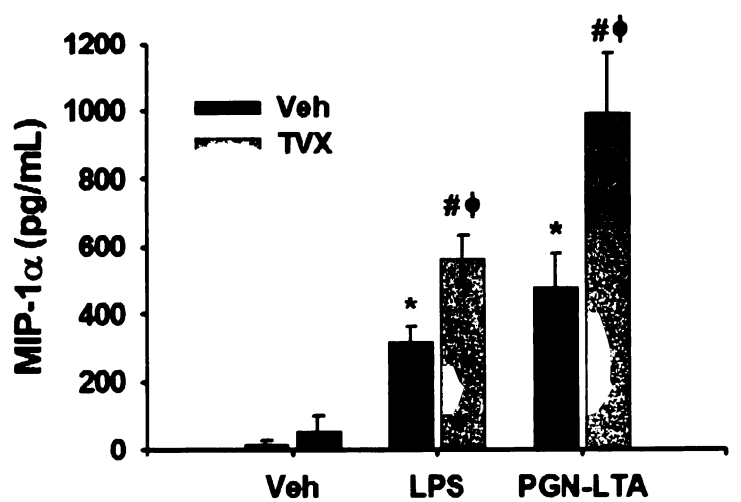

Veh
TVX
MCP-1 (ng/mL)
IL-10 (pg/mL)
VEGF (pg/mL)
Interferon γ (pg/mL)
Veh
LPS
PGN-LTA

Fig. 3.4. Effect of TVX pretreatment on LPS- and PGN-LTA- induced increases in chemokines. Mice were treated with TVX or Veh and then LPS, PGN-LTA or Veh as described in Section 3.3.3. Mice were killed at 4.5 h, and plasma concentrations of KC, MIP-2 and MIP-1α were measured. *n* = 4-6 animals/group. * significantly different from Veh/Veh-treated group. # significantly different from TVX/Veh-treated mice. $^{\Phi}$ significantly different from Veh-treated mice within the same treatment group.


KC (ng/mL)
300
250
200
150
100
50
0
Veh
TVX
#ϕ
*
Veh
LPS
PGN-LTA


MIP-2 (ng/mL)
100
80
60
40
20
0
Veh
TVX
#ϕ
*
Veh
LPS
PGN-LTA


MIP-1α (pg/mL)
1200
1000
800
600
400
200
0
Veh
TVX
#ϕ
*
Veh
LPS
PGN-LTA

3.4.3 Effect of TVX on microbial stimuli-induced hepatic neutrophil accumulation

Hepatic neutrophil accumulation was evaluated at 4.5 h after LPS or PGN-LTA. Both LPS and PGN-LTA alone caused a significant increase in neutrophils present in the liver (Fig. 3.5). TVX alone did not cause hepatic neutrophil accumulation. In addition, TVX pre-treatment did not affect the number of neutrophils present in the liver after either inflammatory stimulus (Fig. 3.5).

3.4.4 Effect of CD18 neutralization on TVX/LPS- and TVX/PGN-LTA-induced liver injury and inflammation

CD18 antiserum was administered as described in Section 3.3.3. CD18 neutralization protected mice from TVX/LPS- and TVX/PGN-LTA-induced liver injury as measured by plasma ALT activity (Fig. 3.6).

CD18 antiserum administration significantly attenuated TVX/PGN-LTA-induced hepatic neutrophil accumulation (Fig. 3.7). In addition, CD18 neutralization reduced the TVX/PGN-LTA-induced increases in TNFα and MCP-1 (Fig. 3.8). In contrast, CD18 neutralization did not affect TVX/LPS-induced hepatic neutrophil accumulation (Fig. 3.7). Similarly, CD18 neutralization did not affect any cytokines induced by TVX/LPS coexposure (Fig. 3.8).

Fig. 3.5. Effect of TVX pretreatment on LPS- and PGN-LTA- induced hepatic neutrophil accumulation. Mice were treated with TVX or Veh and then LPS, PGN-LTA or Veh as described in Section 3.3.3. Mice were killed at 4.5 h. Paraffin-embedded liver lobes were cut and stained for PMNs. *n* = 4-6 animals/group. * significantly different from Veh/Veh-treated group. # significantly different from TVX/Veh-treated mice.

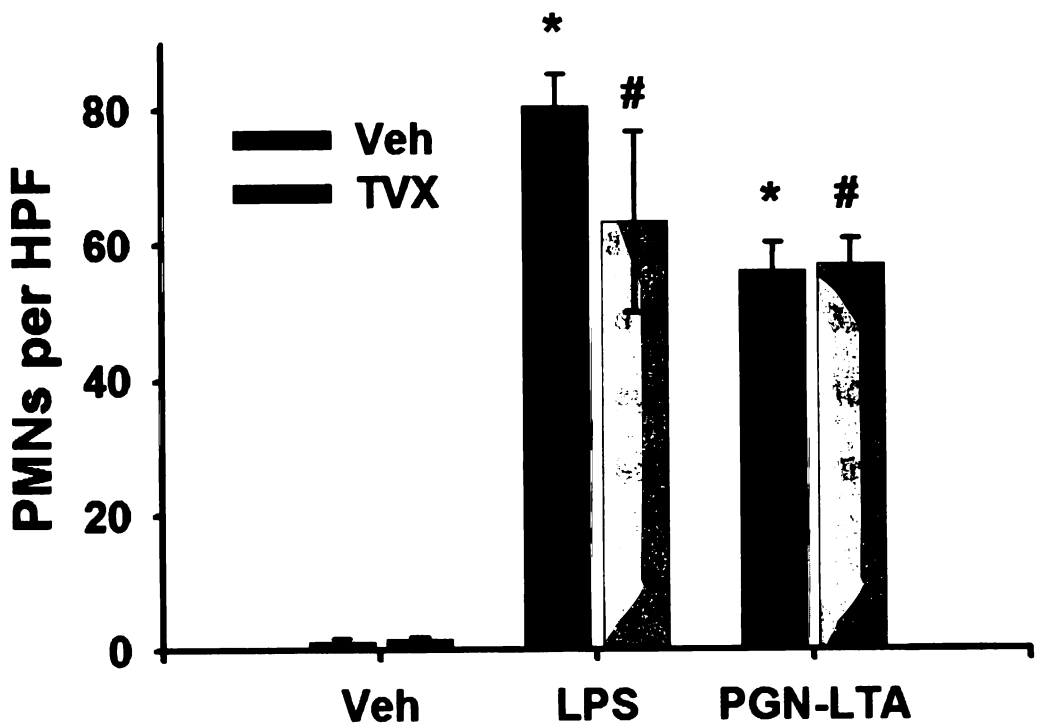

PMNs per HPF
80
60
40
20
0
Veh
TVX
*
#
*
#
Veh
LPS
PGN-LTA

Fig. 3.6. Effect of CD18 neutralization on TVX/LPS- and TVX/PGN-LTA-induced liver injury. Mice were treated with TVX/LPS or TVX/PGN-LTA and CD18 antiserum or control serum as described in Section 3.3.3. Mice were killed at 15 h and plasma ALT activity was measured. *n* = 6-10 animals/group. # significantly different from control serum treated mice within the same treatment group.

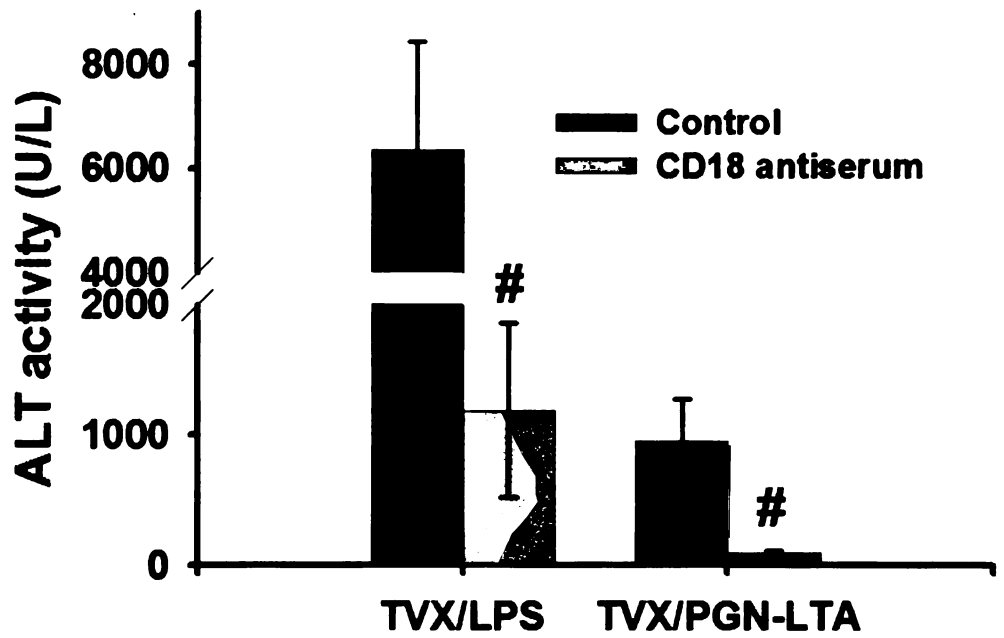

ALT activity (U/L)
8000
6000
4000
2000
1000
0
Control
CD18 antiserum
#
#
TVX/LPS
TVX/PGN-LTA

Fig. 3.7. Effect of CD18 neutralization on TVX/LPS- and TVX/PGN-LTA-induced hepatic neutrophil accumulation. Mice were treated with TVX/LPS or TVX/PGN-LTA in addition to CD18 antiserum or control serum as described in Section 3.3.3. Mice were killed at 4.5 h. Paraffin-embedded liver lobes were stained for PMNs. *n* = 6-10 animals/group. # significantly different from control serum treated mice within the same treatment group.

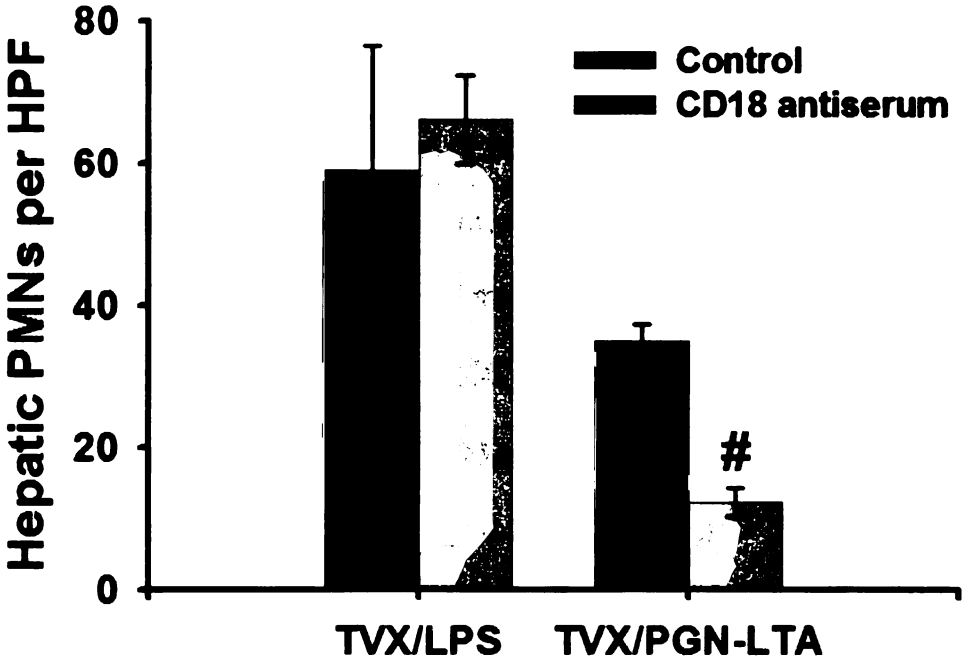

Hepatic PMNs per HPF
80
60
40
20
0
Control
CD18 antiserum
#
TVX/LPS
TVX/PGN-LTA

Fig. 3.8. Effect of CD18 neutralization on TVX/LPS- and TVX/PGN-LTA-induced cytokine increases. Mice were treated with TVX/LPS or TVX/PGN-LTA in addition to CD18 antiserum or control serum as described in Section 3.3.3. Mice were killed at 4.5 h and plasma cytokine concentrations were measured. Control and CD18 antiserum control mice had equivalent baseline concentrations of IL-6 (0.05 $\pm$ 0.005 ng/mL), IL-10 (36 $\pm$ 6 pg/mL), TNFα (411 $\pm$ 120 pg/mL), IL-1β (75 $\pm$ 15 pg/mL), KC (0.04 $\pm$ 0.006 ng/mL), MIP-1α (541 $\pm$ 56 pg/mL), IFNγ (9 $\pm$ 2 pg/mL) and MCP-1 (0.14 $\pm$ 0.03 ng/mL). *n* = 6-10 animals/group. # significantly different from control serum treated mice within the same treatment group.

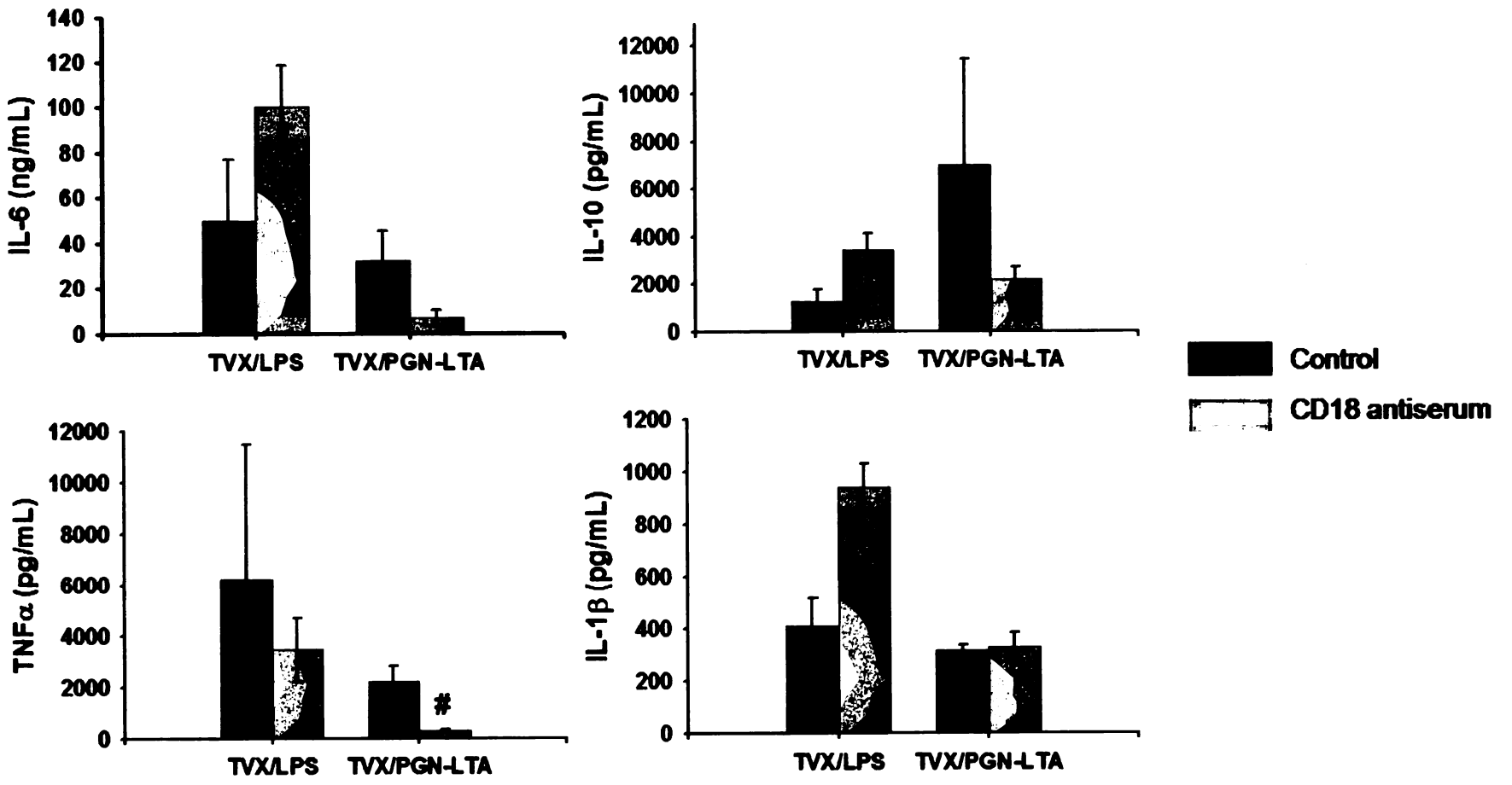
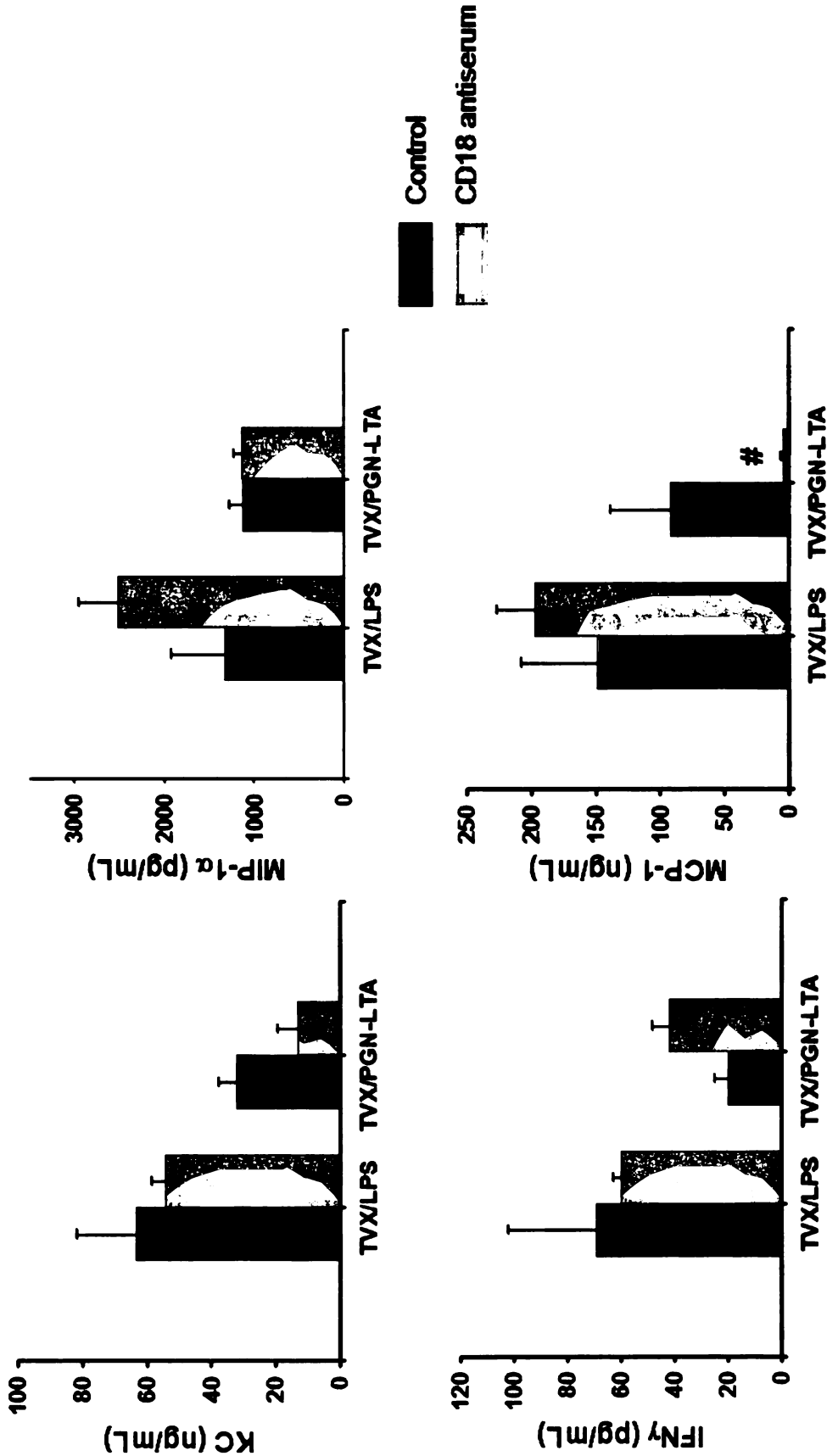

IL-6 (ng/mL)
140
120
100
80
60
40
20
0
TVX/LPS
TVX/PGN-LTA
IL-10 (pg/mL)
12000
10000
8000
6000
4000
2000
0
TVX/LPS
TVX/PGN-LTA
Control
CD18 antiserum
TNFα (pg/mL)
12000
10000
8000
6000
4000
2000
0
#
TVX/LPS
TVX/PGN-LTA
IL-1β (pg/mL)
1200
1000
800
600
400
200
0
TVX/LPS
TVX/PGN-LTA

Fig. 3.8 (cont'd).


Control
CD18 antiserum
KC (ng/mL)
MIP-1α (pg/mL)
MCP-1 (ng/mL)
IFNγ (pg/mL)
TVX/LPS
TVX/PGN-LTA
#

3.5 Discussion

Inflammatory episodes are commonplace and occur sporadically. We hypothesized that an inflammatory stress can decrease the toxicity threshold to certain drugs, precipitating an idiosyncratic toxicity. Previous studies showed that TVX enhanced the LPS-induced plasma TNFα increase and that TVX/LPS coexposure resulted in hepatotoxicity (216). The studies presented here examined whether TVX enhanced the LPS-induced increases in other cytokines. In addition, they explored whether TVX enhanced the cytokine response to gram-positive microbial stimuli, PGN and LTA, and if TVX/PGN-LTA coexposure was hepatotoxic to mice.

Inflammation is a complex process which can be initiated by the host's recognition of microbial products by toll-like receptors. Bacterial components can be measured in the plasma and are increased by a number of stressors including alcohol consumption, surgery and gastrointestinal disturbances (61). Bacterial components of both gram-positive bacteria (PGN and LTA) and gram-negative bacteria (LPS) have been measured in the plasma and activate toll-like receptors to induce inflammation. PGN and LTA activate TLR2 and induce NFκB activation through MyD88-dependent mechanisms (217, 220-222). As described extensively in Section 1.2.1, LPS activates TLR4 and induces NFκB activation through both MyD88-dependent and –independent mechanisms.

A nontoxic dose of TVX was rendered hepatotoxic upon coexposure to a nontoxic dose of either LPS or PGN-LTA (Fig. 3.1). The timecourse of hepatotoxicity for TVX/LPS- and TVX/PGN-LTA-induced liver injury was similar,

inasmuch as plasma ALT activity increased as early as 4.5 h and continued to progress until 15 h. That TVX interacted with either TLR2- or TLR4-activating ligands to cause liver injury proves that the TVX/inflammation-induced liver injury shown previously (216) is not specific to TLR4 activation. Indeed, it suggests that TVX interacts with an inflammatory stress, irrespective of its source, to precipitate liver injury. The result suggests that inflammatory stress induced by either gram-positive or gram-negative bacteria might play a role in TVX hepatotoxicity.

Despite a similar timecourse of liver injury, the histopathology differed between TVX/LPS- and TVX/PGN-LTA-induced liver injury (Fig. 3.2). TVX/LPS-treated mice developed lesions of hepatocellular necrosis and apoptosis primarily localized to midzonal regions, whereas TVX/PGN-LTA lesions of hepatocellular necrosis and apoptosis were primarily centrilobular regions. Such a difference in localization might be due to a difference in TLR2 and TLR4 expression in regions of the mouse liver, of which little is known. Another possibility is that the difference in the location of the lesions suggests a difference in the mechanism of pathology. Therefore, to examine possible mechanisms of pathogenesis, inflammatory cytokines were measured at 4.5 h, the onset of liver injury, to determine if TVX enhanced cytokine release in response to bacterial stimuli.

TVX enhanced the LPS- and PGN-LTA-induced increases of several cytokines: IL-1β, IL-6, IL-18, VEGF, MCP-1 and IL-10. In contrast, only the LPS-induced increase of TNFα and IFNγ was enhanced by TVX, and it did not affect the PGN-LTA induction of these cytokines (Fig. 3.3). These cytokines were only

measured at 4.5 h, therefore it is possible that TVX enhanced PGN-LTA-induced increases in TNFα and IFNγ at other times. Indeed, TVX pretreatment caused a trend towards an increase in TNFα in PGN-LTA-treated mice, but the difference was not statistically significant at this time. IFNγ induction by LPS, but not PGN-LTA, was enhanced by TVX, a result possibly related to the differences in TLR2- and TLR4-activation. TLR4, but not TLR2 activation induces dendritic cells to produce IL-12 (223, 224), which directly stimulates natural killer and T cells to produce IFNγ (225). It is thus possible that TVX acts to enhance a number of the steps in this pathway of IFNγ production induced by LPS which would not be activated by PGN-LTA.

Similar to a number of cytokines, TVX enhanced the LPS- and PGN-LTA-induced increase in KC, MIP-2 and MIP-1α (Fig. 3.4), all of which can be upregulated as a result of NFκB activation (226). It is thus likely that the common upregulation of cytokines by both stimuli was mediated through NFκB activation. KC, MIP-2 and MIP-1α all have chemotactic activity for neutrophils; therefore, hepatic PMN accumulation was quantified to determine if the TVX enhancement of chemokines was associated with an increase in LPS- and/or PGN-LTA-induced PMN accumulation. Despite an increase in several chemokines, TVX pretreatment did not affect LPS- or PGN-LTA-induced neutrophil accumulation in the liver (Fig. 3.5). After LPS/galactosamine treatment, neutrophils roll and adhere in hepatic postsinusoidal venules independently of KC or MIP-2, but extravasation of neutrophils into the parenchyma was significantly reduced by MIP-2 or KC neutralization (227). It is thus possible that TVX enhances LPS- and

PGN-LTA-induced PMN extravasation into the parenchyma and in turn PMN activation. This would not be detected by PMN staining.

To determine if PMN activation is involved in TVX/LPS- or TVX/PGN-LTA-induced liver injury, mice were pretreated with a neutralizing antibody to CD18, a β_2-integrin critical for PMN activation (102, 228). CD18 neutralization attenuated hepatotoxicity induced by either TVX/LPS or TVX/PGN-LTA coexposure (Fig. 3.6); therefore, PMN activation appears to be a common pathway required for the progression of liver injury. Neutrophil activation is required for TVX/inflammation-induced liver injury in both rats and mice (58), which suggests that this requirement is not species-specific. It therefore might be an important pathway in TVX-induced hepatotoxicity in people.

The mechanism of hepatic neutrophil accumulation after TVX/LPS or TVX/PGN-LTA coexposure is different, inasmuch as CD18 neutralization reduced TVX/PGN-LTA- but not TVX/LPS-induced PMN accumulation (Fig. 3.7). This finding that LPS-induced hepatic neutrophil accumulation is CD18-independent is consistent with previous reports (229). However, that PGN-LTA-induced hepatic neutrophil accumulation is CD18-dependent has not been reported to our knowledge. Further studies are required to understand the difference in mechanisms of PMN accumulation between these two stimuli.

TVX enhanced the induction of cytokines by either LPS or PGN-LTA, therefore cytokines were measured to determine if CD18 neutralization affected the induction of proinflammatory cytokines. CD18 neutralization did not significantly affect the TVX/LPS induction of IL-6, IL-10, TNFα, IL-1β, KC, MIP-1α,

IFNγ or MCP-1 (Fig. 3.8). Previously, TNFα was found to be critical for TVX/LPS-induced liver injury (216). The finding that CD18 neutralization did not affect TNFα induction suggests that PMN activation and TNFα activity either represent separate hepatotoxic pathways or that PMN activation is downstream of TNFα in a pathway involved in the pathogenesis in TVX/LPS-cotreated mice.

Similar to TVX/LPS, CD18 neutralization did not affect the TVX/PGN-LTA induction of IL-6, IL-10, IL-1β, KC, MIP-1α or IFNγ. However, it did attenuate the TVX/PGN-LTA-induced increase in TNFα and MCP-1 (Fig. 3.8). Since IL-6, KC and IL-10 were unchanged by CD18 neutralization, Kupffer cell activation was probably not affected, and the attenuation of TNFα and MCP-1 may be neutrophil-dependent. Neutrophils can produce and release MCP-1, and the depletion of neutrophils significantly reduced MCP-1 production induced by the injection of apoptotic cells into the peritoneal cavity of mice (230, 231). Therefore, it is likely that the attenuation of MCP-1 induction by CD18 neutralization is due to the reduction in hepatic neutrophil accumulation. Similarly, the reduction in TNFα concentration by CD18 neutralization might also be due to the attenuation of hepatic neutrophil accumulation, inasmuch as neutrophils express TACE on their extracellular membrane. This enzyme is critical for TNFα cleavage and release (232). Whether TVX/PGN-LTA-induced liver injury requires TNFα is unknown, therefore protection by CD18 neutralization could be due to PMN inactivation or to reduced concentrations of TNFα.

In summary, TVX synergized with a modest inflammatory stress induced by either a gram-negative or a gram-positive stimulus to cause liver injury in mice.

TVX enhanced the LPS- and PGN-LTA-induced increases in proinflammatory cytokines. However, TVX did not enhance the hepatic neutrophil accumulation driven by either of these stimuli. CD18 neutralization attenuated TVX/LPS- and TVX/PGN-LTA-induced liver injury, suggesting that PMN activation plays a critical role in injury progression in both models. CD18 neutralization attenuated TVX/PGN-LTA induced increases in hepatic neutrophil accumulation and TNFα and MCP-1 plasma concentrations. In contrast, it did not affect TVX/LPS-induced hepatic neutrophil accumulation or proinflammatory cytokine increases. The results suggest that inflammatory stress induced by either a gram-positive or gram-negative bacterial products could play a role in the development of TVX-induced hepatotoxicity and that the pathogenesis is CD18-dependent.

CHAPTER 4

Shaw, P.J., Ganey, P.E. and Roth, R.A. (2008). TNFα acting at both p55 and p75 receptors is essential for synergistic hepatotoxicity from TVX/LPS coexposure. ***Submitted to JPET***

4.1 Abstract

The use of trovafloxacin (TVX), a fluoroquinolone antibiotic, was severely restricted due to an association of TVX therapy with idiosyncratic hepatotoxicity in patients. The mechanisms underlying idiosyncratic toxicity are unknown; however, one hypothesis is that an inflammatory stress can render an individual sensitive to the drug. Previously, we reported that treatment of mice with TVX and lipopolysaccharide (LPS) induced tumor necrosis factor α (TNFα)-dependent liver injury, whereas TVX or LPS treatment alone was nontoxic. The goal of this study was to elucidate the role of TNFα in TVX/LPS-induced liver injury. $p55^{-/-}$ (TNFR1) and $p75^{-/-}$ (TNFR2) mice were protected from hepatotoxicity caused by TVX/LPS coexposure, suggesting that TVX/LPS-induced liver injury requires both TNF receptors. TNFα inhibition using etanercept significantly reduced the TVX/LPS-induced increases in the plasma concentrations of several cytokines around the time of onset of liver injury. However, despite the reduction in chemokines, etanercept treatment did not affect the TVX/LPS-induced hepatic accumulation of neutrophils. In addition, etanercept treatment attenuated TVX/LPS-induction of plasminogen activator inhibitor-1 (PAI-1), and this was associated with a reduction in hepatic fibrin deposition. Mice treated with TVX and a nontoxic dose of TNFα also developed liver injury. In summary, TNFα acts through p55 and p75 receptors to precipitate an innocuous inflammatory cascade. TVX enhances this cascade, converting it into one that results in hepatocellular injury.

4.2 Introduction

Trovafloxacin (TVX), a fluoroquinolone antibiotic, is one example of a drug for which use was restricted severely due to IADRs. TVX was approved for use in the U.S. in 1997, and by 1999 its use was associated with 152 cases of serious hepatic events. Of these, 14 resulted in acute liver failure, 5 patients required liver transplants and 4 died (233).

One hypothesis regarding the cause of IADRs is that inflammatory stress alters the toxicity threshold of an individual, rendering a normally therapeutic dose of a drug toxic (55, 73). In accordance with this hypothesis, nontoxic doses of TVX and bacterial lipopolysaccharide (LPS) synergized to cause acute liver injury in both rats and mice (58, 216). In this animal model, TVX pretreatment enhanced the LPS-induced peak in plasma TNFα concentration. In addition, TNFα neutralization completely protected mice from TVX/LPS-induced liver injury (216).

TNFα is a pleiotropic cytokine that stimulates a number of cellular responses, including proliferation, production of inflammatory mediators, upregulation of adhesion molecules and programmed cell death. Large amounts of TNFα are produced in response to several microbial products, including LPS. TNFα is a key mediator of inflammatory responses, which can result in both tissue damage and host defense (74, 75). The main cellular source of TNFα is macrophages, but several other cell types produce TNFα including mast cells, hepatic stellate cells, endothelial cells, fibroblasts and neuronal cells (80, 81).

TNFα plays a critical role in several models of liver injury caused by viral hepatitis, ischemia/reperfusion or hepatotoxic doses of LPS (76, 78, 79, 209).

The biological effects of TNFα are elicited via two high affinity cell surface receptors, p55 (TNF-R1) and p75 (TNF-R2) (83). The role of each receptor has been evaluated in several models of liver injury. The p55 receptor has been studied more extensively and is important in hepatotoxicity caused by LPS, acetaminophen or carbon tetrachloride (77, 95, 96). In contrast, critical roles for both receptors have been shown only in a few models of hepatotoxicity, such as that induced by concanavalin A, *Pseudomonas aeruginosa* exotoxin A or adenovirus (234-236). The study presented here was designed to determine the importance of each receptor in TVX/LPS-induced liver injury and to evaluate the influence of TNFα on other proinflammatory factors in this IADR model.

4.3 Materials and Methods

4.3.1 Materials

Recombinant murine TNFα was purchased from R&D Systems (Minneapolis, MN). Please refer for Section 2.3.1 for additional information on this topic.

4.3.2 Animals

$p55^{-/-}$, $p75^{-/-}$, and C57/Bl6 wild-type controls were purchased from Jackson Laboratory (Bay Harbor, ME). Please refer to Section 2.3.2 for additional information on this topic.

4.3.3 Experimental protocols

Mice fasted for 12 h were given TVX (150 mg/kg) or Veh (saline) by oral gavage. They were then given LPS (2.0×10^6 EU/kg), TNFα (50 μg/kg)) or Veh (saline) by intraperitoneal injection 3 h later. Food was returned immediately after this dosing. Mice were anesthetized with sodium pentobarbital (50 mg/kg; i.p.) and killed at designated times after LPS, TNFα or Veh for various measurements. Blood was drawn from the vena cava into a syringe containing sodium citrate, resulting in a final concentration of 0.76%. The left lateral lobe was fixed in 10% neutral buffered formalin and paraffin blocked.

4.3.4 Histopathology

Please refer to Section 2.3.4 for information on this topic.

4.3.5 Cytokine measurements

Please refer to Section 3.3.5 for information on this topic.

4.3.6 Neutrophil staining

Please refer to Section 3.3.6 for information on this topic.

4.3.7 Hemostatic system measurements

Plasma thrombin:antithrombin III (TAT) dimers were measured using the Enzygnost TAT ELISA kit purchased from Dade Behring (Marburg, Germany). Active PAI-1 plasma concentration was measured using an ELISA kit purchased from Molecular Innovations, Inc. (Novi, MI). Hepatic fibrin immunohistochemistry and estimation of deposition was done following the protocol described previously with a slight modification (237), i.e., artifactual fibrin staining seen within vessel lumens in all treatment groups was removed from quantification calculations.

4.3.8 Statistical analyses

Results are presented as mean $\pm$ S.E.M. A student's t-test or a 2-way analysis of variance (ANOVA) was used as appropriate after data normalization.

All pairwise comparisons were made using a Tukey test with the criterion for significance at $p < 0.05$.

4.4 Results

4.4.1 $p55^{-/-}$ and $p75^{-/-}$ mice are protected from TVX/LPS-induced liver injury

To determine the contribution of each TNF receptor to TVX/LPS-induced liver injury, $p55^{-/-}$ and $p75^{-/-}$ mice were treated with TVX/LPS as described in Materials and Methods. TVX/LPS coexposure caused significant liver injury at 15 h in control (wild-type) mice. Both $p55^{-/-}$ and $p75^{-/-}$ mice were resistant to TVX/LPS-induced liver injury (Fig. 4.1). $p75^{-/-}$ mice were completely protected from TVX/LPS-induced liver injury and had significantly reduced plasma ALT activity compared to control and $p55^{-/-}$ mice (Fig. 4.1). Histopathologic examination of livers corroborated this result, inasmuch as lesions of hepatocellular necrosis were decreased in $p55^{-/-}$ compared to control mice, and were completely absent in $p75^{-/-}$ mice (Fig. 4.1).

4.4.2 TNFα neutralization attenuates TVX/LPS-induced inflammatory cytokines and chemokines

In a previous study, treatment with etanercept, which is a mimic of the soluble p75 receptor, reduced TVX/LPS-induced increase in plasma TNFα concentration and protected mice from TVX/LPS-induced liver injury (216). TVX/LPS-treated mice were dosed with etanercept to determine the effects of TNFα on the induction of proinflammatory cytokines and chemokines at 4.5 h, a time near the onset of liver injury (see Fig. 3.1). TNFα inhibition attenuated the TVX/LPS-mediated induction of IFNγ, IL-6, IL-10, MCP-1 and VEGF (Fig. 4.2).

Fig. 4.1. The role of TNF receptors in TVX/LPS-induced liver injury. Wild-type, $p55^{-/-}$ and $p75^{-/-}$ mice were treated with TVX 3 h before LPS as described in Materials and Methods. Mice were killed 15 h after LPS, and plasma ALT activity was measured. Photomicrographs were taken of livers from representative mice from each group. *n* = 5-8 animals/group. *significantly different from wild-type group; # significantly different from $p55^{-/-}$ group.

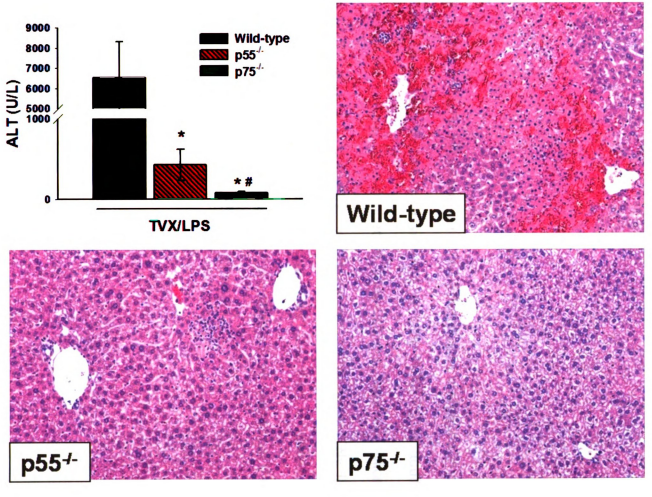
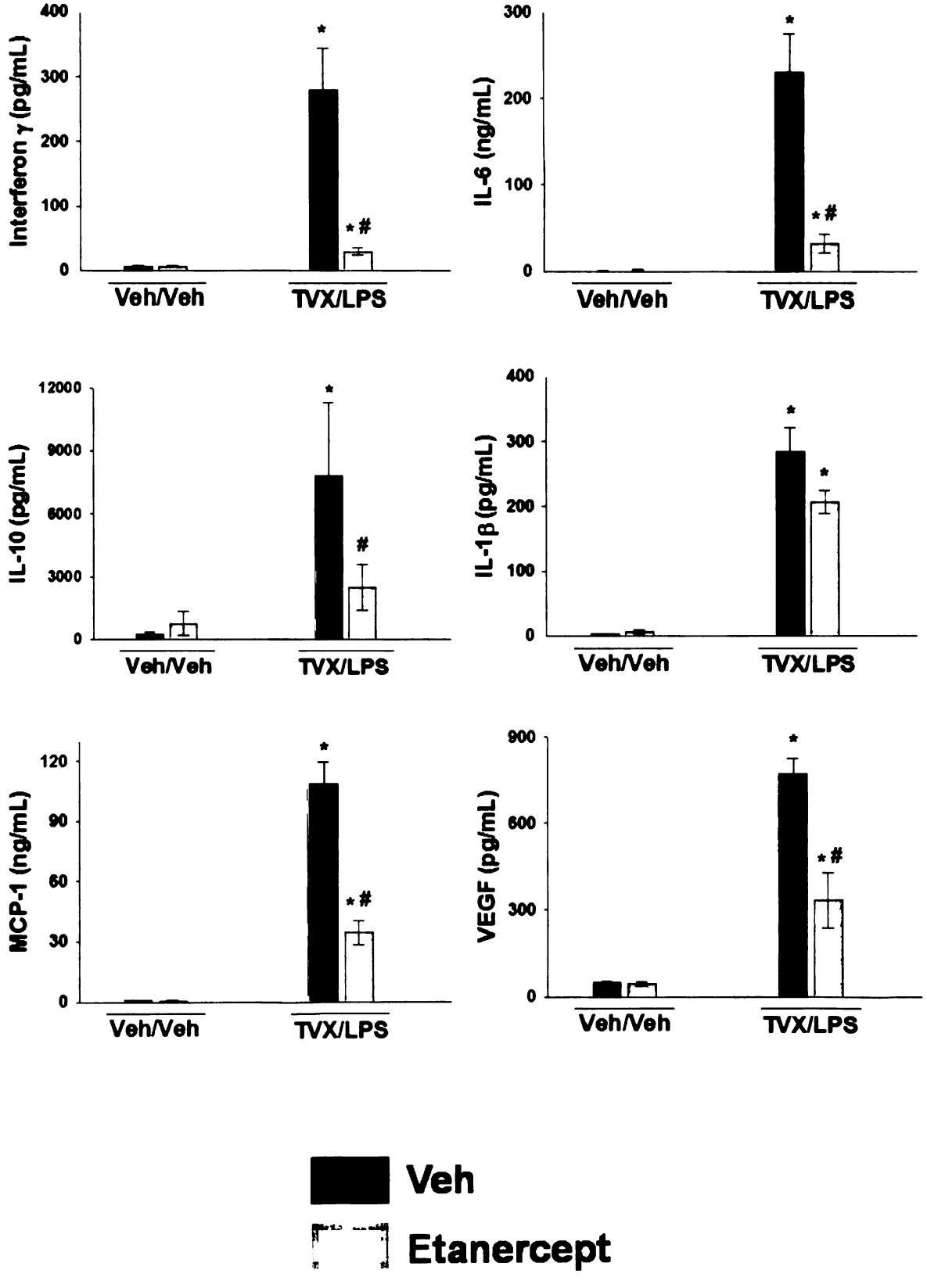

9000
8000
7000
6000
5000
1000
0
ALT (U/L)
Wild-type
p55-/-
p75-/-
*
*#
TVX/LPS
Wild-type
p55-/-
p75-/-

Fig. 4.2. Effect of TNFα inhibition on TVX/LPS-induced increases in plasma cytokines. Mice were treated with vehicles or with TVX/LPS in addition to etanercept or its vehicle as described in Materials and Methods. Mice were sacrificed 4.5 h after LPS administration. Plasma concentrations of IFNγ, IL-6, IL-10, IL-1β, MCP-1 and VEGF were measured as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh; # significantly different from TVX/LPS/Veh group.


Interferon γ (pg/mL)
400
300
200
100
0
*
*#
Veh/Veh
TVX/LPS
IL-6 (ng/mL)
300
200
100
0
*
*#
Veh/Veh
TVX/LPS
IL-10 (pg/mL)
12000
9000
6000
3000
0
*
#
Veh/Veh
TVX/LPS
IL-1β (pg/mL)
400
300
200
100
0
*
*
Veh/Veh
TVX/LPS
MCP-1 (ng/mL)
120
90
60
30
0
*
*#
Veh/Veh
TVX/LPS
VEGF (pg/mL)
900
600
300
0
*
*#
Veh/Veh
TVX/LPS
Veh
Etanercept

The increase in IL-1β plasma concentration following TVX/LPS treatment was not changed by etanercept treatment (Fig. 4.2). TNFα neutralization significantly reduced the TVX/LPS induction of chemokines MIP-2, KC and MIP-1α (Fig. 4.3).

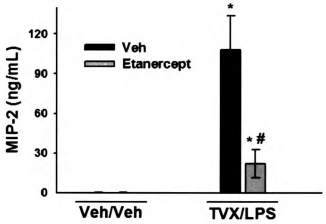
4.4.3 TVX/LPS-induced hepatic neutrophil accumulation is independent of TNFα

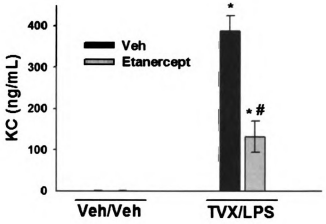
Previous results pointed to a role for PMNs in the pathogenesis of TVX/LPS-induced liver injury (Chapter 3). Despite causing a reduction in chemokines (Fig. 4.3), etanercept treatment did not reduce hepatic neutrophil accumulation induced by TVX/LPS coexposure. In fact, etanercept treatment slightly increased neutrophil accumulation (Fig. 4.4).

4.4.4 TNFα neutralization attenuates TVX/LPS-induced hemostatic system activation

The coagulation system plays an important role in TVX/LPS-induced pathogenesis (presented in Chapter 6). To determine if TNFα plays a role in TVX/LPS-induced coagulation system activation, TVX/LPS-treated mice were treated with etanercept and killed at 4.5 h. The dose of etanercept markedly reduced the TVX/LPS-induced release of TNFα in this model (216). Plasma thrombin-antithrombin (TAT) dimers, measured as a biomarker of coagulation system activation, were significantly increased in TVX/LPS-treated mice (Fig. 4.5A). Etanercept treatment caused a trend toward reduction in plasma TAT dimers, but this difference was not statistically significant. The plasma

Fig. 4.3. Effect of TNFα inhibition on TVX/LPS-induced increases in plasma chemokines. Mice were treated with vehicles or with TVX/LPS in addition to etanercept or its vehicle as described in Materials and Methods. Mice were sacrificed 4.5 h after LPS administration. Plasma concentrations of MIP-2, KC and MIP-1α were measured as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group; # significantly different from TVX/LPS/Veh group.


MIP-2 (ng/mL)
120
90
60
30
0
Veh
Etanercept
*
*#
Veh/Veh
TVX/LPS


KC (ng/mL)
400
300
200
100
0
Veh
Etanercept
*
*#
Veh/Veh
TVX/LPS

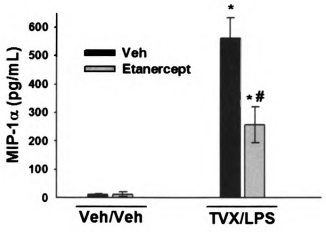

MIP-1α (pg/mL)
600
500
400
300
200
100
0
Veh
Etanercept
*
*#
Veh/Veh
TVX/LPS

Fig. 4.4. Effect of TNFα inhibition on TVX/LPS-induced hepatic PMN accumulation. Mice were treated with vehicles or with TVX/LPS in addition to etanercept or its vehicle as described in Materials and Methods. Mice were sacrificed 4.5 h after LPS administration. Paraffin-embedded livers were stained for neutrophils, and the number of neutrophils was quantified as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group; # significantly different from TVX/LPS/Veh group.

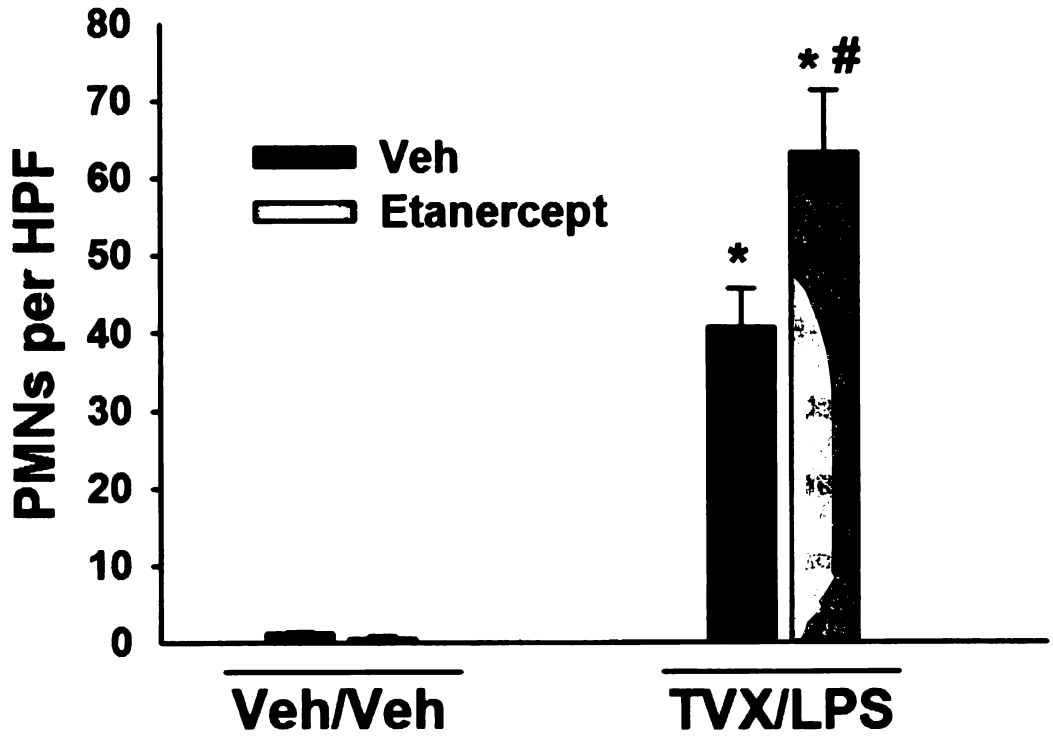
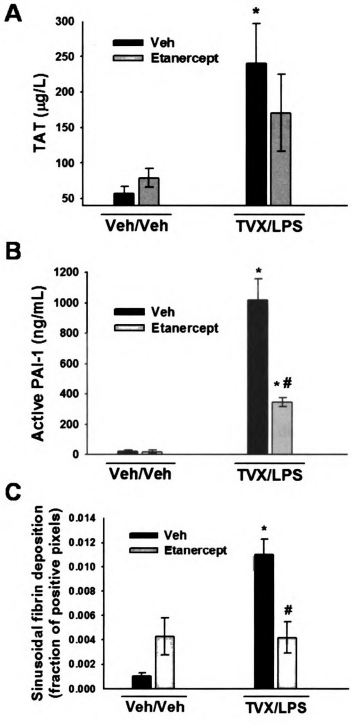

80
70
60
50
40
30
20
10
0
PMNs per HPF
Veh
Etanercept
*
* #
Veh/Veh
TVX/LPS

Fig. 4.5. Effect of TNFα inhibition on hemostatic system dysregulation mediated by TVX/LPS coexposure. Mice were treated with vehicles or with TVX/LPS in addition to etanercept or its vehicle as described in Materials and Methods. They were sacrificed 4.5 h after LPS administration. Plasma concentrations of (A) TAT dimers and (B) active PAI-1 were measured as described in Materials and Methods. (C) Hepatic fibrin deposition was stained immunohistochemically and quanitified as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group; #significantly different from TVX/LPS/Veh group.


A
TAT (μg/L)
300
250
200
150
100
50
Veh
Etanercept
*
Veh/Veh
TVX/LPS
B
Active PAI-1 (ng/mL)
1200
1000
800
600
400
200
0
Veh
Etanercept
*
*#
Veh/Veh
TVX/LPS
C
Sinusoidal fibrin deposition (fraction of positive pixels)
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Veh
Etanercept
*
#
Veh/Veh
TVX/LPS

concentration of active PAI-1, an inhibitor of the fibrinolytic system, was increased by TVX/LPS coexposure (Fig. 4.5B). Etanercept significantly reduced the TVX/LPS induction of plasma active PAI-1 (Fig. 4.5B). Fibrin deposition in tissue occurs if the rate of coagulation system activation exceeds the rate of fibrinolysis. TVX/LPS coexposure caused a significant increase in sinusoidal fibrin deposition in the liver at 4.5 h, which was significantly reduced by etanercept treatment (Fig. 4.5C).

4.4.5 TVX and TNFα coexposure causes hepatotoxicity

TVX/LPS-induced liver injury is dependent on TNFα (216), but to determine if TNFα alone could interact with TVX, mice were treated with TVX and recombinant murine TNFα as described in Materials and Methods. They were killed 15 h after TNFα treatment, the time of maximal plasma ALT activity in TVX/LPS-treated mice. TVX or TNFα treatment alone did not cause an increase in plasma ALT activity (Fig. 4.6). However, TVX/TNFα coexposure increased plasma ALT activity. Histopathological evaluation of liver sections corroborated the lack of injury from TVX or TNFα alone (Fig. 4.7). In contrast, TVX/TNFα coexposure caused hepatocellular necrotic and apoptotic lesions primarily in centrilobular and midzonal regions of liver lobules, and these lesions extended to periportal regions in some severely affected mice.

Fig. 4.6. TVX/TNFα coexposure-induced liver injury. Mice were treated with TVX 3 h before recombinant murine TNFα as described in Materials and Methods. Mice were killed 15 h after TNFα administration, and plasma ALT activity was measured. *n* = 4-5 animals/group. *significantly different from TVX/Veh group; # significantly different from Veh/TNFα group.

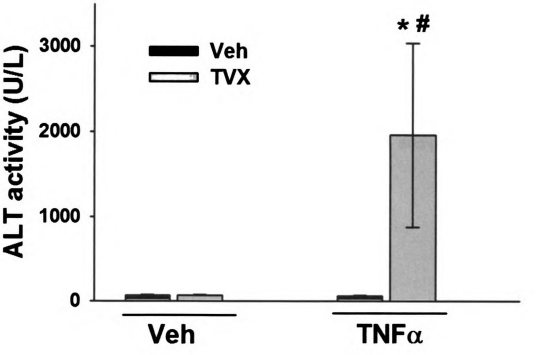
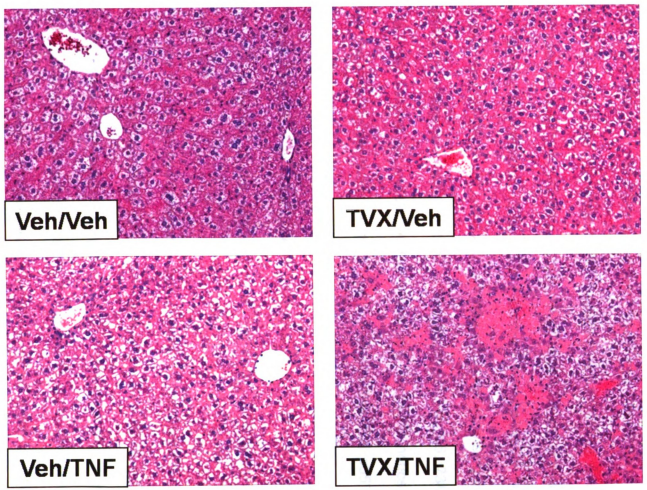

ALT activity (U/L)
3000
2000
1000
0
Veh
TVX
* #
Veh
TNFα

Fig. 4.7. Histopathology of TVX/TNFα-induced liver injury. Mice were treated with TVX 3 h before recombinant murine TNFα as described in Materials and Methods. Mice were killed 15 h after TNFα administration, and photomicrographs were taken of representative livers.


Veh/Veh
TVX/Veh
Veh/TNF
TVX/TNF

4.5 Discussion

Previously, we reported that a nontoxic dose of TVX interacts with a nontoxic dose of LPS to cause TNFα-dependent liver injury in mice (216). The critical role of TNFα in TVX/LPS-induced liver injury was based upon TNFα neutralization, however the role of each TNF receptor was not studied. Activation of the p55 receptor results in two main signals: NFκB activation and activation of caspases leading to apoptosis (238, 239). Inasmuch as ligation of the p55 receptor can result in NFκB activation and cell death, it is not surprising that the p55 receptor is critical to several models of liver injury (77, 95, 96, 234-236). Similar to other models dependent on TNFα and p55, TVX/LPS-induced liver injury was significantly attenuated in $p55^{-/-}$ mice. It is possible that $p55^{-/-}$ mice have reduced liver injury due to decreased plasma TNFα concentrations, since TNFα induction by LPS was found to be attenuated in $p55^{-/-}$ mice (96). However, p55 was not involved in the production of TNFα by galactosamine/LPS coexposure (240).

The function of the p75 receptor is less understood compared to the p55 receptor. Similar to p55, activation of the p75 receptor causes NFκB activation, but it does not result in caspase activation (241). The critical role of the p75 receptor in hepatotoxicity is unclear: it is not involved in some models of liver injury that are dependent on TNFα (96, 240) but is involved in others (234-236). Indeed, $p75^{-/-}$ mice were completely protected from TVX/LPS-induced liver injury and had significantly reduced hepatocellular injury compared to $p55^{-/-}$ mice.

Why $p75^{-/-}$ mice are completely protected from TVX/LPS-induced hepatotoxicity is unclear. It is possible that the p75 receptor is playing two roles in the progression of TVX/LPS-induced hepatotoxicity. It has been suggested that the p75 receptor acts to bind TNFα and transfer it to the p55 receptor, resulting in p55 activation at lower concentrations of TNFα (242). Therefore, without the p75 receptor present, the threshold for TNFα-dependent liver injury might be higher than the concentration that is achieved. Additionally, the p75 receptor can cooperate with the p55 receptor to enhance necrotic cell death in response to TNFα (243). To account for the minor hepatotoxicity seen in $p55^{-/-}$ mice, p75 activation must also cause minor hepatocellular injury independent of p55 following TVX/LPS coexposure. Therefore, it is possible that combined activation of p55 and p75 synergize to cause extensive hepatocellular necrosis which is not seen when either receptor is absent. In addition, the p75 receptor is involved in the LPS-induced production of TNFα (96). It is possible that the complete protection in $p75^{-/-}$ mice resulted from a combination of these mechanisms, inculding a reduction in LPS-induced TNFα production.

TNFα was involved in the TVX/LPS-mediated increases in several inflammatory cytokines: IFNγ, IL-6, MCP-1, VEGF, MIP-2, KC and MIP-1α. The attenuation of IL-6 and MIP-2 after TNFα neutralization was also seen in another model of drug/LPS coexposure-induced hepatotoxicity (244). The reduction of such a large number of cytokines might be due to a decrease in TNFα-driven NFκB activation mediated through p55 and p75 receptor activation (238, 241).

Several of the cytokines that required TNFα for their release have chemotactic properties. Therefore, we measured PMN accumulation in livers of these mice. The hepatic accumulation of PMNs induced by TVX/LPS was not decreased by TNFα inhibition. It is thus likely that the TVX/LPS-induced hepatic PMN accumulation is mediated by selectins and other adhesion molecules as seen in endotoxemia or by TNFα-independent sinusoidal contraction (227, 245). It is possible that TNFα is not involved in PMN accumulation but is needed for PMN activation, since TNFα can promote neutrophil activation *in vitro* (246). If TNFα enhances PMN activation and degranulation, it might explain the slight increase in hepatic PMN accumulation in TVX/LPS/etanercept-treated mice, since when TNFα is present the accumulated PMNs might be activated, degranulate and undergo clearance from the tissue.

In addition to being critical to TVX/LPS upregulation of cytokines, it is possible that TNFα is involved in the progression of liver injury by enhancing hemostasis. TVX/LPS coexposure caused fibrin deposition in liver sinusoids, and treatment with anticoagulant heparin significantly reduced TVX/LPS-induced liver injury (presented in Chapter 6). TNFα has the potential to interact with the hemostatic system in several ways. It can induce tissue factor which activates the coagulation system, but it also increases PAI-1 expression which could depress fibrinolysis (244, 247, 248). Indeed, TVX/LPS-induced increases in active PAI-1 and hepatic fibrin deposition were TNFα-dependent, whereas coagulation system activation showed a trend but was not significantly reduced following TNFα inhibition. The results suggest that if tissue factor induction

occurs during TVX/LPS coexposure, it is TNFα-independent, but that a slight reduction in coagulation system activation by etanercept along with a more pronounced reduction in active PAI-1 was able to prevent hepatic fibrin deposition.

Based on the importance of TNFα in TVX/LPS-induced liver injury, we examined whether TVX can interact with a dose of TNFα to induce similar hepatocellular damage. Indeed, TVX treatment prior to a nonhepatotoxic dose of recombinant murine TNFα resulted in significant liver injury. Other studies have shown that TNFα by itself does not cause liver injury in mice but can when administered with galactosamine or a DNA synthesis inhibitor (249, 250). It is unclear from these results whether TVX sensitized mice to TNFα-induced liver injury or vice versa. However, the hepatocellular lesions in TVX/TNFα-treated mice appear similar to those seen after galactosamine/TNFα coexposure, suggesting commonalities in mechanisms (251). Recently, cytochrome P450 2E1 (CYP2E1) induction by pyrazole was shown to sensitize mice to TNFα-induced liver injury (252). It is unlikely that TVX/TNFα-induced liver injury is related to an effect on CYP2E1 activity by TVX, as TVX treatment alone did not have any effect on CYP2E1 expression (unpublished results). However, to exclude this possibility CYP2E1 activity would need to be measured following TVX exposure. It is also possible that TVX treatment reduced TNFα clearance, and that the prolonged presence of TNFα resulted in cell death. This is consistent with the prolonged presence of TNFα in the plasma of TVX/LPS-treated mice compared to LPS treatment alone (216). TNFα inactivation and clearance is mediated by

soluble forms of the two receptors (253). It is possible that TVX reduces the cleavage or expression of these receptors, in turn reducing TNFα clearance. Further studies are required to better understand the mechanism by which TVX and TNFα interact to cause hepatocellular damage.

In summary, TVX/LPS-induced liver injury depended on the presence of both TNF receptors, p55 and p75. The p75 receptor may play an even more important role than the p55 receptor in the progression of TVX/LPS-induced hepatotoxicity. At the onset of liver injury, the TVX/LPS coexposure-related increase in several cytokines, active PAI-1 and hepatic fibrin was TNFα-dependent. However, despite the observation that the induction of chemokines was TNFα-dependent, the hepatic PMN accumulation was independent of TNFα. The critical role of TNFα in TVX/LPS-induced liver injury was likely through upregulation of cytokines and activation of the hemostatic system. The observation that the liver injury could be reproduced by substituting TNFα administration for LPS supports the critical importance of this cytokine in the pathogenesis.

CHAPTER 5

Shaw, P.J., Ditewig, A.C., Waring, J.F., Liguori, M.J., Blomme, E.A., Ganey, P.E. and Roth, R.A. (2008). Coexposure of mice to trovafloxacin and lipopolysaccharide, a model of idiosyncratic hepatotoxicity, results in a unique gene expression profile and interferon gamma-dependent liver injury. *Submitted to Toxicological Sciences.*

5.1 Abstract

The antibiotic trovafloxacin (TVX) has caused severe idiosyncratic hepatotoxicity in people, whereas levofloxacin (LVX) has not. Mice cotreated with TVX and lipopolysaccharide (LPS), but not with LVX and LPS, develop severe hepatocellular necrosis. Mice were treated with TVX and/or LPS, and hepatic gene expression changes were measured before liver injury using gene array. Hepatic gene expression profiles from mice treated with TVX/LPS clustered differently from those treated with LPS or TVX alone. Several of the probesets expressed differently in TVX/LPS-treated mice were involved in interferon signaling and the JAK/STAT pathway. A timecourse of plasma concentrations of interferon gamma (IFNγ) and interleukin-18 (IL-18), which directly induced IFNγ production, revealed that both cytokines were selectively increased in TVX/LPS-treated mice. Both IL-18$^{-/-}$ and IFNγ$^{-/-}$ mice were significantly protected from TVX/LPS-induced liver injury. In addition, IFNγ$^{-/-}$ mice had decreased plasma concentrations of TNFα, IL-18 and IL-1β when compared to wild-type mice. In conclusion, the altered expression of genes involved in type II interferon signaling in TVX/LPS-treated mice led to the finding that IL-18 and IFNγ play a critical role in TVX/LPS-induced liver injury.

5.2 Introduction

Despite the rare occurrence of IADRs, they represent a serious hazard to public health and are an important issue for pharmaceutical companies and drug-regulatory agencies. Current preclinical testing protocols fail to identify drugs that cause IADRs because predictive animal or *in vitro* models are lacking. Despite the prevalence and threat of IADRs, the mechanisms underlying them are still unknown. As described in Section 1.1.2, we and others have hypothesized that an episode of inflammatory stress can render an individual susceptible to a normally nontoxic drug dose, thereby precipitating an IADR (55, 73, 254). In concordance with this hypothesis, an inflammatory stress renders mice sensitive to TVX-induced hepatotoxicity (56-59, 216).

TVX is a fluoroquinolone antibiotic which has seen limited use due to its association with idiosyncratic hepatotoxicity in people. LVX is a fluroroquinolone antibiotic not associated with hepatotoxicity. In accordance with the lack of hepatotoxicity seen in people, coexposure to TVX/LPS, but not to LVX/LPS, was hepatotoxic to mice and rats (58, 216).

Global gene expression analysis of livers at a time of maximal hepatotoxicity revealed distinct clustering of LPS/TVX-treated rats. We showed recently that mice cotreated with TVX and LPS also develop hepatocellular necrosis. Similar to the rat model, LVX did not interact with LPS to cause liver injury (216). Here we test the hypothesis that at a time before the onset of liver injury, TVX/LPS treatment of mice leads to a distinct pattern of gene expression.

In testing this hypothesis, we identified several genes involved in type II interferon signaling that were changed by TVX/LPS coexposure. IFNγ is a proinflammatory cytokine that plays a key role in both the innate immune system and modulation of the adaptive immune system (255). It is a critical mediator of liver injury from several xenobiotic agents (132, 256, 257). Accordingly, we hypothesized that IFNγ plays a critical role in TVX/LPS-induced liver injury and investigated its influence on the induction of other proinflammatory cytokines.

5.3 Materials and Methods

5.3.1 Materials

Please refer to Section 2.3.1 for information on this topic.

5.3.2 Animals

Please refer to Section 2.3.2 for information on this topic. In addition, IL-$18^{-/-}$ mice and C57/Bl6 wild-type controls were purchased from Jackson Laboratory (Bay Harbor, ME). IFN$\gamma^{-/-}$ female Balb/C mice were a kind gift from Dr. Alison Bauer (MSU, East Lansing, MI), and corresponding female Balb/C control mice were purchased from Jackson Laboratory (Bay Harbor, ME).

5.3.3 Experimental protocol

In a previous study, mice cotreated with nonhepatotoxic doses of TVX and LPS developed liver injury (216). The dose of LVX was chosen to approximate the ratio of TVX/LVX doses used clinically in humans. Mice were fasted for 12 h before each experiment. TVX (150 mg/kg), LVX (375 mg/kg) or their saline vehicle was administered to mice by oral gavage and then given LPS (2.0 X 10^6 EU/kg, i.p.) 3 h later. Food was returned immediately after LPS administration. Mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) at various times, and sacrificed by exsanguination. The left lateral liver lobe was fixed in 10% neutral buffered formalin and blocked in paraffin within 72 h. The right medial liver lobe was flash frozen in liquid nitrogen for total RNA isolation.

5.3.4 ALT activity and histopathology

Plasma ALT activity was measured spectrophotometrically using Infinity ALT reagent purchased from Thermo Electron Corp. (Louisville, CO). Formalin-fixed liver lobes were processed and embedded in paraffin. Paraffin sections were cut at 5 μm and stained with hematoxylin and eosin.

5.3.5 RNA isolation

Frozen liver samples (50 mg of tissue per sample) were immediately added to 2 mL of TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California). One mL of the tissue homogenate was transferred to a microfuge tube, and total RNA was extracted with chloroform followed by nucleic acid precipitation with isopropanol. The pellet was washed with 80% ethanol and resuspended in molecular biology grade water. Nucleic acid concentration was determined spectrophotometrically at 260 nm (Smart-Spec, Bio-Rad Laboratories, Hercules, CA), and RNA integrity was evaluated using an Agilent bioanalyzer (Agilent Technologies, Model 2100, Foster City, CA).

5.3.6 Gene array analysis

Microarray analysis was performed using the standard protocol provided by Affymetrix, Inc. (Santa Clara, CA). Briefly, approximately 5 ug of total RNA was reversed transcribed into cDNA using a Superscript II Double-Strand cDNA synthesis kit (Invitrogen Life Technologies, Carlsbad, California). The primer used for the reverse transcription reaction was a modified T7 primer with 24

thymidines at the 5' end (Affymetrix). The sequence was 5'GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3'. cDNA was purified via phenol/chloroform/isoamylalcohol (Invitrogen Life Technologies, Carlsbad, California) extraction and ethanol precipitation. Biotin-labeled cRNA was synthesized according to the manufacturer's instructions from the cDNA using the Enzo RNA Transcript Labeling Kit (Affymetrix). The labeled cRNA was then purified using RNeasy kits (Qiagen, Valencia, CA). cRNA concentration and integrity were evaluated. Approximately 20 μg of cRNA was then fragmented in a solution of 40 mM Tris-acetate, pH 8.1, 100 mM potassium acetate, and 30 mM magnesium acetate at 94°C for 35 minutes. Fragmented, labeled cRNA was hybridized to an Affymetrix mouse genome array, 430A 2.0 which contains sequences corresponding to roughly 22,600 transcripts, at 45°C overnight using an Affymetrix Hybridization Oven 640. The array was subsequently washed and stained twice with strepavidin-phycoerythrin (Molecular Probes, Eugen, OR) using a Gene-Chip Fluidics Workstation 400 (Affymetrix). The array was then scanned using the Affymetrix GeneChip® Scanner 3000.

5.3.7 Hepatic neutrophil accumulation

Please refer to Section 3.3.6 for information on this topic.

5.3.8 Plasma cytokine measurements

The plasma concentrations of IFNγ, IL-18, IL-6, IL-10, MIP-1α, KC, MIP-2, MCP-1, VEGF, TNFα and IL-1β were measured using bead-plex kits purchased

from Bio-Rad Laboratories and measured using a Bio-Plex 200 system (Hercules, CA).

5.3.9 Statistical methods

ALT activity and plasma protein concentration results are presented as mean ± S.E.M. A 1-,2-, or 3-way analysis of variance (ANOVA) was used as appropriate after data normalization. All pairwise comparisons were made using Dunn's method. The criterion for significance was $p < 0.05$.

The results of the microarrays were analyzed using Rosetta Resolver error models, and ratios were built for each treatment array compared to the Veh/Veh control using the Resolver System. This analysis calculated a *p*-value for every gene's fold change relative to Veh/Veh using the Rosetta Resolver error model (258). The gene expression change was considered significant if it had a *p*-value <0.001. Genes were considered regulated if the *p*-value was <0.001 for 2 of 3 Veh/Veh-, 2 of 3 TVX/Veh-, 2 of 3 LVX/Veh-, 4 of 5 Veh/LPS-, 3 of 4 LVX/LPS-, 5 of 7 TVX/LPS-treated mice. Average link heuristic criteria and the Euclidean distance metric for similarity measure were used to perform the agglomerative cluster analysis of the treatment arrays using Rosetta Resolver software. Gene expression profile analysis was done using Ingenuity Pathway Analysis purchased from Ingenuity Systems (Redwood, CA).

5.4 Results

5.4.1 Development of hepatocellular injury after TVX/LPS-, but not LVX/LPS-coexposure

The timecourse of hepatocellular injury was examined to determine the time of the onset of liver injury. Treatment with TVX, LVX, LPS, or LVX/LPS did not increase plasma ALT activity (Fig. 5.1). TVX/LPS coexposure, however, caused a significant increase in plasma ALT activity as early as 4.5 h which continued to increase through 15 h (Fig. 5.1).

5.4.2 Hepatic global gene expression changes before the onset of hepatotoxicity

Hepatic gene expression was evaluated at 3 h after LPS, a time prior to the onset of liver injury. Analysis of Affymetrix Genechip 430 2.0 Array data identified probesets defined as regulated relative to Veh/Veh-treated mice, and these were subjected to hierarchical clustering analysis (Fig. 5.2). Within the cluster of LPS-treated mice, LVX/LPS- and Veh/LPS-treated mice were distinguished from TVX/LPS-treated mice (Fig. 5.2). Thus, hierarchical clustering applied to gene expression analysis was able to distinguish TVX/Veh from LVX/Veh-treated mice. In addition, at a time before liver injury, a separate cluster occurred for TVX/LPS-treated mice, the only treatment that produced hepatotoxicity.

Fig.5.1. Development of TVX/LPS-induced liver injury. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg) or Veh (saline) orally and then 3 h later with either LPS (2 x 10^6 EU/kg; i.p.) or Veh (saline). Mice were sacrificed at various times after LPS dosing, and plasma ALT activity was measured. *n*= 4-6 animals/group. * significantly different from 0 h respective control group. # significantly different from all other treatment groups at the same time.

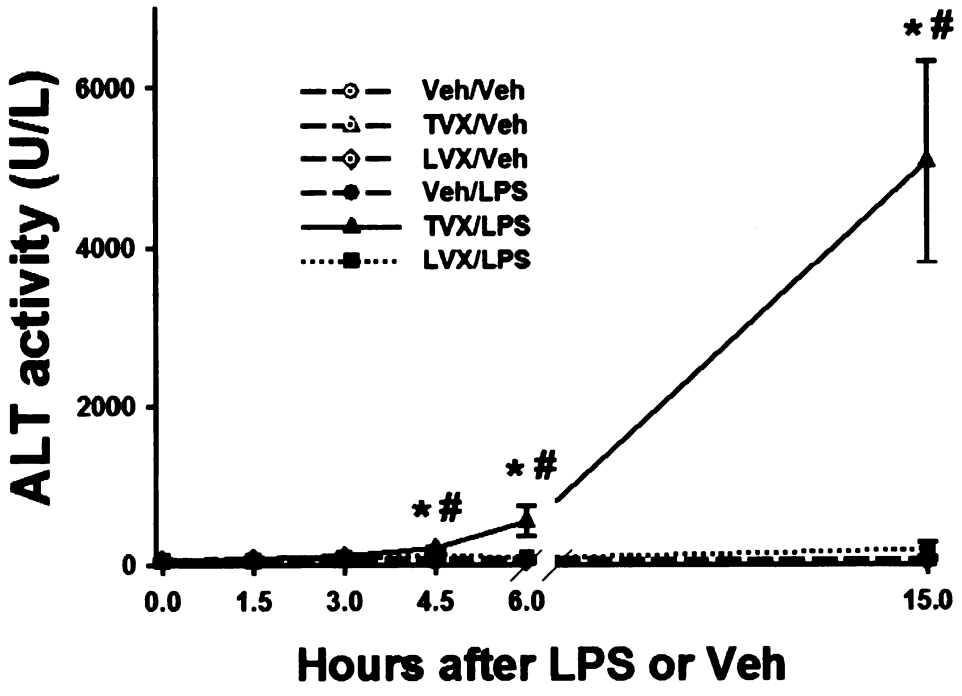
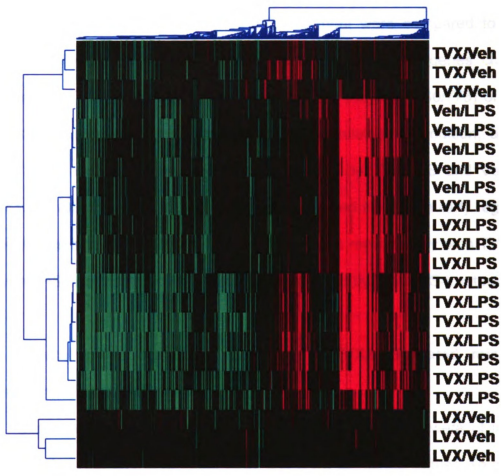

ALT activity (U/L)
6000
4000
2000
0
Veh/Veh
TVX/Veh
LVX/Veh
Veh/LPS
TVX/LPS
LVX/LPS
*#
*#
*#
0.0
1.5
3.0
4.5
6.0
15.0
Hours after LPS or Veh

Fig. 5.2. Hierarchical clustering of hepatic gene expression profiles. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg) or Veh (saline) orally and then 3 h later with either LPS (2 x 10^6 EU/kg; i.p.) or Veh (saline). Mice were sacrificed 3 h after LPS or Veh dosing, and total RNA was isolated from the liver. Gene expression was evaluated using Affymetrix Genechip 430 2.0 Arrays. RNA from each mouse was analyzed using a separate array. Gene expression profiles are analyzed relative to Veh/Veh-treated mice. Green represents probesets downregulated, whereas red represents probesets upregulated with respect to Veh/Veh-treated mice.


TVX/Veh
TVX/Veh
TVX/Veh
Veh/LPS
Veh/LPS
Veh/LPS
Veh/LPS
Veh/LPS
LVX/LPS
LVX/LPS
LVX/LPS
LVX/LPS
TVX/LPS
TVX/LPS
TVX/LPS
TVX/LPS
TVX/LPS
TVX/LPS
TVX/LPS
LVX/Veh
LVX/Veh
LVX/Veh

p≤0.001; n= 6345

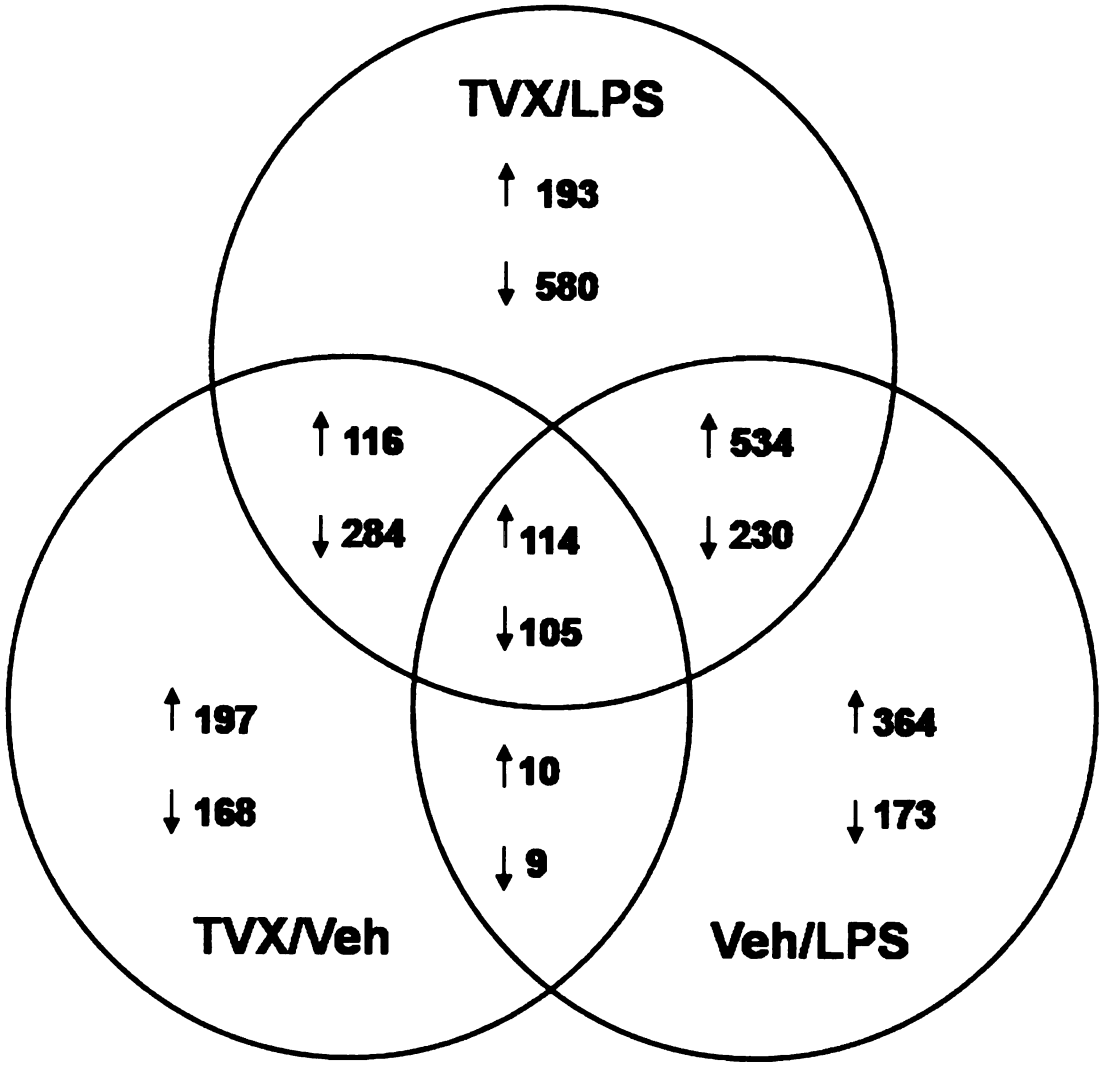
5.4.3 Gene expression changes within TVX/LPS, TVX/Veh and Veh/LPS treatment groups

The gene expression changes in the TVX/LPS-treated group are important because they can be anchored to hepatotoxicity. TVX/LPS treatment resulted in 2156 gene expression changes compared to Veh/Veh-treated mice. The gene expression changes induced by TVX/LPS treatment were compared to those induced by TVX or LPS alone revealing a large number of genes changed selectively in TVX/LPS-treated mice (Fig. 5.3). The majority (580/773) of these TVX/LPS treatment-selective genes were downregulated. In addition, a large number of genes (983) were commonly regulated by TVX/LPS and Veh/LPS treatment. TVX/LPS and TVX/Veh treatments resulted in 619 similarly regulated genes. As expected, there were relatively few genes regulated by both TVX/Veh and Veh/LPS treatments which were not also affected by TVX/LPS-treatment.

5.4.4 Several gene expression changes in TVX/LPS-treated mice are involved in interferon signaling

Gene expression changes in the TVX/LPS-treated mice were of interest due to the development of hepatotoxicity and were examined further. To do this, a list of genes expressed differently ($p<0.001$) in the TVX/LPS-treated mice compared to all other treatment groups (Veh/Veh, TVX/Veh, LVX/Veh, Veh/LPS, and LVX/LPS) was created using TVX/LPS-treatment as the baseline for ratio building. 254 probesets were selectively altered by TVX/LPS treatment; of these probesets, 142 represented functional genes as determined by Ingenuity

Fig. 5.3. Venn diagram depiction of probeset regulation of TVX/Veh-, Veh/LPS- and TVX/LPS-treated mice relative to Veh/Veh-treated controls. Mice were treated with TVX and/or LPS as described in Methods. Three hours after LPS administration, total RNA was isolated from the liver, and gene expression was evaluated using Affymetrix Genechip 430 2.0 Arrays. The number of probesets increased or decreased relative to Veh/Veh-treated mice is shown. Probesets were defined as regulated if $p < 0.001$.


TVX/LPS
↑ 193
↓ 580
↑ 116
↓ 284
↑ 534
↓ 230
↑ 114
↓ 105
↑ 197
↓ 168
↑ 10
↓ 9
↑ 364
↓ 173
TVX/Veh
Veh/LPS

Systems analysis. A full list of these 142 TVX/LPS-selective genes can be found in Supplemental Table 5.3.

The 142 genes selectively changed in expression by TVX/LPS were analyzed using Ingenuity Pathway Analysis to determine highly affected pathways. Table 5.1 is a list of pathways impacted by the genes selectively altered by TVX/LPS treatment in order of increasing *p*-values. Pathways with *p*-values > 0.1 were excluded from the list. In addition to the *p*-values, the fraction (ratio) of genes affected within each pathway is reported.

Of the pathways affected, two which had the highest ratios, low p-values and are involved in several models of liver injury were JAK/Stat signaling and interferon signaling (Table 5.1). Type I and II interferons signal via specific receptors through JAK/Stat signaling within cells (259). To examine further the potential role of interferons in TVX/LPS-induced gene expression changes, genes in the set of 142 TVX/LPS-selective genes were examined for their relationship to interferons. Of the 142 genes, 26 (18.3%) are regulated by interferons (Table 5.2). Of these IFN-regulated genes, all but 3 were increased in expression. A number of the genes have potential importance in the development of hepatotoxicity by being pro-apoptotic, chemokines, pro-inflammatory or involved in immune responses (Table 5.2). The majority of these genes were regulated by IFNγ. Accordingly, we examined the role of IFNγ in TVX/LPS-induced liver injury.

Table 5.1. Pathways highly affected by the 142 functional genes selectively changed by TVX/LPS-treatment compared to all other treatment groups. Mice were treated as described in methods. Three hours after LPS administration, RNA was isolated from the liver, and gene expression was evaluated using Affymetrix Genechip 430 2.0 Array. Probesets were defined as regulated if $p<0.001$. The 254 gene-associated probesets changed by TVX/LPS-coexposure when compared to all other treatment groups were analyzed by Ingenuity Pathway Analysis. Of these 254 probesets, 142 were identified as functional genes. The pathways highly affected ($p<0.1$) are listed along with the ratio of the number of genes changed selectively by TVX/LPS to the total number of genes in the pathway as identified by the Ingenuity database. The specific genes selectively changed by TVX/LPS that are in each pathway are listed. Some of these genes are involved in more than one pathway and are, therefore, listed more than once.

Table 5.1

Pathway	p-value	Ratio	Genes
Glucocorticoid receptor signaling	9.12E-06	0.049	ADRB2, ICAM1, CEBPA, STAT3, UBE2I, CDKN1A, MAP2K7, NFKBIA, FOS, CXCL3, TAT, SELE, SERPINE1
Acute phase response signaling	9.55E-06	0.064	NFKBIA, MYD88, SOCS3, FOS, SOD2, SERPINA3, STAT3, MAP2K7, SOCS2, HMOX1, SERPINE1
Protein ubiquitination	7.24E-05	0.044	MED20, BIRC4, B2M, BIRC3, NEDD4L, PSMA5, HLA-A, MDM2, UBE2I
IL-10 signaling	9.33E-04	0.074	NFKBIA, SOCS3, FOS, STAT3, HMOX1
Hypoxia signaling in the cardiovascular system	1.48E-03	0.070	NFKBIA, VEGFA, MDM2, UBE2I, PTEN
EGF signaling	2.51E-03	0.085	FOS, STAT3, MAP2K7, EGFR
FXR/RXR activation	4.27E-03	0.052	MLXIPL, G6PC, CYP7A1, SLCO1B3, FASN
P53 signaling	6.03E-03	0.058	THBS1, MDM2, CDKN1A, PTEN, FASN
Death receptor signaling	6.46E-03	0.066	NFKBIA, BIRC4, BIRC3, MAP2K7
JAK/Stat signaling	**7.41E-03**	**0.068**	**SOCS3, STAT3, CDKN1A, SOCS2**
NRF2-mediated oxidative stress response	7.59E-03	0.039	JUNB, FOS, SOD2, GCLC, MAP2K7, HMOX1, DNAJB2
LXR/RXR activation	1.02E-02	0.049	APOA5, CYP7A1, LDLR, FASN
NF-κB signaling	1.02E-02	0.042	NFKBIA, MYD88, TNFAIP3, MAP2K7, EGFR, MAP3K8
Toll-like receptor signaling	2.19E-02	0.059	NFKBIA, MYD88, FOS
Hepatic fibrosis/hepatic stellate cell activation	2.19E-02	0.038	VEGFA, ICAM1, CXCL3, MMP13, EGFR
Neuregulin signaling	2.45E-02	0.025	CRK, EGFR, ERRFI1, PTEN
IL-6 signaling	2.69E-02	0.044	NFKBIA, FOS, STAT3, MAP2K7
Apoptosis signaling	2.88E-02	0.037	NFKBIA, BIRC4, BIRC3, MAP2K7, MAP3K8
Hepatic cholestasis	2.88E-02	0.031	NFKBIA, MYD88, CYP7A1, SLCO1B3, CGCR

Table 5.1 (cont'd).

Aryl hydrocarbon receptor signaling	3.39E-02	0.033	FOS, MDM2, TGM2, CDKN1A, FASN
Interferon signaling	**5.37E-02**	**0.069**	**IRF1, PTPN2**
Antigen presentation	5.62E-02	0.051	B2M, HLA-A
PI3K/Akt signaling	5.62E-02	0.028	NFKBIA, MDM2, CDKN1A, PTEN, MAP3K8
Glutamate metabolism	6.03E-02	0.026	GCLC, GNPNAT1
PDGF signaling	6.17E-02	0.041	CRK, FOS, STAT3
Leukocyte extravasation signaling	7.08E-02	0.027	CLDN14, CRK, ICAM1, RDX, MMP13
VDR/RXR activation	8.32E-02	0.038	IGFBP1, CEBPA, CDKN1A
TGF-β signaling	8.32E-02	0.036	FOS, SERPINE1, TGIF1
Cell cycle: G2/M DNA damage checkpoint regulation	9.55E-02	0.047	MDM2, CDKN1A
PTEN signaling	9.55E-02	0.033	CDKN1A, EGFR, PTEN
Amyotrophic lateral sclerosis signaling	9.77E-02	0.029	VEGFA, BIRC4, BIRC3
G-protein coupled receptor signaling	9.77E-02	0.025	ADRB2, NFKBIA, PDE4B, STAT3, MAP3K8

Table 5.2. Genes selectively altered in expression by TVX/LPS that are regulated by interferons Mice were treated as described in methods. Three hours after LPS administration, RNA was isolated from the liver, and gene expression was evaluated using Affymetrix Genechip 430 2.0 Array. Probesets were defined as regulated if $p<0.001$. Of the 142 genes changed selectively by TVX/LPS-coexposure, the 26 listed have been reported to be regulated by interferons as determined by Ingenuity Pathway analysis.

Table 5.2

Sequence code	Gene symbol	Gene name	Regulation	Fold change	Potential importance in model
1427511_at	B2M	β2-microglobulin	▲	10.6	pro-apoptotic
1418025_at	BHLHB2	Basic helix-loop-helix domain containing, class B, 2	▲	6.1	
1434529_x_at	CHFR	Checkpoint with forkhead and ring finger domains	▼	3.8	cell division, cell viability
1419209_at	CXCL2	Chemokine (C-X-C motif) ligand 2	▲	86.4	MIP-2α, neutrophil chemokine
1449984_at	CXCL3	Chemokine (C-X-C motif) ligand 3	▲	100	MIP-2β, neutrophil chemokine
1416268_at	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	▲	7.8	pro-apoptotic
1451931_x_at	H2-LD	Histocompatibility 2, D region	▲	1.4	MHC I antigen presentation, immune response
1426324_at	HLA-A	Major histocompatibility complex, class I, A	▲	12.4	MHC I antigen presentation, immune response
1424067_at	ICAM1	Intercellular adhesion molecule 1 (CD54)	▲	47.8	leukocyte recruitment and activation
1417141_at	IGTP	Interferon gamma induced GTPase	▲	6.5	
1420905_at	IL17RA	Interleukin 17 receptor A	▲	8.0	pro-inflammatory
1448436_a_at	IRF1	Interferon regulatory factor 1	▲	19.7	pro-apoptotic
1419569_a_at	ISG20	Interferon stimulated exonuclease gene	▲	11.7	
1419208_at	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	▲	58.4	activates MAPK & JNK pathways, pro-inflammatory
1419272_at	MYD88	Myeloid differentiation primary response gene	▲	5.6	TLR signaling, inflammatory response
1418835_at	PHLDA1	Pleckstrin homology-like domain	▲	3.9	involved in FasL synthesis

Table 5.2 (cont'd).

		A1			
1434356_a_at	PSMA5	Proteasome subunit, alpha type, 5	▲	1.2	processing of MHC I peptides
1425197_at	PTPN2	Protein tyrosine phosphatase, non-receptor type 2	▲	9.0	negative feedback response to JAK/STAT signaling
1419100_at	SERPINA3	Alpha-1 antiproteinase	▲	1.9	
1426441_at	SLC11A2	Proton-coupled divalent metal ion transporters	▲	1.7	heavy metal ion transport
1449109_at	SOCS2	Suppressor of cytokine signaling 2	▲	10.3	Negative feedback response to JAK/STAT signaling
1455899_x_at	SOCS3	Suppressor of cytokine signaling 3	▲	26.1	Negative feedback response to JAK/STAT signaling
1448610_a_at	SOD2	Superoxide dismutase 2, mitochondrial	▲	1.8	response to oxidative stress
1426587_a_at	STAT3	Signal transducer and activator of transcription 3	▲	4.7	pro-inflammatory cytokine & chemokine production
1415781_a_at	SUMO2	SMT3 suppressor of mif two 3 homolog 2	▼	1.7	
1420909_at	VEGFA	Vascular endothelial growth factor A	▼	3.5	pro-inflammatory, leukocyte migration

5.4.5 Timecourse of plasma concentrations of IL-18 and IFNγ

IL-18 induces the production of IFNγ in several cell types. Neither TVX nor LVX increased plasma concentrations of IL-18 or IFNγ when given alone. LPS treatment caused a significant increase in both IL-18 and IFNγ plasma concentrations at all times measured (Fig. 5.4A, B). TVX treatment prior to LPS caused a further increase in plasma concentrations of IL-18 and IFNγ at both 4.5 and 6 h (Fig. 5.4A, B). In contrast, LVX did not affect either plasma IL-18 or IFNγ induction by LPS (Fig. 5.4A, B).

5.4.6 TVX/LPS-induced hepatocellular injury in IL-18$^{-/-}$ mice

To explore the role of IL-18 in TVX/LPS-induced liver injury in mice, wild-type and IL-18$^{-/-}$ mice were treated with TVX/LPS. IL-18$^{-/-}$ mice had significantly reduced plasma ALT activity compared to the wild-type controls (Fig. 5.5A). In corroboration with the ALT values, TVX/LPS treatment resulted in midzonal hepatocellular necrosis in wild-type control mice (Fig. 5.5B), which was less extensive in IL-18$^{-/-}$ mice (Fig 5.5C).

5.4.7 TVX/LPS-induced liver injury in IFNγ $^{-/-}$ mice

To examine the role of IFNγ in TVX/LPS-induced liver injury, wild-type and IFNγ$^{-/-}$ female Balb/C mice were treated with TVX/LPS. TVX or LPS treatment alone did not cause hepatocellular injury in female Balb/C mice (data not shown). TVX/LPS-treatment resulted in significant liver injury in the female Balb/C mice, as seen in male C57/Bl6 mice. In IFNγ$^{-/-}$ mice, the TVX/LPS-induced increase in

Fig. 5.4. Timecourse of IL-18 and IFNγ plasma concentrations. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg) or Veh (saline) orally and then 3 h later with either LPS (2 x 10^6 EU/kg; i.p.) or Veh (saline). Mice were killed at various times, and plasma concentrations of IL-18 **(A)** or IFNγ **(B)** were measured. *n* = 4-6 animals/group. * significantly different from the respective treatment group at 0 h. # significantly different from Veh/LPS-treated mice at the same time.

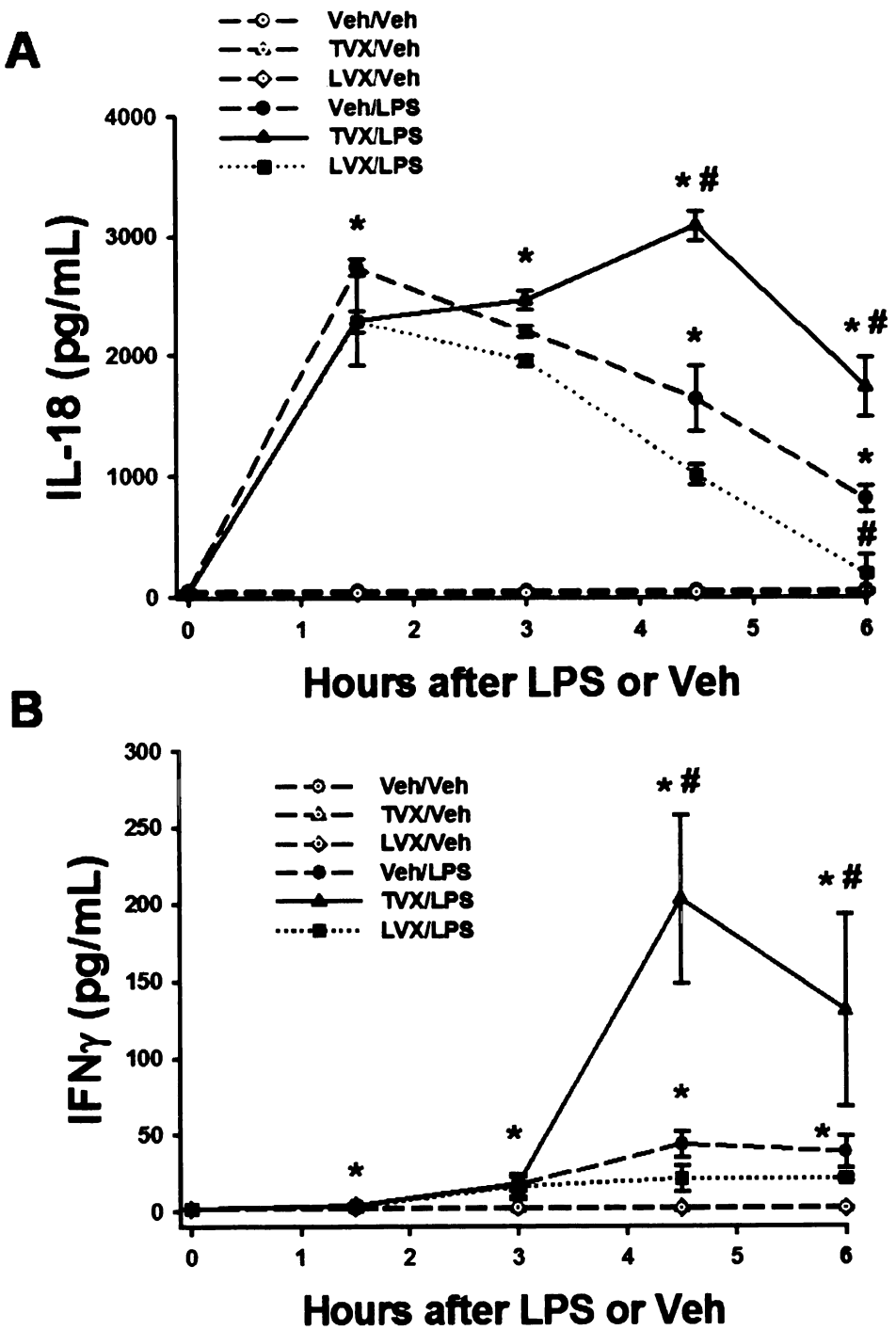
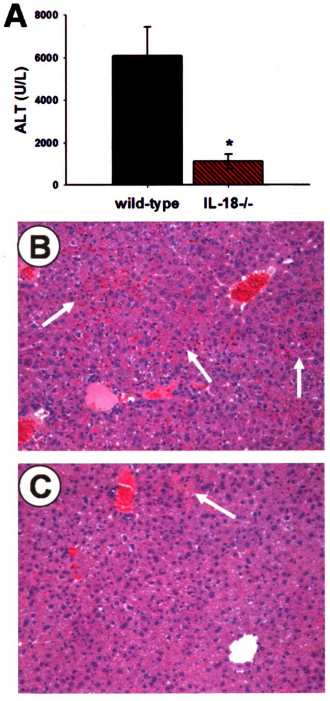

A
Veh/Veh
TVX/Veh
LVX/Veh
Veh/LPS
TVX/LPS
LVX/LPS
IL-18 (pg/mL)
4000
3000
2000
1000
0
0
1
2
3
4
5
6
Hours after LPS or Veh
B
IFNγ (pg/mL)
300
250
200
150
100
50
0
Hours after LPS or Veh

Fig. 5.5. TVX/LPS-induced liver injury is attenuated in IL-18$^{-/-}$ mice. Male, C57Bl/6J wild-type or IL-18 $^{-/-}$ mice were treated with TVX/LPS as described in Methods. **A:** Mice were sacrificed 15 h after LPS treatment, and plasma ALT activity was measured. Wild-type controls and IL-18$^{-/-}$ mice had equivalent baseline values of 55 $\pm$ 15 U/L. *n* = 6-9 animals/group. * significantly different from wild-type control. **B:** Representative photomicrograph of liver from a wild-type mouse sacrificed 15 h after TVX/LPS-treatment. Midzonal lesions of coagulative necrosis were observed. Arrows highlight necrotic lesions. **C:** Representative photomicrograph of liver from an IL-18$^{-/-}$ mouse sacrificed 15 h after TVX/LPS-treatment. Few lesions of necrosis were observed. Arrows highlight necrotic lesions.

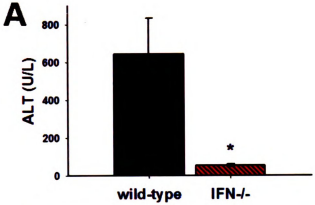

A
8000
6000
4000
2000
0
ALT (U/L)
*
wild-type
IL-18-/-
B
C

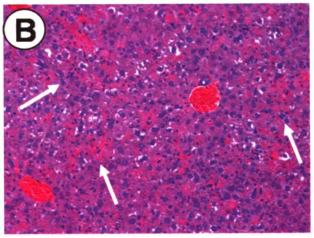
plasma ALT activity was markedly attenuated compared to wild-type controls (Fig. 5.6A). Midzonal coagulative necrotic lesions obvious in wild-type mice were not seen in IFN$\gamma^{-/-}$ mice (Fig. 5.6B and 5.6C).

5.4.8 TVX/LPS-induced hepatic neutrophil accumulation and pro-inflammatory cytokines in IFN$\gamma^{-/-}$ mice

Hepatic PMN accumulation and plasma concentrations of several cytokines and chemokines were measured at a time before the onset of liver injury in wild-type controls and in IFN$\gamma^{-/-}$ mice. Plasma ALT activity values were equivalent to baseline for both wild-type and IFN$\gamma^{-/-}$ mice (data not shown). Hepatic PMN accumulation was also similar in IFN$\gamma^{-/-}$ mice compared to wild-type mice 6 h after TVX/LPS treatment (Fig. 5.7). The plasma concentrations of IL-6, MCP-1, IL-10, KC, MIP-2, MIP-1α, and VEGF were similar in wild-type and IFN$\gamma^{-/-}$ mice given TVX/LPS (data not shown). In contrast, TVX/LPS-treated IFN$\gamma^{-/-}$ mice had significantly reduced plasma concentrations of TNFα, IL-18, and IL-1β compared to TVX/LPS-treated wild-type mice (Fig. 5.8A, B, C).

Fig. 5.6. IFNγ$^{-/-}$ mice are resistant to TVX/LPS-induced liver injury. Female, Balb/CJ wild-type or IFNγ$^{-/-}$ mice were treated as decribed in Methods. **A:** Mice were sacrificed 15 h after LPS treatment, and plasma ALT activity was measured. Wild-type controls and IFNγ$^{-/-}$ mice had equivalent baseline values of 55 ± 15 U/L. *n* = 6-10 animals/group. * significantly different from wild-type control. **B:** Representative photomicrograph of liver from a wild-type mouse sacrificed 15 h after TVX/LPS-treatment. Midzonal lesions of coagulative necrosis were observed. Arrows highlight necrotic lesions. **C:** Representative photomicrograph of liver from an IFNγ$^{-/-}$ mouse sacrificed 15 h after TVX/LPS-treatment. Few lesions of necrosis were observed.


A
800
600
400
200
0
ALT (U/L)
*
wild-type
IFN-/-


B

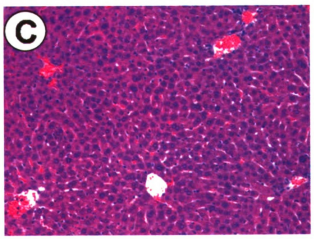

C

Fig. 5.7. TVX/LPS-induced hepatic PMN accumulation is unchanged in IFNγ$^{-/-}$ mice. Female Balb/C wild-type and IFNγ $^{-/-}$ mice were treated with TVX and LPS as described in Methods. Mice were sacrificed 6 h after LPS administration, and hepatic PMN accumulation was quantified as described in methods. Wild-type controls and IFNγ$^{-/-}$ mice had equivalent baseline values of 1 $\pm$ 0.5 PMNs per HPF. *n* = 5 animals/group.

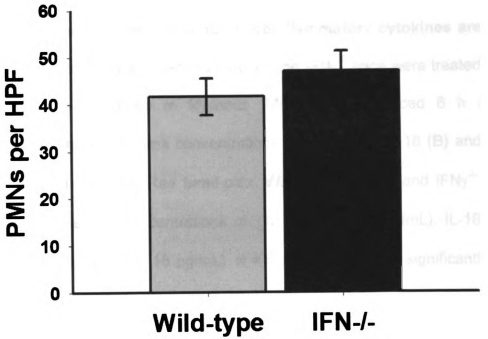
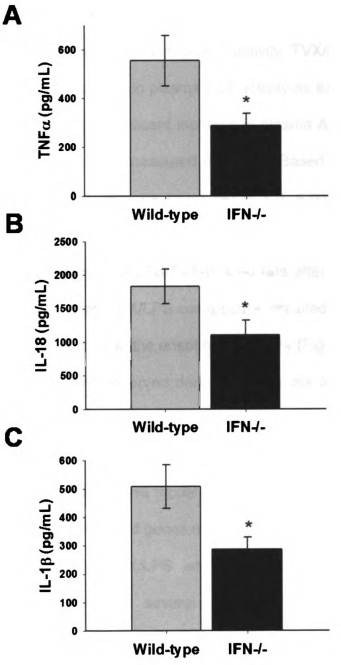

PMNs per HPF
60
50
40
30
20
10
0
Wild-type
IFN-/-

Fig. 5.8. Plasma concentrations of proinflammatory cytokines are reduced in IFN$\gamma^{-/-}$ mice. Female, Balb/C wild-type and IFN$\gamma^{-/-}$ mice were treated with TVX and LPS as described in Methods. Mice were sacrificed 6 h after LPS administration, and plasma concentrations of TNFα **(A)**, IL-18 **(B)** and IL-1β **(C)** were measured by Bio-Rad bead-plex. Wild-type controls and IFN$\gamma^{-/-}$ mice had equivalent baseline concentrations of TNFα (210 $\pm$ 41 pg/mL), IL-18 (25 $\pm$ 10 pg/mL) and IL-1β (75 $\pm$ 16 pg/mL). *n* = 5 animals/group. * significantly different from wild-type controls.


A
TNFα (pg/mL)
600
400
200
0
*
Wild-type
IFN-/-
B
IL-18 (pg/mL)
2500
2000
1500
1000
500
0
*
Wild-type
IFN-/-
C
IL-1β (pg/mL)
600
500
400
300
200
100
0
*
Wild-type
IFN-/-

5.5 Discussion

This study confirmed previous results that TVX-pretreatment, but not LVX-pretreatment, interacted with an inflammatory stress induced by LPS to cause liver injury in mice. We extended this to define the early timecourse of hepatocellular injury as measured by plasma ALT activity. TVX/LPS-treated mice had a small but significant increase in plasma ALT activity as early as 4.5 h after LPS, whereas there was not a significant increase in plasma ALT activity in any other treatment group at any time measured (Fig. 5.1). Based on these results, hepatic gene expression changes were examined at 3 h, a time just before the onset of liver injury.

Similar to results obtained in LPS/TVX-treated rats after the onset of liver injury (58), we observed that TVX/LPS-coexposure resulted in unique gene expression changes in mice prior to the onset of liver injury (Fig. 5.2). The distinct gene expression profiles were observed despite differences in species, timing, and routes of LPS and TVX administrations in the two studies (58). These results in mice suggest that global gene expression change is an earlier marker of liver toxicity than plasma ALT activity in this model.

Although, numerous groups of genes might be involved in injury, the set of genes selectively affected by TVX/LPS was chosen as a starting point for mechanistic analysis. There were several pathways affected by TVX/LPS-treatment that suggest that TVX magnifies the inflammatory response induced by LPS treatment (Table 5.1). This finding is consistent with the finding that TVX pretreatment enhances the plasma concentrations of several pro-inflammatory

cytokines induced by LPS (See Fig. 3.2). Of the pathways impacted by TVX/LPS-treatment, JAK/STAT and interferon signaling pathways had among the highest ratios and low p-values.

The actions of IFNγ are exerted through the activation of the JAK/STAT pathway (259). Of the 142 TVX/LPS-selectively expressed genes, 26, or 18%, can be regulated by interferons (Table 5.2). Furthermore, a significant number of these genes have roles in functions related to hepatotoxicity, such as apoptosis, leukocyte migration, pro-inflammatory cytokine production and immune response. Most (23/26) of these IFN-regulated genes were enhanced in expression in TVX/LPS-treated mice, suggesting an increase in interferon signaling. The majority of these are known to be regulated by IFNγ, rather than by IFNα or IFNβ. IRF-1, one of the genes selectively upregulated by TVX/LPS, encodes for a transcription factor activated as a result of JAK/STAT activation by IFNγ (259). This pathway is activated in several other models of liver injury, including concavalin A, acetaminophen, ischemia/reperfusion and LPS/galactosamine (115, 116, 131, 132). However, the role of IFNγ or IL-18 has not been examined in a model of liver injury from drug/inflammation interaction; therefore, the role of IL-18 and IFNγ was explored in TVX/LPS-induced injury.

The first step was to measure the plasma concentrations of IL-18 and IFNγ. IL-18 stimulates IFNγ production (260). The plasma concentrations of both IL-18 and IFNγ were selectively increased by TVX/LPS treatment (Fig. 5.4A, B). This enhancement and prolongation of the IFNγ induction by LPS has not been shown previously in any drug/LPS-coexposure model of liver injury. The mechanism by

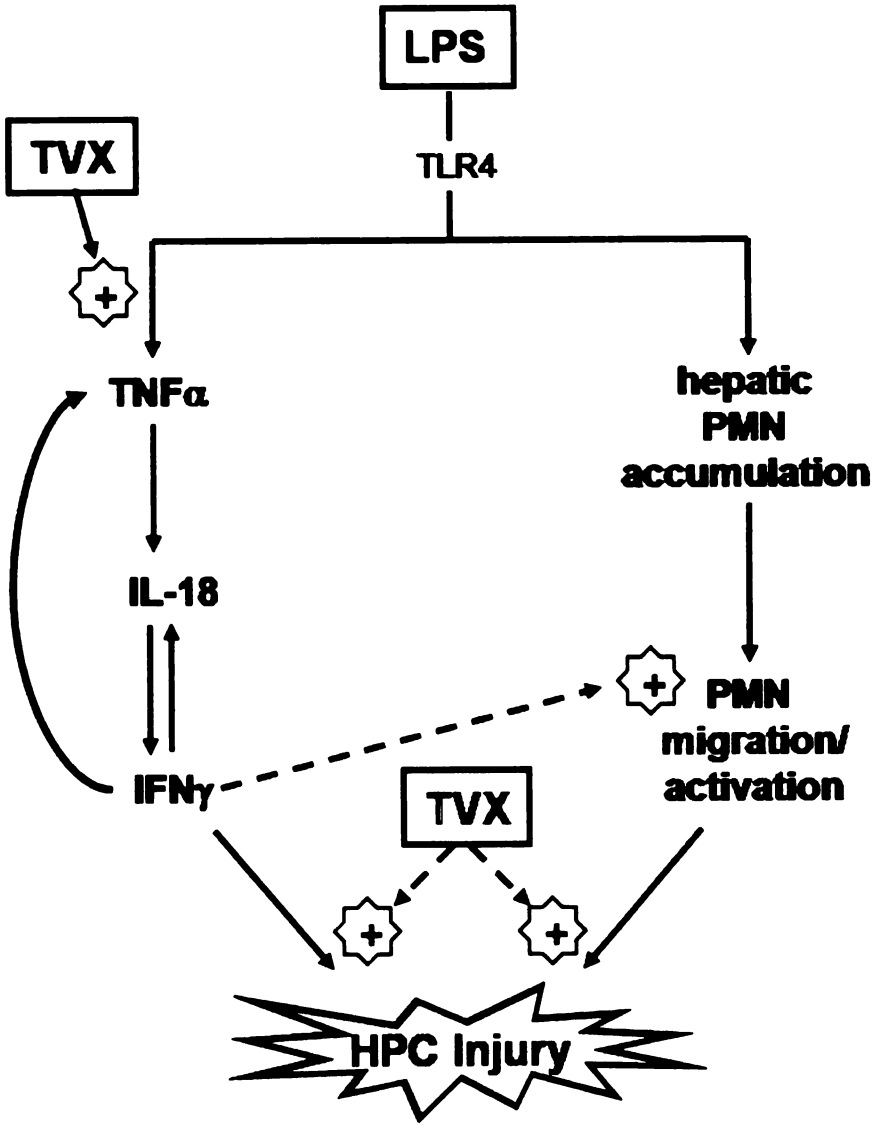
which this interaction occurs is unknown. However, we reported previously that TVX pretreatment prolongs the LPS-induced plasma TNFα peak (216). It is thus possible that this prolongation of the TNFα peak drives the prolongation of the plasma IFNγ peak.

Based on the selective increases in IL-18 and IFNγ in TVX/LPS-treated mice, we examined the role each cytokine plays in the development of TVX/LPS-induced hepatotoxicity using transgenic mice. IL-18 and IFNγ are components of the same signaling pathway. Both IL-18$^{-/-}$ mice and IFNγ$^{-/-}$ mice had significantly reduced hepatocellular injury following TVX/LPS-treatment compared to their respective wild-type controls (Fig. 5.5 and 5.6), implicating pathophysiological roles for IL-18 and IFNγ. Female Balb/C mice were used to generate the data for Fig. 5.6-8. The reason for the gender and strain change related to the availability of IFNγ$^{-/-}$ mice of this background. Despite the wild-type controls having lower plasma ALT activity in response to TVX/LPS-treatment, the extent of histopathologically evident hepatocellular injury was similar to that seen in male C57Bl/6 mice. It is unknown whether the difference seen in the magnitude of ALT activity increase in response to TVX/LPS is due to a strain or gender difference. Regardless, the pathway by which IL-18 increases IFNγ is common to both male C57Bl/6 and female Balb/C mice. Thus, the observation that both male C57Bl/6 IL-18$^{-/-}$ mice and female Balb/C IFNγ$^{-/-}$ mice were protected from TVX/LPS-induced liver injury suggests that the importance of this pathway in TVX/LPS-induced liver injury is not gender- or strain-specific.

Several inflammatory cytokines and chemokines were measured in wild-type and IFNγ$^{-/-}$ mice treated with TVX/LPS at a time before the onset of liver injury. This time was selected to exclude the possibility that a difference seen was a result of injury. The plasma concentrations of several cytokines were unchanged, but TNFα, IL-1β, and IL-18 were significantly reduced in IFNγ$^{-/-}$ mice. Previously, TNFα inhibition attenuated TVX/LPS-induced liver injury (216). IFNγ enhances the production and release of TNFα, as shown *in vitro* (121). In vivo, IFNγ induction of TNFα production is mediated by IRF-1 (261), a transcription factor selectively upregulated in TVX/LPS-treated mice (Table 5.2). Interestingly, TNFα inhibition reduced the TVX/LPS-induced increase in IFNγ (see Fig. 4.2), suggesting that each of these cytokines regulates the expression of the other. Additionally, IFNγ$^{-/-}$ mice had reduced levels of IL-1β and IL-18. These cytokines exist as pro-forms of the proteins in cells and are cleaved by caspase 1 to their active forms. The mRNA levels of IL-1β and IL-18 were unchanged in gene expression analysis. Therefore, we hypothesize that IFNγ positively feeds back to increase caspase 1 activity either directly or indirectly. The consequent increase in IL-18 could then cause more production of IFNγ, potentially resulting in a proinflammatory vicious cycle.

IFNγ causes apoptosis in primary hepatocytes (262), and it is possible that TVX directly sensitizes hepatocytes to IFNγ-induced cell death. Additionally, TVX enhanced the LPS-induced increases in both TNFα (216) and IFNγ, which synergize to cause primary hepatotocyte cell death (263). It is also possible that

Fig. 5.9. Hypothesized role of IFNγ in TVX/LPS-induced liver injury. Dashed arrows represent untested hypotheses. LPS causes hepatic neutrophil accumulation and TNFα release. TVX enhances the LPS-induced increase in TNFα (216). TNFα can increase IL-18, which in turn induces IFNγ production. In addition, IFNγ can feedback to increase both IL-18 and TNFα (Fig. 8). This has the potential to create a vicious, proinflammatory cycle of IFNγ and TNFα production. In addition, PMNs migrate into tissues and become activated, causing death of hepatic parenchymal cells. It is possible that IFNγ enhances PMN activation. In addition, TVX might act directly on hepatocytes to sensitize them to cell death induced directly by IFNγ, IFNγ/TNFα coexposure or toxic factors released from PMNs.


LPS
TVX
TLR4
TNFα
hepatic
PMN
accumulation
IL-18
PMN
migration/
activation
IFNγ
TVX
HPC Injury

TVX acts directly on hepatocytes to enhance TNFα/IFNγ-induced cell death. Fig. 9 illustrates the proposed pathway to TVX/LPS-induced liver injury and the interaction between TNFα and IFNγ.

In addition to effects on other proinflammatory cytokines, IFNγ can play a direct role in the regulation and activation of many cell types that might be involved in liver injury. For example, PMN activation is critical for TVX/LPS-dependent liver injury (see Fig. 3.6) and IFNγ can increase PMN activation *in vitro* as measured by oxidative burst and differential gene expression (118, 264, 265). Although PMN accumulation after TVX/LPS-cotreatment was unchanged in IFNγ$^{-/-}$ mice, it is possible that activation of accumulated PMNs was decreased in these mice, and this could be a reason for the reduced hepatotoxicity in response to TVX/LPS (as depicted in Fig. 5.9). In addition, IFNγ plays a role in the activation of CD8+ T cells and macrophages (104, 266). Accordingly, it is possible that IFNγ is involved in hepatotoxicity by activating one or more of these cell types.

In summary, TVX interacts with LPS, but not LVX, to cause liver injury in mice. That hepatotoxicity only occurred for the drug with IADR-potential in humans raises the possibility that inflammatory stress might play a role in the pathogenesis of idiosyncratic liver injury caused by TVX in people. Gene expression analysis revealed distinct clustering by treatment at a time before the onset of liver injury. A small group of genes was identified which changed in expression only after TVX/LPS-treatment compared to all other treatment groups. Of these genes, a large number were related to interferon signaling. This

observation led to experiments that demonstrated the involvement of IL-18 and IFNγ in the pathogenesis of TVX/LPS-induced liver injury in mice.

Supplemental Table 5.3. Functional genes selectively changed by TVX/LPS-treatment compared to all other treatment groups. Mice were treated as described in methods. Three hours after LPS administration, RNA was isolated from the liver, and gene expression was evaluated using Affymetrix Genechip 430 2.0 Array. Probesets were defined as regulated if $p<0.001$. The 254 gene-associated probesets changed by TVX/LPS-coexposure when compared to all other treatment groups were analyzed by Ingenuity Pathway Analysis. Of these 254 probesets, 142 were identified as functional genes. The specific genes selectively changed by TVX/LPS are listed.

Supplemental Table 5.3

Sequence code	Gene symbol	Gene name	Regulation	Fold change	Potential importance in model
143702_at	ADRB2	β2 adrenergic receptor	▲	28.8	activates MAPK cascade
1417130_s_at	ANGPTL4	Angiopoietin-like 4	▲	3.8	response to hypoxia
1416135_at	APEX1	APEX nuclease	▲	2.7	
1417610_at	APOA5	Apolipoprotein A-V	▼	2.8	
1420973_at	ARID5B	AT rich interactive domain 5B	▲	9.2	
1428112_at	ARMET	Arginine-rich, mutated in early stage tumors	▼	2.5	MHC I antigen presentation, immune response
1427511_at	B2M	β2-microglobulin	▲	10.6	pro-apoptotic
1418025_at	BHLHB2	Basic helix-loop-helix domain containing, class B, 2	▲	6.1	
1425223_at	BIRC3	Baculoviral IAP repeat-containing 3	▲	5.7	
1437533_at	BIRC4	Baculoviral IAP repeat-containing 4	▼	1.7	
1448347_at	CAPRIN1	Cell cycle associated protein 1	▼	2.5	
1451980_at	CASD1	CAS1 domain containing 1	▼	1.8	
1423811_at	CCDC6	Coiled-coil domain containing 6	▲	1.8	
1425837_a_at	CCRN4L	CCR4 carbon catabolite repression 4-like	▲	7.0	
1448569_at	CD8B	CD8b molecule	▼	4.8	immune response, cytotoxic T cell activation
1424638_at	CDKN1A	Cyclin-dependent kinase inhibitor 1A	▲	11.8	pro-apoptotic, response to DNA damage
1418982_at	CEBPA	C/EBP alpha	▼	2.9	
1434529_x_at	CHFR	Checkpoint with forkhead and ring finger domains	▼	3.8	cell division, cell viability

Supplemental Table 5.3 (cont'd).

1450264_a_at	CHKA	Choline kinase alpha	▲	2.6	
1420345_at	CLDN14	Claudin 14	▲	4.6	
1452152_at	CLINT1	Clathrin interactor 1	▼	2.0	
1418701_at	COMT	Catechol-O-methyltransferase	▼	5.1	catecholamine metabolism
1448248_at	CRK	v-crk sarcoma virus CT10 oncogene homolog	▼	2.0	
1419209_at	CXCL2	Chemokine (C-X-C motif) ligand 2	▲	86.4	MIP-2α, neutrophil chemokine
1449984_at	CXCL3	Chemokine (C-X-C motif) ligand 3	▲	100	MIP-2β, neutrophil chemokine
1434681_at	CXORF15	Chromosome X open reading frame 15	▼	3.3	
1422100_at	CYP7A1	Cytochrome P450 7A1	▼	2.2	
1452155_a_at	DDX17	DEAD box polypeptide 17	▼	3.2	
1435101_at	DERL2	Der1-like domain family member 2	▼	35.4	positive regulation of cell growth
1448657_a_at	DNAJB2	DnaJ (HSP40) homolog B2	▼	7.5	
1418401_a_at	DUSP16	Dual specificity phosphatase 16	▲	4.6	negative feedback of MAPK cascade
1418714_at	DUSP8	Dual specificity phosphatase 8	▲	12.9	negative feedback of MAPK cascade
1451530_at	EGFR	Epidermal growth factor receptor	▲	12.3	MAPK/ERK activation, activates STAT3
1441023_at	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2 beta	▲	2.9	
1416659_at	EIF3A	Eukaryotic translation initiation factor 3, subunit A	▼	3.0	
1427578_a_at	EIF6	Eukaryotic translation factor 6	▲	1.8	

Supplemental Table 5.3 (cont'd).

1416129_at	ERRFI1	ERBB receptor feedback inhibitor 1	▲	2.9	
1416268_at	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	▲	7.8	pro-apoptotic
1423828_at	FASN	Fatty acid synthase	▼	9.1	
1417480_at	FBXO9	F-box protein 9	▼	2.3	
1423100_at	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	▲	11.34	pro-apoptotic, pro-inflammatory
1450044_at	FZD7	Frizzled homolog 7	▼	6.9	
1417880_at	G6PC	Glucose-6-phosphatase, catalytic subunit	▼	4.4	
1450127_a_at	GCGR	Glucagon receptor	▼	2.1	
1455959_s_at	GCLC	Glutamate-cysteine ligase, catalytic subunit	▼	4.7	
1422489_at	GCS1	Glucosidase 1	▼	26.0	
1437992_x_at	GJA1	Gap junction protein, alpha 1	▲	12.6	positive regulation of NFκB pathway
1423156_at	GNPNAT1	Glucosamine-phosphate N-acetyltransferase 1	▼	1.9	
1448625_at	GOLGA2	Golgi autoantigen, golgin subfamily a, 2	▼	4.2	
1417674_s_at	GOLGA4	Golgi autoantigen, golgin subfamily a, 4	▼	1.7	
1451931_x_at	H2-LD	Histocompatibility 2, D region	▲	1.4	MHC I antigen presentation, immune response
1426324_at	HLA-A	Major histocompatibility complex, class I, A	▲	12.4	MHC I antigen presentation, immune response
1448239_at	HMOX1	Heme oxygenase 1	▲	24.4	LPS-inducible, oxidative stress

Supplemental Table 5.3 (cont'd).

1456664_x_at	HNRP4	Heterogeneous nuclear ribonucleoprotein F	▼	1.3	
1424067_at	ICAM1	Intercellular adhesion molecule 1 (CD54)	▲	47.8	leukocyte recruitment and activation
1422537_a_at	ID2	Inhibitor of DNA binding 2	▼	4.6	
1416067_at	IFRD1	Interferon-related developmental regulator 1	▲	2.6	
1418918_at	IGFBP1	Insulin-like growth factor binding protein 1	▲	24.5	
1417141_at	IGTP	Interferon gamma induced GTPase	▲	6.5	
1420905_at	IL17RA	Interleukin 17 receptor A	▲	8.0	pro-inflammatory
1448436_a_at	IRF1	Interferon regulatory factor 1	▲	19.7	pro-apoptotic
1419569_a_at	ISG20	Interferon stimulated exonuclease gene	▲	11.7	
1420961_a_at	IVNS1ABP	Influenza virus NS1A binding protein	▲	11.5	
1415899_at	JUNB	Jun B proto-oncogene	▲	27.6	
1439185_x_at	KIAA1271	Virus-induced signaling adapter	▼	3.0	regulates IFNα and IFNβ
1421821_at	LDLR	Low density lipoprotein receptor	▼	1.7	
1416303_at	LITAF	LPS-induced TNF factor	▲	2.9	positive regulation of NFκB pathway
1451736_a_at	MAP2K7	Mitogen-activated protein kinase kinase 7	▼	3.0	
1419208_at	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	▲	58.4	activates MAPK & JNK pathways, pro-inflammatory
1423605_a_at	MDM2	Mdm2, p53 binding protein	▲	3.1	
1416826_a_at	MED20	Mediator complex subunit 20	▼	5.6	

Supplemental Table 5.3 (cont'd).

1419185_a_at	MLXIPL	MLX interacting protein-like	▼	5.9	
1417256_at	MMP13	Matrix metallopeptidase 13	▲	12.4	extracellular matrix degradation, leukocyte migration
1451612_at	MT1E	Metallothionein 1E	▲	40.7	oxidative stress response
1419272_at	MYD88	Myeloid differentiation primary response gene	▲	5.6	TLR signaling, inflammatory response
1423269_a_at	NEDD4L	Neural precursor cell expressed	▲	4.0	
1450767_at	NEDD9	Neural precursor cell expressed	▲	3.4	
1448306_at	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	▲	17.8	negative regulation of NFκB pathway
1448728_a_at	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	▲	38.6	
1453299_a_at	NP	Nucleoside phosphorylase	▲	3.4	
1460648_at	NR2F6	Nuclear receptor 2F6	▼	78.0	
1431302_a_at	NUDT7	Nudix-type motif 7	▼	4.8	
1438625_s_at	PCTK1	PCTAIRE protein kinase 1	▼	10.5	
1422473_at	PDE4B	Phosphodiesterase 4B, cAMP-specific	▲	42.1	cAMP degradation, increases NFκB activation
1418835_at	PHLDA1	Pleckstrin homology-like domain A1	▲	3.9	involved in FasL synthesis
1418144_a_at	PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase I α	▲	3.1	
1426387_x_at	PISD	Phosphatidylserine decarboxylase	▼	7.4	
1434496_at	PLK3	Polo-like kinase 3	▲	22.7	
1437377_a_at	POLRMT	Mitochondrial polymerase	▼	5.2	responsible for mitochondrial gene

Supplemental Table 5.3 (cont'd).

					expression
1448325_at	PPP1R15A	Protein phosphatase 1, regulatory subunit 15A	▲	4.8	pro-apoptotic, response to DNA damage
1456037_x_at	PREB	Prolactin regulatory element binding	▼	2.8	
1434356_a_at	PSMA5	Proteasome subunit, alpha type, 5	▲	1.2	processing of MHC I peptides
1454722_at	PTEN	Phosphatase and tensin homolog	▼	1.7	pro-apoptotic
1455002_at	PTP4A1	Protein tyrosine phosphatase type IVA, 1	▲	3.2	
1425197_at	PTPN2	Protein tyrosine phosphatase, non-receptor type 2	▲	9.0	negative feedback response to JAK/STAT signaling
1420711_a_at	PXMP3	Peroxisomal membrane protein 3	▼	3.4	
1416601_a_at	RCAN1	Regulator of calcineurin 1	▲	11.3	
1416179_a_at	RDX	radixin	▼	1.5	
1420710_at	REL	v-rel reticuloendotheliosis viral oncogene homolog	▲	9.3	positive regulation of NFκB pathway
1416701_at	RND3	Rho family GTPase 3	▲	15.3	
1427898_at	RNF6	Ring finger protein 6	▼	2.9	
1460351_at	S100A11	S100 calcium binding protein A11	▲	1.9	
1421712_at	SELE	E-selectin	▲	18.8	leukocyte adhesion and extravasation
1425466_at	SENP2	SMT3 specific peptidase 2	▼	2.4	
1419100_at	SERPINA3	Alpha-1 antiproteinase	▲	1.9	

Supplemental Table 5.3 (cont'd).

1419149_at	SERPINE1	Plasminogen activator inhibitor 1	▲	98.7	PAI-1, inhibition of fibrinolysis
1434972_x_at	SFRS1	Splicing factor, arginine/serine-rich 1	▼	1.8	
1419543_a_at	SFRS10	Splicing factor, arginine/serine-rich 10	▲	2.8	
1448778_at	SFRS4	Splicing factor, arginine/serine-rich 4	▲	2.6	
1423130_a_at	SFRS5	Splicing factor, arginine/serine-rich 5	▼	2.8	
1452214_at	SKIL	SKI-like oncogene	▲	4.9	
1426441_at	SLC11A2	Proton-coupled divalent metal ion transporters	▲	1.7	heavy metal ion transport
1418923_at	SLC17A3	Sodium phosphate transporter	▼	2.2	
1425649_at	SLC17A14	Zinc transporter	▲	8.3	
1426008_a_at	SLC7A2	Cationic amino acid transporter	▲	3.5	
1450982_at	SLC9A3R	Sodium/hydrogen exchanger	▲	2.7	
1449394_at	SLCO1B3	Organic anion transporter	▼	1.5	
1448400_a_at	SMARCD2	Actin dependent regulator of chromatin d2	▼	8.1	
1429043_at	SMNDC1	Survival motor neuron domain containing 1	▲	2.7	
1449109_at	SOCS2	Suppressor of cytokine signaling 2	▲	10.3	Negative feedback response to JAK/STAT signaling
1455899_x_at	SOCS3	Suppressor of cytokine signaling 3	▲	26.1	Negative feedback response to JAK/STAT signaling
1448610_a_at	SOD2	Superoxide dismutase 2, mitochondrial	▲	1.8	response to oxidative stress

Supplemental Table 5.3 (cont'd).

1434403_at	SPRED2	ECH1 domain containing 2	▲	3.4	activates MAPK cascade
1421849_at	STAG2	Stromal antigen 2	▼	3.1	
1450769_s_at	STARD5	StAR-related lipid transfer domain containing 5	▼	4.5	
1426587_a_at	STAT3	Signal transducer and activator of transcription 3	▲	4.7	pro-inflammatory cytokine & chemokine production
1434442_at	STBD1	Starch binding domain 1	▼	3.3	
1415781_a_at	SUMO2	SMT3 suppressor of mif two 3 homolog 2	▼	1.7	
1422768_at	SYNCRIP	Synaptotagmin binding	▲	2.6	
1451557_at	TAT	Tyrosine aminotransferase	▲	1.7	
1415887_at	TFG	TRK-fused gene	▼	1.7	
1422286_a_at	TGIF-1	TGFβ-induced factor homeobox 1	▲	3.4	
1417500_a_at	TGM2	Transglutaminase 2	▲	4.4	leukocyte adhesion
1460302_at	THBS1	Thrombospondin 1	▲	27.3	pro-inflammatory, platelet aggregation
1452125_at	THRAP3	Thyroid hormone receptor associated protein 3	▲	2.6	
1450829_at	TNFAIP3	TNFα-induced protein 3	▲	50.0	
1423641_at	TUBB2C	Tubulin, beta 2C	▼	4.1	
1422714_at	UBE2I	Ubiquitin-conjugating enzyme E2I	▼	4.2	
1448562_at	UPP1	Uridine phosphorylase 1	▲	7.1	
1420909_at	VEGFA	Vascular endothelial growth factor A	▼	3.5	pro-inflammatory, leukocyte migration
1451006_at	XDH	Xanthine dehydrogenase	▲	2.8	
1416085_s_at	ZFAND5	Zinc finger, AN1-type domain 5	▲	2.1	

CHAPTER 6

Shaw, P.J., Fullerton, A.F., Ganey, P.E. and Roth, R.A. (2008). The role of the hemostatic system in a murine model of idiosyncratic liver injury induced by trovafloxacin and lipopolysaccharide coexposure.

6.1 Abstract

The use of the fluoroquinolone antibiotic TVX was severely restricted in 1999 due to its association with idiosyncratic hepatotoxicity. Previously, we reported that a nontoxic dose of TVX interacts with a nontoxic dose of LPS to cause robust hepatocellular injury in mice. This interaction with LPS was not seen in mice treated with LVX, a fluoroquinolone not associated with hepatotoxicity in people. TVX/LPS-coexposure caused an increase in plasma ALT activity as early as 4.5 h after LPS administration and which progressed through 15 h. We examined the role of the hemostatic system in TVX/LPS-induced liver injury. At the onset of liver injury, coexposure to TVX/LPS, but not exposure to TVX, LVX, LPS or LVX/LPS, caused increased plasma concentration of thrombin-antithrombin dimers and decreased plasma circulating fibrinogen. LPS treatment induced a small increase in plasma plasminogen activator inhibitor-1 (PAI-1) concentration, and TVX pretreatment enhanced this effect. TVX/LPS coexposure also resulted in hepatic fibrin deposition. Anticoagulant heparin administration reduced TVX/LPS-induced hepatic fibrin deposition and liver injury. PAI-$1^{-/-}$ mice treated with TVX/LPS exhibited similar fibrin deposition to wild-type mice but had significantly reduced hepatocellular injury. PAI-$1^{-/-}$ mice, but not heparin-treated mice, had reduced plasma concentrations of several cytokines compared to TVX/LPS-treated controls. In summary, TVX/LPS-coexposure caused an imbalance in the hemostatic system, resulting in increased thrombin activation, plasma concentrations of PAI-1 and hepatic fibrin deposition. Both thrombin activation and PAI-1 play a critical role in

the progression of TVX/LPS-induced liver injury, but through different modes of action.

6.2 Introduction

The hemostatic system encompasses a number of factors involved in the complex interactions of platelets, von Willebrand factor, the coagulation system, anticoagulants and the fibrinolytic system. The activation of blood coagulation occurs predominantly through the tissue factor pathway, which leads to the formation of thrombin from prothrombin. Thrombin is a protease with a number of biological activities including the cleavage of circulating fibrinogen to fibrin, which polymerizes to form insoluble fibrin. Dissolution of fibrin is mediated by plasmin. The fibrinolytic system is controlled primarily by plasminogen activator inhibitor-1 (PAI-1), which inhibits the production of active plasmin. In addition to its role in fibrinolysis, PAI-1 has several other proinflammatory properties (146, 147).

The coagulation and fibrinolytic systems exist in a delicate balance to prevent widespread blood loss while controlling fibrin deposition. If the balance of these two components is altered, a possible outcome is unregulated activation of the hemostatic system, which could lead to fibrin deposition and production of occlusive thrombi. These in turn have the potential to alter blood flow and result in local tissue hypoxia, thereby contributing to tissue injury (103, 152, 154).

Whether an alteration in the hemostatic system occurs in the TVX/LPS-coexposure model of liver injury in mice has not been determined. The studies presented here were designed to test the hypothesis that TVX/LPS-coexposure in mice results in hemostatic system dysregulation and that this plays a role in the development of TVX/LPS-induced liver injury. To examine this hypothesis, biomarkers of thrombin activation, active PAI-1 concentrations and hepatic fibrin

deposition were evaluated at the time of onset of liver injury. To determine the importance of the hemostatic system in the development of TVX/LPS-induced liver injury, mice were treated with anticoagulant heparin. The importance of PAI-1 in TVX/LPS-induced hepatotoxicity was determined using PAI-$1^{-/-}$ mice. Heparin and PAI-1 can have profound effects on inflammation. Heparin attenuates ischemia/reperfusion-induced inflammatory responses (153), whereas PAI-1 induces proinflammatory cytokine expression (147). Therefore, we looked to determine what role thrombin activation and PAI-1 play in the TVX/LPS-induction of proinflammatory cytokines, some of which contribute to the pathogenesis in this model (216).

6.3 Materials and Methods

6.3.1 Materials

Please refer for Section 2.3.1 for information on this topic.

6.3.2 Animals

Please refer to Section 2.3.2 for information on this topic. In addition, PAI-$1^{-/-}$ and C57Bl/6J mice were purchased from Jackson Laboratory (Bay Harbor, ME).

6.3.3 Experimental protocols

Mice fasted for 12 h were given various doses of TVX or Veh (saline) by oral gavage. They were then given LPS (2.0×10^6 EU/kg) or Veh (saline) by intraperitoneal injection 3 h later. Food was returned immediately after this dosing. Mice were anesthetized with sodium pentobarbital (50 mg/kg; i.p.) and killed at designated times after the dose of LPS or Veh for various measurements. Blood was drawn from the vena cava into a syringe containing sodium citrate resulting in a final concentration of 0.76%. The left lateral lobe was fixed in 10% neutral buffered formalin and paraffin blocked.

6.3.4 Heparin treatment

Heparin (3000 U/kg, s.c.) was administered at 2, 6 and 10 h after LPS administration. Mice were killed at 15 h for experiments used in the generation of

Figs. 6.5A, 6.7A, and 6.7C. Mice sacrificed at 4.5 h (Figs. 6.5B, 6.5C, 6.8) were treated with heparin only at 2 h after LPS dosing.

6.3.5 Histopathology

Please refer to section 2.3.4 for information on this topic.

6.3.6 Hemostatic system measurements

Please refer to Section 4.3.7 for information on this topic.

6.3.7 Cytokine measurements

Please refer to Section 3.3.5 for information on this topic.

6.3.8 Statistical analyses

All bar graph results are presented as mean $\pm$ S.E.M. A 1- or 2-way analysis of variance (ANOVA) was used as appropriate after data normalization. All multiple pairwise comparisons were done using Tukey's Test. The criterion for significance was $p < 0.05$.

6.4 Results

6.4.1 Changes in the hemostatic system in TVX/LPS-induced liver injury

TVX/LPS-coexposure causes hepatotoxicity in mice, whereas treatment with TVX, LVX, LPS, or LVX/LPS is nontoxic (216). In a preliminary study in TVX/LPS-treated mice, the plasma concentration of TAT dimers, a biomarker of coagulation system activation, peaked at 4.5 h after LPS (data not shown). This corresponds to the onset of liver injury (see Fig. 3.1). In further studies at 4.5 h, treatment with TVX, LVX, LPS or LVX/LPS did not significantly affect the plasma concentration of TAT dimers, whereas TVX/LPS-treated mice had a significant elevation (Fig. 6.1A).

Circulating fibrinogen is consumed during coagulation system activation. Treatment with either TVX or LVX alone did not affect the plasma fibrinogen concentration (Fig. 6.1B). Administration of LPS alone caused a small increase in plasma fibrinogen concentration (acute phase response) which was unaffected by pretreatment with LVX. In contrast, TVX/LPS-coexposure caused a marked decrease in plasma fibrinogen concentration (Fig. 6.1B), suggesting coagulation system activation.

The plasma concentration of active PAI-1 was measured as a marker of fibrinolytic system downregulation. No effect on active PAI-1 concentration was seen when mice were treated with TVX or LVX alone (Fig. 6.2). LPS treatment caused a small increase in plasma active PAI-1 concentration, which was

Fig. 6.1. Effect of fluoroquinolones and LPS on coagulation system activation. Mice were treated as described in Materials and Methods with TVX, LVX or Veh and then 3 h later with LPS or Veh. They were sacrificed 4.5 h after LPS administration, and plasma concentrations of TAT (A) and fibrinogen (B) were measured. *n* = 4-6 animals/group.*significantly different from respective control group without LPS treatment. # significantly different from Veh/LPS group.

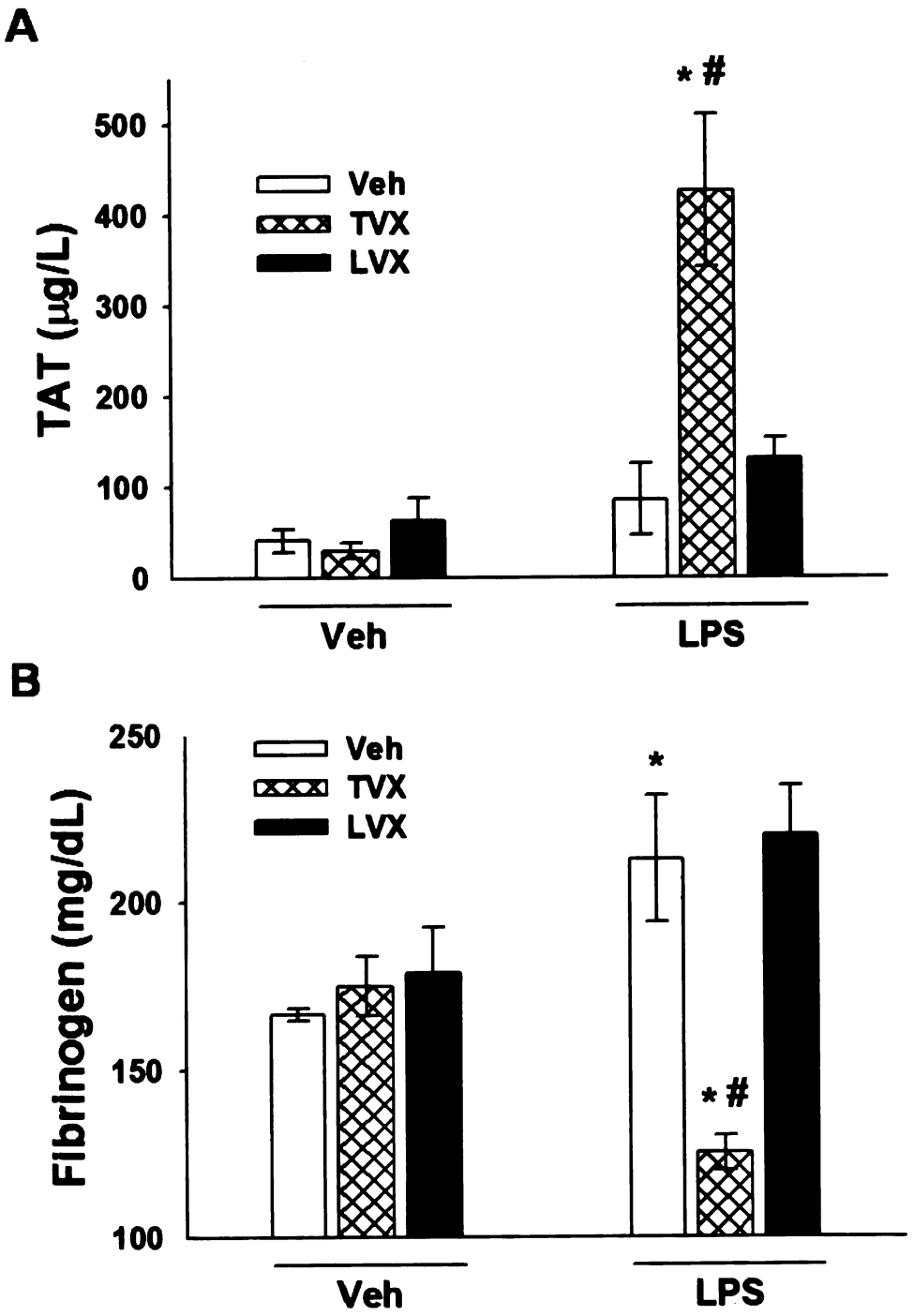
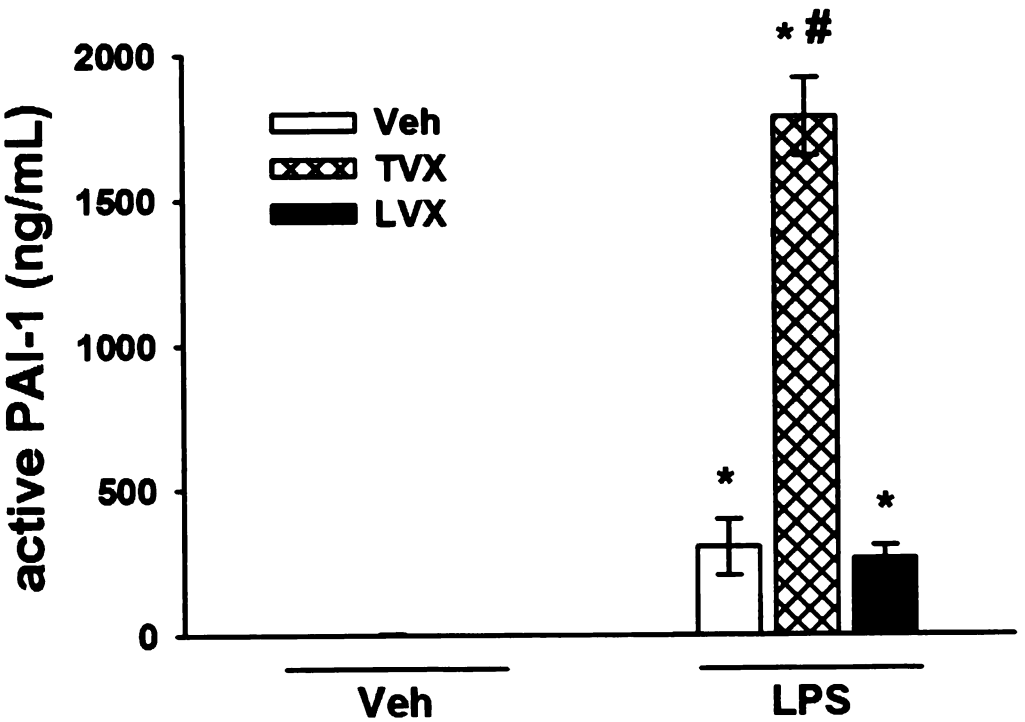

A
TAT (µg/L)
500
400
300
200
100
0
Veh
TVX
LVX
* #
Veh
LPS
B
Fibrinogen (mg/dL)
250
200
150
100
Veh
TVX
LVX
*
* #
Veh
LPS

Fig. 6.2. Effect of fluoroquinolones and LPS on plasma concentration of active PAI-1. Mice were treated as described in Materials and Methods with TVX, LVX or Veh and then 3 h later with LPS or Veh. They were sacrificed 4.5 h after LPS administration, and plasma concentration of PAI-1 was measured. *n* = 4-6 animals/group.*significantly different from respective control group without LPS treatment. # significantly different from Veh/LPS group.


active PAI-1 (ng/mL)
2000
1500
1000
500
0
Veh
TVX
LVX
* #
*
*
Veh
LPS

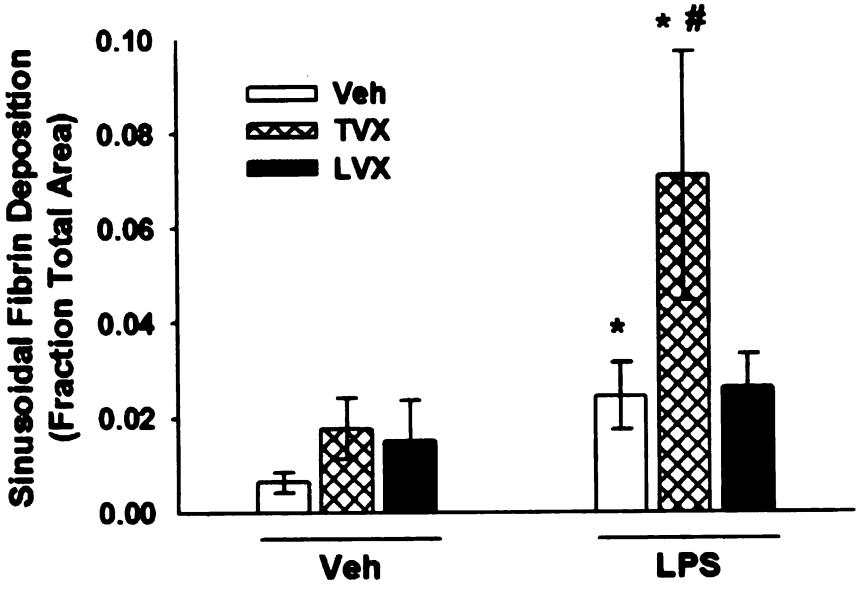
markedly enhanced by TVX pretreatment (Fig. 6.2). In contrast, LVX pretreatment did not affect the LPS-induced increase in active PAI-1 (Fig. 6.2).

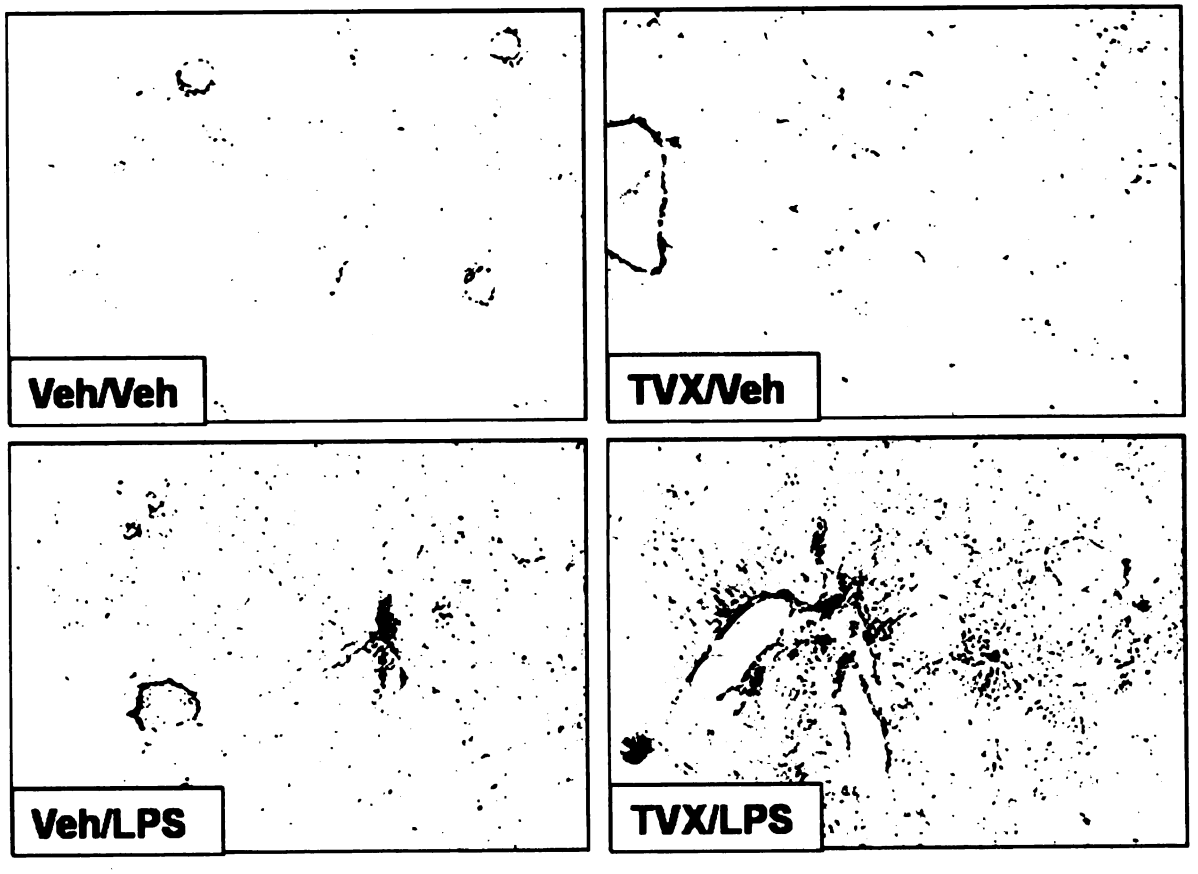
When the rate of fibrin polymerization exceeds the rate of fibrinolysis, fibrin deposition occurs. Administration of TVX or LVX alone did not result in hepatic fibrin deposition (Fig. 6.3). Treatment with LPS alone caused a slight, but significant increase in sinusoidal fibrin when compared to Veh/Veh-treated mice (Fig. 6.3). LVX pretreatment did not affect the LPS-induced fibrin deposition, but TVX/LPS-cotreated mice had significantly increased hepatic sinusoidal fibrin compared to both Veh/Veh- and LPS/Veh-treated mice (Fig. 6.3).

6.4.2 Anticoagulant heparin protects from TVX/LPS-induced liver injury

To determine if the activation of the coagulation system seen in TVX/LPS-treated mice plays a critical role in the progression of liver injury, mice were treated with anticoagulant heparin. Heparin inhibits coagulation system activation by increasing the affinity of endogenous antithrombin for thrombin and other serine proteases of the coagulation pathway. Extensive fibrin deposition observed in the livers of TVX/LPS/Veh-treated mice at 4.5 h was significantly reduced by heparin treatment (Fig. 6.4A). Treatment with heparin also reduced the increase in plasma ALT activity in mice given TVX/LPS (Fig. 6.4B). This protection was confirmed by histopathological examination. TVX/LPS/Veh-treated mice developed large lesions of necrosis with evidence of apoptosis in some, whereas TVX/LPS/heparin-treated mice had less frequent and smaller necrotic lesions (Fig. 6.5, top).

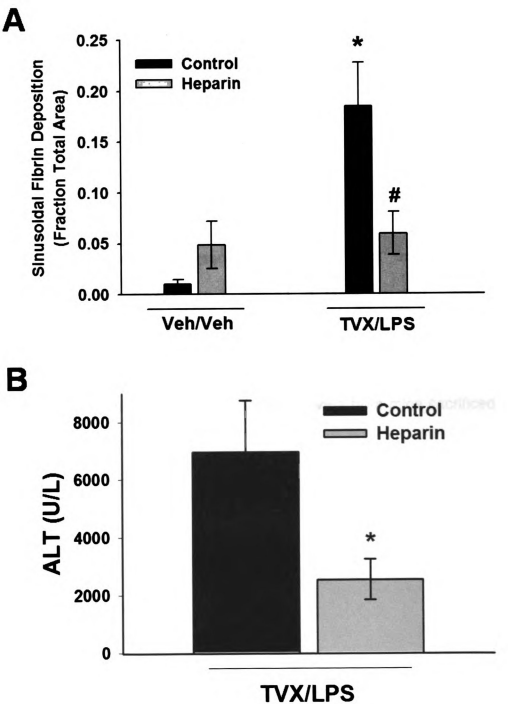
Fig. 6.3. Effect of fluoroquinolones and LPS on hepatic sinusoidal fibrin deposition. Mice were treated as described in Materials and Methods with TVX, LVX or Veh and then 3 h later with LPS or Veh. They were sacrificed 4.5 h after LPS administration, and hepatic fibrin deposition was quantified immunohistochemically as described in Materials and Methods. *n* = 4-6 animals/group.*significantly different from respective control group without LPS treatment. # significantly different from Veh/LPS group.





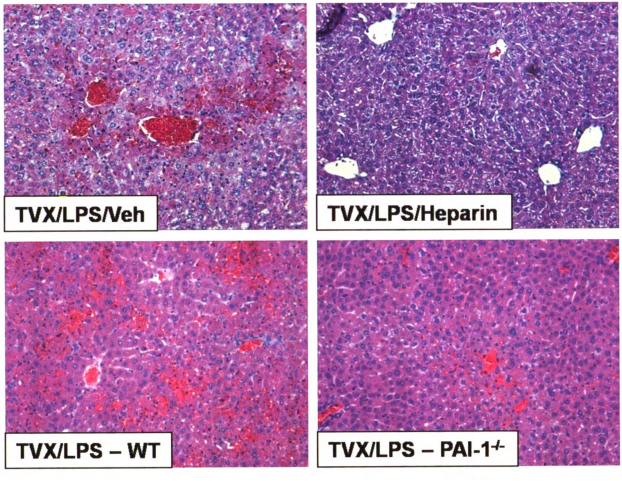
These data were generated by Aaron Fullerton.

Fig. 6.4. Effect of heparin treatment on TVX/LPS-induced hepatic fibrin deposition and liver injury. Mice were treated as described in Materials and Methods with TVX/LPS and heparin or saline vehicle. (A) They were sacrificed at 4.5 h, and fibrin deposition was quantified as described in Materials and Methods. *n* = 4-6 animals/group.*significantly different from respective control group without TVX/LPS treatment. # significantly different from TVX/LPS group not treated with heparin. (B) Mice were sacrificed 15 h after LPS dosing, and plasma ALT activity was measured. *n* = 8-12 animals/group.*significantly different from TVX/LPS/Veh-treated mice.



These data were generated by Aaron Fullerton.

Fig. 6.5. Histopathology of heparin-treated and PAI-1$^{-/-}$ mice treated with TVX/LPS. Mice were treated as described in Materials and Methods with TVX/LPS/Veh or TVX/LPS/heparin (top). In addition, wild-type or PAI-1$^{-/-}$ mice were treated with TVX/LPS as described in Materials and Methods (bottom). Photomicrographs were taken of representative livers from mice sacrificed at 15 h after LPS administration.


TVX/LPS/Veh
TVX/LPS/Heparin
TVX/LPS – WT
TVX/LPS – PAI-1-/-

6.4.3 PAI-1$^{-/-}$ mice are protected from TVX/LPS-induced liver injury

To determine if PAI-1 plays a role in hepatic fibrin deposition at the onset of liver injury, wild-type and PAI-1$^{-/-}$ mice were treated with TVX/LPS and killed at 4.5 h. TVX/LPS-coexposure caused significant fibrin deposition in wild-type mice as well as in PAI-1$^{-/-}$ mice (Fig. 6.6A). To confirm that PAI-1$^{-/-}$ mice do not have reduced fibrin deposition at a different time, mice were killed at 15 h and hepatic fibrin deposition was quantified. At 15 h, PAI-1$^{-/-}$ mice had the same amount of fibrin deposition as wild-type mice (Fig. 6.6B) but had significantly reduced hepatocellular injury compared to wild-type mice after TVX/LPS-coexposure (Fig. 6.6C). Histopathologic evaluation of liver sections confirmed this protective effect. Midzonal lesions of hepatocellular necrosis and apoptosis observed in TVX/LPS-treated wild-type mice (Fig. 6.5, bottom) were less frequent and smaller in PAI-1$^{-/-}$ mice (Table 6.1). TVX/LPS coexposure caused grade 4 or 5 (most severe) necrosis in 8/14 wild-type mice, whereas none of the 9 PAI-1$^{-/-}$ mice were grade 4 or 5. PAI-1$^{-/-}$ mice showed a trend toward decreased inflammation and hemorrhage that did not reach statistical significance.

6.4.4 Role of coagulation system activation and PAI-1 in TVX/LPS-induction of cytokines

Heparin-treated, wild-type and PAI-1$^{-/-}$ mice were cotreated with TVX/LPS and killed at 4.5 h to determine what roles heparin and PAI-1 have in the TVX/LPS-induction of inflammatory cytokines. Heparin treatment did not change the TVX/LPS-induced increases in IL-18, IL-6, KC, IL-10, MCP-1, VEGF, or

Fig. 6.6. Effect of PAI-1 deficiency on TVX/LPS-induced hepatic fibrin deposition and liver injury. Wild-type and PAI-$1^{-/-}$ mice were treated as described in Materials and Methods with TVX and then 3 h later with LPS. They were sacrificed at 4.5 h (A) or 15 (B), and fibrin deposition was quantified as described in Materials and Methods. *n* = 4-6 animals/group.*significantly different from Veh/Veh. (C) Mice were sacrificed 15 h after LPS dosing, and plasma ALT activity was measured. *n* = 13-22 animals/group.*significantly different from wild-type.

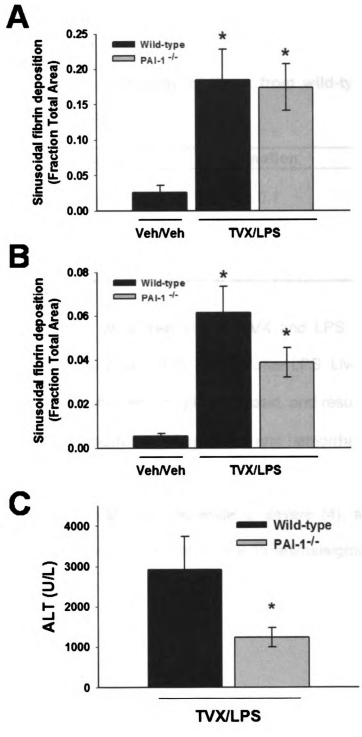

A
Sinusoidal fibrin deposition (Fraction Total Area)
0.25
0.20
0.15
0.10
0.05
0.00
Wild-type
PAI-1 -/-
*
*
Veh/Veh
TVX/LPS
B
Sinusoidal fibrin deposition (Fraction Total Area)
0.08
0.06
0.04
0.02
0.00
Wild-type
PAI-1 -/-
*
*
Veh/Veh
TVX/LPS
C
ALT (U/L)
4000
3000
2000
1000
0
Wild-type
PAI-1-/-
*
TVX/LPS

Table 6.1. Scoring of histopathology of livers from wild-type and PAI-1$^{-/-}$ mice cotreated with TVX/LPS.

Mouse strain	Necrosis	Inflammation	Hemorrhage
Wild-type	3.2 ± 0.4	1.4 ± 0.1	0.6 ± 0.2
PAI-1$^{-/-}$	2.0 ± 0.2 *	1.0 ± 0	0 ± 0

Wild-type and PAI-1$^{-/-}$ mice were treated with TVX and LPS as described in Materials and Methods. They were sacrificed 15 h after LPS. Liver sections were cut 5 μm thick and stained with hematoxylin and eosin, and resultant slides were scored by a pathologist for necrosis, inflammation and hemorrhage. The scoring scale was set from 0-5 with the following criteria: no observation (0), mild (1), mild to moderate (2), moderate (3), moderate to severe (4), and severe (5). Scores are reported as average ± S.E.M. *n* = 9-15 animals/group.*significantly different from wild-type group.

TNFα. However, it enhanced the TVX/LPS-induction of IL-1β and IFNγ (Fig. 6.7). In contrast, PAI-$1^{-/-}$ mice had significantly reduced levels of several cytokines after TVX/LPS treatment. These included IL-1β, IL-6, KC, IL-10, MCP-1, and IFNγ (Fig. 6.7). There was a trend toward decreased plasma concentration of TNFα in PAI-$1^{-/-}$ mice, but this was not statistically significant. Of the cytokines measured, only IL-18 and VEGF were increased to the same degree in wild-type and PAI-$1^{-/-}$ mice by TVX/LPS-coexposure (Fig. 6.7).

Fig. 6.7. Effect of heparin treatment or PAI-1 deficiency on TVX/LPS-induced increases in plasma cytokines. Mice were treated with vehicles or with TVX/LPS and sacrificed 4.5 h after LPS administration. Plasma concentrations of IL-1β, IL-18, IL-6, KC, IL-10, MCP-1, VEGF, IFNγ, and TNFα were measured as described in Materials and Methods. *n* = 4-6 animals/group.*significantly different from Veh/Veh. # significantly different from TVX/LPS control group.

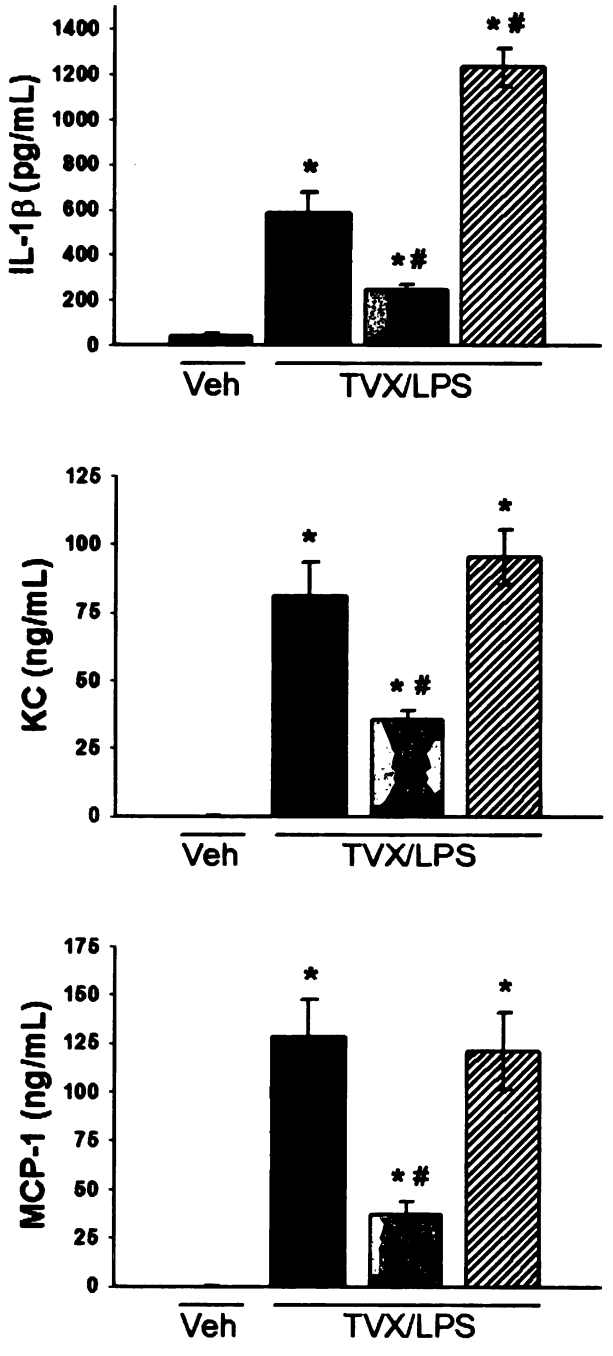

IL-1β (pg/mL)
1400
1200
1000
800
600
400
200
0
Veh
TVX/LPS
KC (ng/mL)
125
100
75
50
25
0
Veh
TVX/LPS
MCP-1 (ng/mL)
175
150
125
100
75
50
25
0
Veh
TVX/LPS
Control
PAI-1-/-
Heparin

Fig. 6.7 (cont'd).

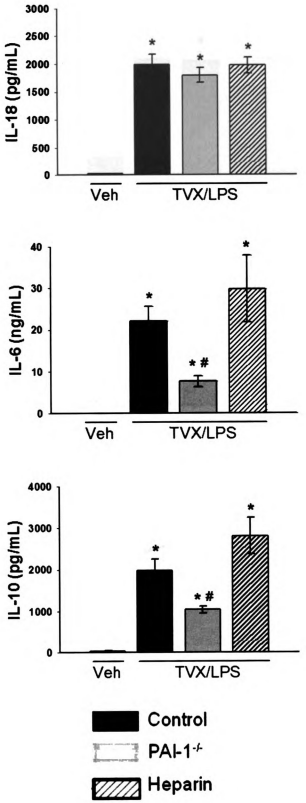
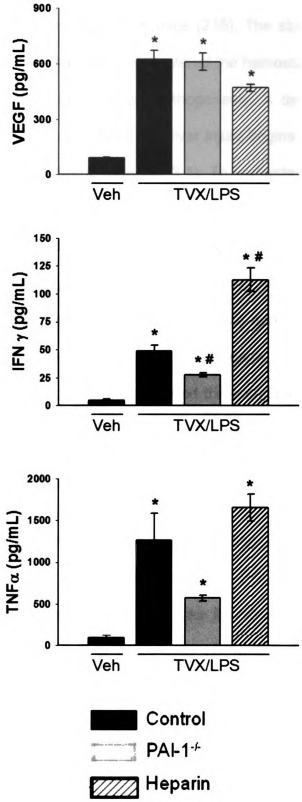

IL-18 (pg/mL)
3000
2500
2000
1500
1000
500
0
Veh
TVX/LPS
IL-6 (ng/mL)
40
30
20
10
0
*#
IL-10 (pg/mL)
4000
3000
2000
1000
Control
PAI-1-/-
Heparin

Fig. 6.7 (cont'd).


VEGF (pg/mL)
900
600
300
0
Veh
TVX/LPS
IFN γ (pg/mL)
150
125
100
75
50
25
0
Veh
TVX/LPS
TNFα (pg/mL)
2000
1500
1000
500
0
Veh
TVX/LPS
Control
PAI-1-/-
Heparin

6.5 Discussion

Previous studies showed that TVX, but not LVX, interacts with a nontoxic dose of LPS to cause hepatotoxicity in mice (216). The studies presented here examined whether TVX/LPS-coexposure altered the hemostatic system and, if so, what role the alteration played in the pathogenesis. A detailed timecourse of TVX/LPS-induced liver injury showed that liver injury begins at ~4.5 h and peaks at 15 h after LPS administration (see Fig. 3.1). To understand the importance of the hemostatic system in the progression of liver injury, hemostatic system biomarkers were measured at the onset of liver injury.

The hemostatic system normally functions as a balance between prothrombotic and antithrombotic factors to maintain homeostasis. Based on an increase in thrombin activation and a decrease in plasma fibrinogen concentration, TVX/LPS-coexposure activated the coagulation system, but TVX, LVX, LPS or LVX/LPS treatment did not (Fig. 6.1). LPS treatment alone induced a small, but statistically significant increase in plasma fibrinogen. This response has been seen previously and likely reflects an acute phase response to LPS (267). Coagulation system activation accompanying liver injury occurs after cotreatment with LPS and other agents (268-270). PAI-1 inhibits fibrinolysis via inhibition of plasminogen activators (271). Like its effect on coagulation system activation, TVX, but not LVX, enhanced the LPS-induced increase in plasma concentration of active PAI-1 (Fig. 6.2). Whether TVX acts directly to enhance the LPS induction of coagulation factors and PAI-1 is unknown. It is possible that TVX directly upregulates tissue factor or acts indirectly by enhancing the LPS

induction of cytokines such as TNFα that can induce tissue factor production (272). TVX enhances the LPS-induced production of TNFα, and it is likely that this plays a role in the TVX/LPS-induced coagulation system activation (216). TNFα is required for TVX/LPS-induced liver injury, and the increased TNFα in TVX/LPS-treated mice affected coagulation system activation and PAI-1 production (Chapter 4).

The TVX/LPS-mediated coagulation system activation and impairment of the fibrinolytic system suggests an imbalance in the hemostatic system that favors fibrin formation. Indeed, TVX/LPS-coexposure did result in increased in fibrin deposition compared to all other treatment groups (Fig. 6.3). Sinusoidal fibrin can impair hepatic blood flow, and thus oxygen delivery to hepatocytes, causing tissue hypoxia (103). Hypoxia could play a role in the pathogenesis of TVX/LPS-induced liver injury in a number of ways. For example, it sensitizes hepatocytes to cytotoxicity by proteases and hypochlorous acid (HOCl) released by activated neutrophils (103). In addition, hypoxia can alter cellular homeostasis by inducing oxidative stress and can directly cause cell death (273-275).

To address whether thrombin activation was involved in the pathogenesis of liver injury, heparin was used to inhibit coagulation. Heparin inhibited thrombin activation and decreased the TVX/LPS-induced fibrin deposition which in turn protected mice from TVX/LPS-induced liver injury (Fig. 6.4). This suggests that coagulation system activation plays a critical role in the fibrin deposition and the pathogenesis. However, whether hepatic fibrin deposition, thrombin activation of receptors or both are critical for TVX/LPS-induced liver injury is unknown.

Heparin can attenuate inflammation (153); accordingly, to address if this might be involved in its protective effect, plasma cytokine concentrations were measured. Heparin treatment failed to reduce plasma concentrations of any of the cytokines induced by TVX/LPS-treatment (Fig. 6.7), suggesting that heparin did not provide protection against liver injury by reducing inflammatory cytokines. This result also suggests that coagulation system activation acts downstream of any effects initiated by cytokines. Heparin treatment significantly increased TVX/LPS-induction of IFNγ. This enhancement might have been a result of the inhibition of thrombin, which can drive a Th2 response that downregulates IFNγ expression (142). In addition, after heparin treatment there was a slight, but not statistically significant decrease in the plasma concentration of VEGF, the expression of which can be driven by hypoxia (276).

In other models, there is evidence that PAI-1 can play a role in both fibrin deposition and the progression of liver injury (277-279). However, hepatic fibrin deposition at both 4.5 and 15 h was similar in wild-type and PAI-$1^{-/-}$ mice treated with TVX/LPS, yet both plasma ALT activity and histopathologic evaluation indicated lesser hepatocellular injury in the PAI-$1^{-/-}$ mice (Fig. 6.5 and 6.6). Therefore, the results suggest that protection from TVX/LPS-induced liver injury in PAI-$1^{-/-}$ mice is not related to fibrin deposition.

In addition to its role in fibrinolysis, PAI-1 has proinflammatory properties including induction of IL-6 (147). Moreover, PAI-1 can enhance LPS-induced neutrophil activation (146). Relative to wild-type mice, PAI-$1^{-/-}$ mice treated with TVX/LPS had reduced concentrations of IL-1β, IL-6, IL-10, IFNγ, MCP-1 and KC

at 4.5 h (Fig. 6.7). This result suggests a proinflammatory role for PAI-1 in the regulation of several cytokines. Our result for IFNγ is in contrast to a report that PAI-$1^{-/-}$ mice have an enhanced plasma IFNγ response to a toxic dose of LPS (280). It may be that the induction of IFNγ occurs by a different mechanism after TVX/LPS-coexposure compared to administration of a toxic dose of LPS by itself. It is also possible that the attenuated cytokine response in PAI-$1^{-/-}$ mice is secondary to a reduction in injury; however plasma concentrations were measured at 4.5 h, when there is only a slight increase in plasma ALT activity which is not attenuated in PAI-$1^{-/-}$ mice (data not shown). Therefore, it seems likely that the reduced cytokine response underlies the protection seen in PAI-$1^{-/-}$ mice. However, it has been reported that PAI-$1^{-/-}$ endothelial cells are resistant to apoptosis (281). If hepatocytes are also resistant to apoptosis in these mice, this might play a role that attenuated liver injury in PAI-$1^{-/-}$ mice.

In summary, TVX interacted with LPS to enhance coagulation system activation, increase PAI-1 production and cause liver injury. In contrast, LVX did not alter the hemostatic system or cause liver injury when coadministered with LPS. The TVX/LPS-induced imbalance in the hemostatic system resulted in hepatic fibrin deposition. Anticoagulant heparin inhibited thrombin activation which significantly reduced fibrin deposition and protected mice from TVX/LPS-induced hepatocellular necrosis without decreasing plasma concentrations of proinflammatory cytokines. This suggests that active thrombin, perhaps through enhanced fibrin deposition, contributes to the liver injury. PAI-$1^{-/-}$ mice did not have attenuated hepatic fibrin deposition but did have smaller plasma

concentrations of several inflammatory cytokines and less TVX/LPS-induced liver injury. Accordingly, coagulation system activation was critical for TVX/LPS-induced fibrin deposition, whereas PAI-1 was not, and the important role of PAI-1 in promoting the liver injury appears to be unrelated to its ability to downregulate fibrinolysis. It is likely that thrombin activation and proinflammatory cytokines comprise two independent pathways that contribute to TVX/LPS-induced liver injury, inasmuch as the removal of either one attenuated, but did not eliminate liver injury; in order to achieve complete protection from TVX/LPS-induced liver injury the inhibition of both was required.

CHAPTER 7

Vascular endothelial growth factor has a proinflammatory role which is critical to trovafloxacin and lipopolysaccharide coexposure-induced liver injury.

7.1 Abstract

TVX is a fluoroquinolone antibiotic which has caused idiosyncratic hepatotoxicity in a small fraction of people. Animal models predictive of drugs that cause IADRs are lacking in preclinical safety testing. Previously, we showed that a modest inflammatory stress induced by LPS renders mice sensitive to nonhepatotoxic doses of TVX. In contrast, LVX, a fluoroquinolone antibiotic that has not been associated with IADRs in humans, does not cause hepatotoxicity in mice cotreated with LPS. TVX/LPS-induced liver injury is dependent on both coagulation and TNFα pathways. VEGF is a cytokine with proinflammatory properties which has the potential to be a part of either of the pathways involved in the pathogenesis. The purpose of this study was to explore interactions between VEGF and other proinflammatory mediators of liver injury and, in turn, the role of VEGF in TVX/LPS-induced liver injury. TVX prolonged the LPS-induced increase in plasma VEGF. VEGF neutralization attenuated the TVX/LPS-induced increases in both hepatic fibrin deposition and plasma TNFα concentration. Additionally, VEGF neutralization protected mice from TVX/LPS-induced liver injury, as reflected by both plasma ALT activity and liver histopathology. In summary, VEGF has proinflammatory properties and plays a critical role in the progression of TVX/LPS-induced liver injury.

7.2 Introduction

VEGF has been studied extensively for its angiogenic properties. However, recent work has focused on its proinflammatory properties. Several cytokines can affect VEGF expression; conversely, VEGF can induce TNFα, neutrophil chemokines and tissue factor (92, 179, 180). Inasmuch as VEGF has the potential to interact with several pathways involved in TVX/LPS-induced liver injury, its interactions with various mediators of liver injury were examined. In accordance with its proinflammatory effects, VEGF is involved in the development of liver injury in animal models of endotoxemia and ischemia/reperfusion (179, 180).

The purpose of this study was to test the hypothesis that VEGF is involved in the pathogenesis of TVX/LPS-induced liver injury. Furthermore, the potential interaction of VEGF with neutrophil accumulation, TNFα, IFNγ and hepatic fibrin deposition, all of which play a role in TVX/LPS-induced liver injury, was explored in this study. These pathways involved in the pathogenesis were explored at a time near the onset of liver injury. Additionally, the study explored novel proinflammatory properties of VEGF that might be of importance for inflammatory tissue injury.

7.3 Materials and Methods

7.3.1 Materials

Please refer for Section 2.3.1 for information on this topic. VEGF antiserum and control rabbit serum were a kind gift from Dr. David Briscoe (Harvard Medical School, Boston, MA).

7.3.2 Animals

Please refer to Section 2.3.2 for information on this topic.

7.3.3 Experimental protocols

Mice fasted for 12 h were given TVX (150 mg/kg) or Veh (saline) by oral gavage. They were then given LPS (2.0×10^6 EU/kg) or Veh (saline) by intraperitoneal injection 3 h later. Food was returned immediately thereafter. Mice were anesthetized with sodium pentobarbital (50 mg/kg; i.p.) and killed at designated times after the administration of LPS or Veh for various measurements. Blood was drawn from the vena cava into a syringe containing sodium citrate resulting in a final concentration of 0.76%. The left lateral lobe was fixed in 10% neutral buffered formalin and paraffin blocked.

For VEGF neutralization studies, VEGF antiserum or control serum was administered (0.5 mL, i.p.) 15 h before and (0.8 mL, i.p.) 2 h after LPS.

7.3.4 Histopathology

Please refer to section 2.3.4 for information on this topic.

7.3.5 Cytokine measurements

Please refer to Section 3.3.5 for information on this topic.

7.3.6 Neutrophil staining

Please refer to section 3.3.6 for information on this topic.

7.3.7 Hemostatic system measurements

Please refer to Section 4.3.7 for information on this topic.

7.3.8 Statistical analyses

Results are presented as mean $\pm$ S.E.M. A Student's t-test or a 2-way analysis of variance (ANOVA) was used as appropriate after data normalization. All pairwise comparisons were made using Tukey's test with the criterion for significance at $p < 0.05$.

7.4 Results

7.4.1 Timecourse of plasma concentration of VEGF

TVX alone did not have any effect on plasma VEGF concentration (Fig. 7.1). LPS treatment caused a significant increase in plasma VEGF at all times measured (Fig. 7.1). TVX treatment prior to LPS enhanced the LPS-induced increase in plasma concentration of VEGF at 4.5 (Fig. 7.1).

7.4.2 Effect of VEGF neutralization on TVX/LPS-induced hepatotoxicity

To explore the role of VEGF in TVX/LPS-induced liver injury, VEGF was neutralized by VEGF antiserum, which reduced the TVX/LPS-induced increase in plasma VEGF at 4.5 h (Fig. 7.2A). TVX/LPS-treated mice were treated with control serum or VEGF antiserum and then killed at 15 h. TVX/LPS-induced liver injury was attenuated by VEGF neutralization (Fig. 7.2B). Histopathologic evaluation confirmed that mice were protected from TVX/LPS-induced hepatotoxicity by VEGF neutralization. TVX/LPS coexposure caused hepatocellular necrosis and apoptosis primarily localized to the midzonal regions (Fig. 7.3). VEGF neutralization reduced the size and frequency of lesions induced by TVX/LPS coexposure (Fig. 7.3).

Fig. 7.1 Timecourse of VEGF plasma concentration. Mice were treated with TVX or Veh 3h before LPS or Veh as described in Materials and Methods. They were killed at various times and plasma concentration of VEGF was measured. *n* = 5-7 animals/group. *significantly different from 0 h within same treatment group. #significantly different from Veh/LPS treatment group at same time.

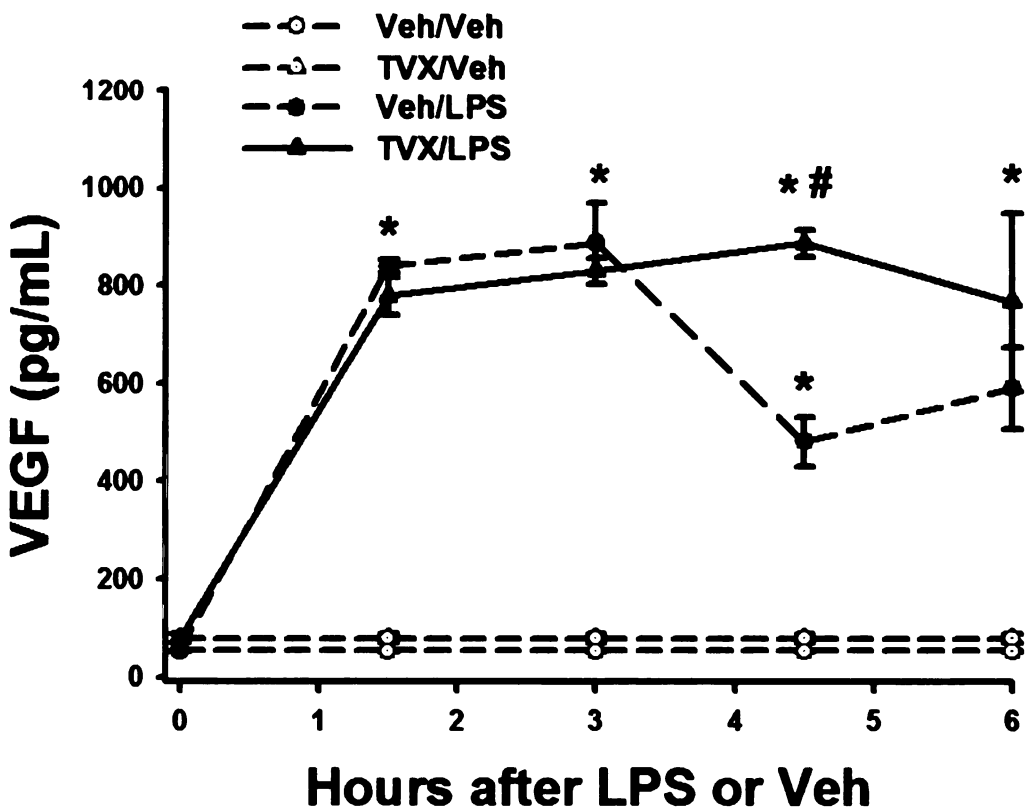

Veh/Veh
TVX/Veh
Veh/LPS
TVX/LPS
VEGF (pg/mL)
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
Hours after LPS or Veh
*
*
*#
*

Fig. 7.2. Effect of VEGF neutralization on TVX/LPS-induced plasma VEGF induction and liver injury. Mice were treated with TVX/LPS or Veh/Veh in addition to VEGF antiserum or control serum as described in Materials and Methods. (A) They were killed 4.5 h after LPS administration. Plasma concentration of VEGF was measured as described in Materials and Methods. *n* = 4-6 animals/group. (B) Mice were killed 15 h after LPS administration and plasma ALT activity was measured. *n* = 6-10 animals/group. *significantly different from respective Veh/Veh group. #significantly different from TVX/LPS/control serum-treated group.

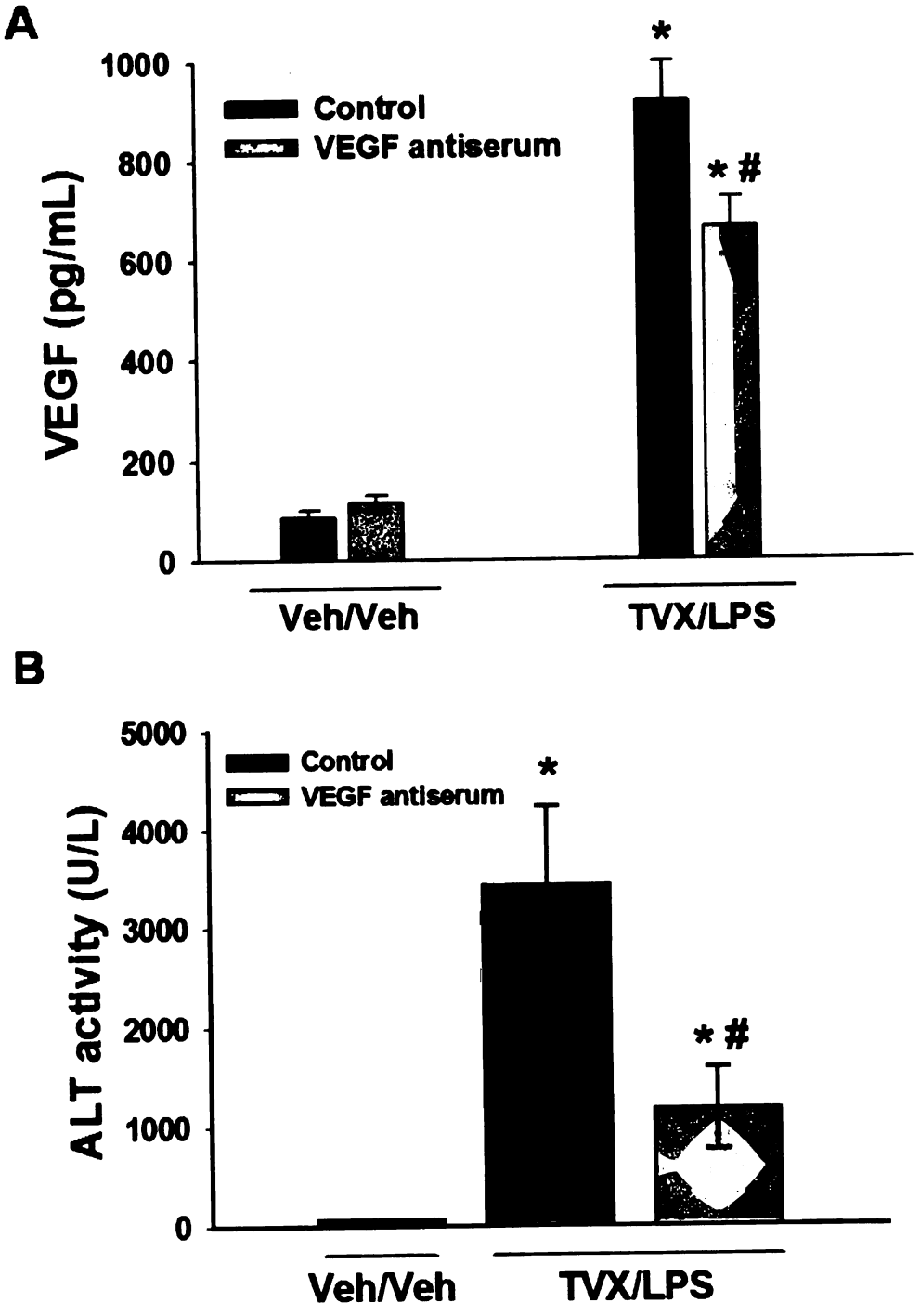
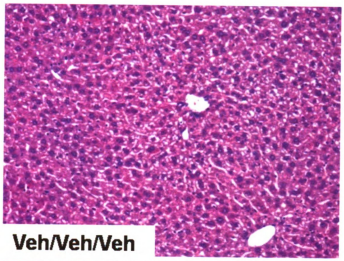
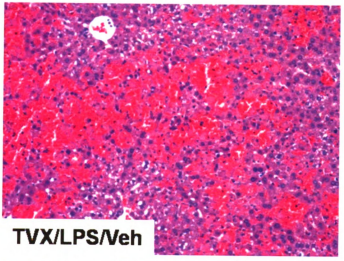
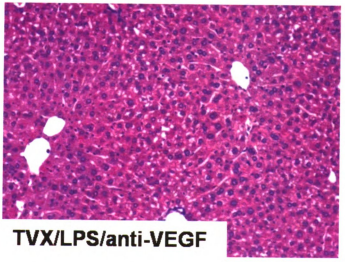

A
1000
800
600
400
200
0
VEGF (pg/mL)
Control
VEGF antiserum
*
* #
Veh/Veh
TVX/LPS
B
5000
4000
3000
2000
1000
0
ALT activity (U/L)
Control
VEGF antiserum
*
* #
Veh/Veh
TVX/LPS

Fig. 7.3. Protection from TVX/LPS-induced liver pathology by VEGF neutralization. Mice were treated with TVX/LPS or Veh/Veh in addition to VEGF antiserum or control serum as described in Materials and Methods. They were killed 15 h after LPS administration, and photomicrographs were taken of representative livers.


Veh/Veh/Veh


TVX/LPS/Veh


TVX/LPS/anti-VEGF

7.4.3 Effect of VEGF neutralization on plasma concentrations of cytokines

Mice were treated as described in Materials and Methods and killed at 4.5 h, a time near the onset of liver injury. VEGF antiserum by itself did not affect the plasma concentrations of any cytokines or chemokines measured (Fig. 7.4 and 7.5). TVX/LPS coexposure caused an increase in the plasma concentrations of TNFα, IL-10, IL-1β, IL-6, IL-18 and IFNγ (Fig. 7.4). VEGF neutralization reduced the TVX/LPS-induced increase in TNFα and IL-10, but did not affect the induction of other cytokines (Fig. 7.4). In addition to inducing a number of cytokines, TVX/LPS coexposure caused an increase in the chemokines MCP-1, KC and MIP-1α, and this increase was unaffected by VEGF neutralization (Fig. 7.5).

7.4.4 Effect of VEGF neutralization on TVX/LPS-induced hepatic neutrophil accumulation

Mice were treated with TVX/LPS and killed at 4.5 to measure hepatic neutrophil accumulation. TVX/LPS coexposure caused neutrophils to accumulate in the liver, and this was unaffected by VEGF neutralization (Fig. 7.6).

7.4.5 Effect of VEGF neutralization on the hemostatic system

TVX/LPS coexposure altered the balance of the hemostatic system to cause hepatic fibrin deposition (See Chapter 6). Hemostatic system biomarkers were measured at 4.5 h, a time at the onset of liver injury. VEGF antiserum by itself did not cause any changes in TAT dimers, active PAI-1 or hepatic fibrin

Fig. 7.4. Effect of VEGF neutralization on TVX/LPS-induced increase of cytokines. Mice were treated with TVX/LPS or Veh/Veh in addition to VEGF antiserum or control serum as described in Materials and Methods. They were killed 4.5 h after LPS administration. Plasma concentrations of TNFα, IL-10, IL-1β, IL-6, IL-18 and IFNγ were measured as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group. #significantly different from TVX/LPS/control serum-treated group.

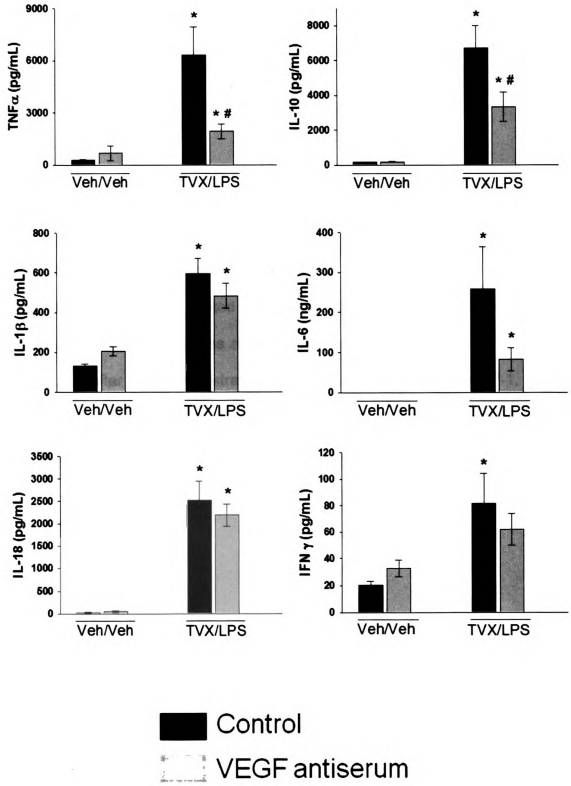

TNFα (pg/mL)
9000
6000
3000
0
Veh/Veh
TVX/LPS
IL-10 (pg/mL)
8000
6000
4000
2000
0
IL-1β (pg/mL)
800
600
400
200
0
IL-6 (ng/mL)
400
300
200
100
0
IL-18 (pg/mL)
3500
3000
2500
2000
1500
1000
500
0
IFN γ (pg/mL)
120
100
80
60
40
20
0
Control
VEGF antiserum

Fig. 7.5. VEGF neutralization did not affect TVX/LPS-induced increases in chemokines. Mice were treated with nTVX/LPS or Veh/Veh in addition to VEGF antiserum or control serum as described in Materials and Methods. They were killed 4.5 h after LPS administration. Plasma concentrations of MCP-1, KC and MIP-1α were measured as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group. #significantly different from TVX/LPS/control serum-treated group.

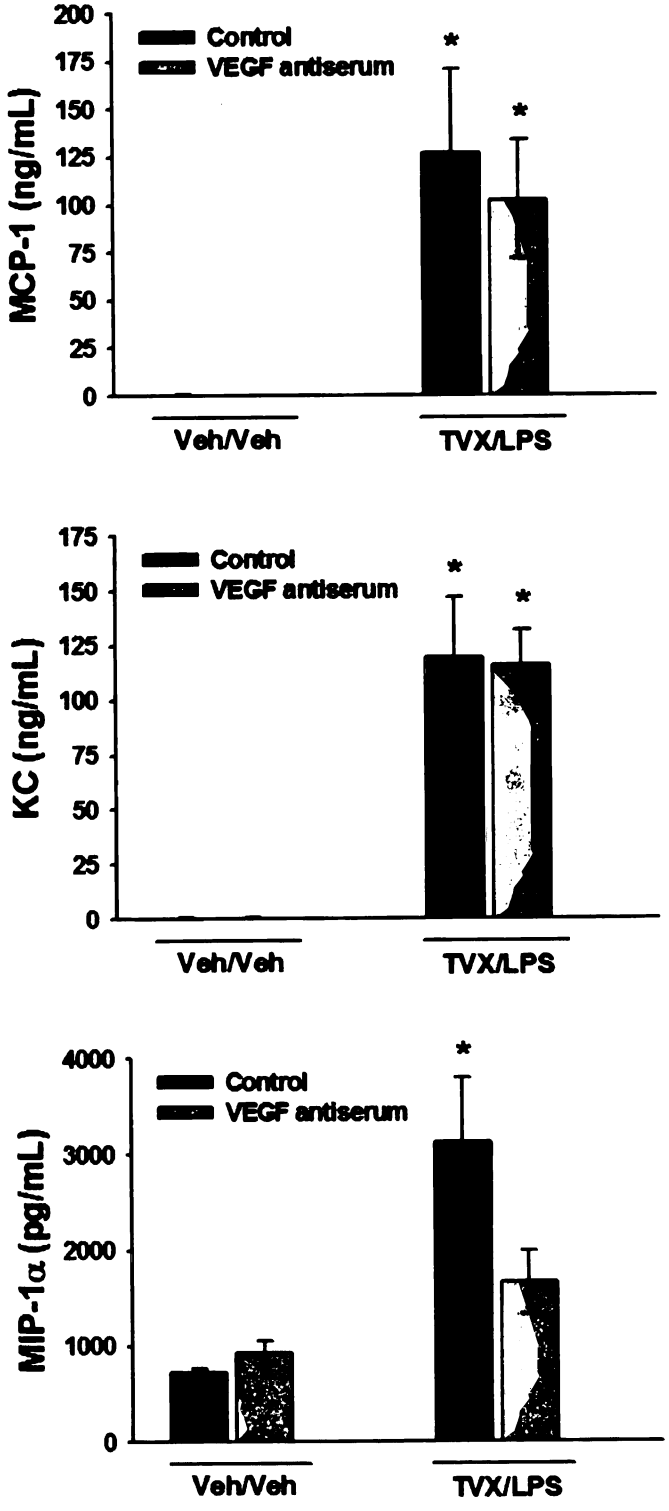
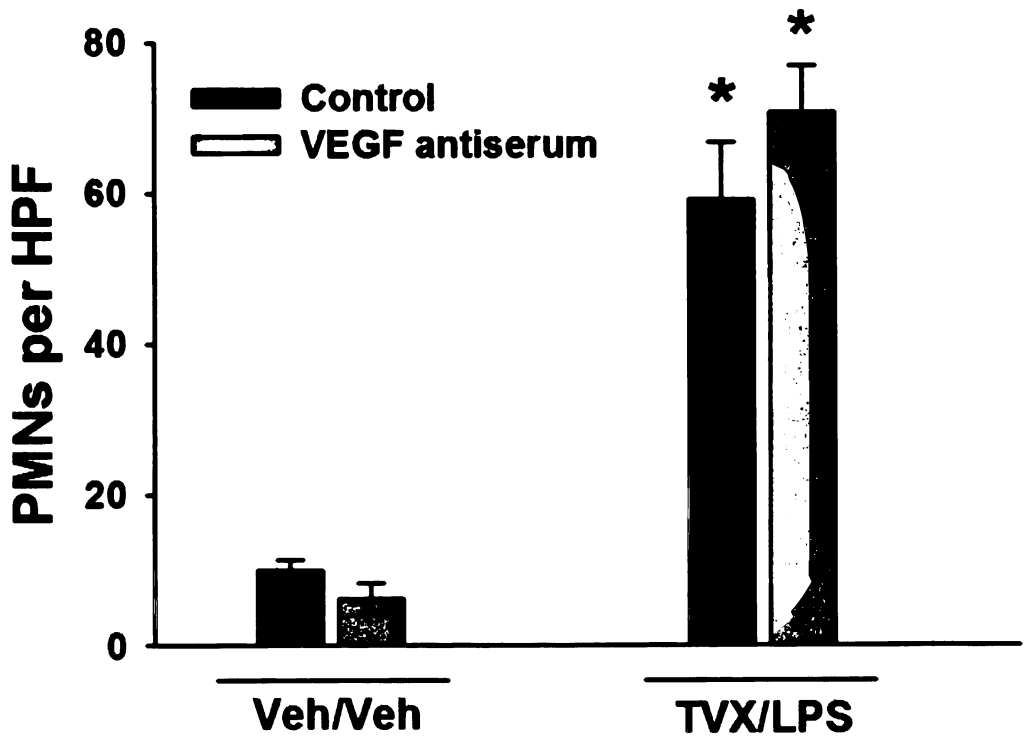
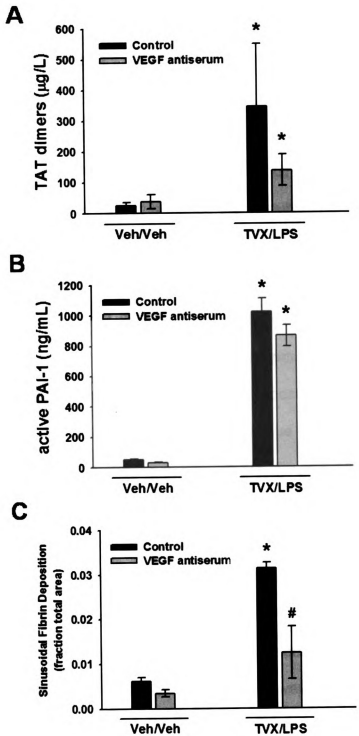

MCP-1 (ng/mL)
200
175
150
125
100
75
50
25
0
Control
VEGF antiserum
*
*
Veh/Veh
TVX/LPS
KC (ng/mL)
175
150
125
100
75
50
25
0
Control
VEGF antiserum
*
*
Veh/Veh
TVX/LPS
MIP-1α (pg/mL)
4000
3000
2000
1000
0
Control
VEGF antiserum
*
Veh/Veh
TVX/LPS

Fig. 7.6. Effect of VEGF neutralization on TVX/LPS-induced hepatic neutrophil accumulation. Mice were treated with TVX/LPS or Veh/Veh in addition to VEGF antiserum or control serum as described in Materials and Methods. They were killed 4.5 h after LPS administration. Liver sections were stained immunohistochemically for neutrophils and quantified as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group.


80
60
40
20
0
PMNs per HPF
Control
VEGF antiserum
*
*
Veh/Veh
TVX/LPS

deposition (Fig. 7.7). VEGF neutralization caused a trend towards a decrease in TVX/LPS-induced increase in TAT dimers (Fig. 7.7A). The plasma concentration of active PAI-1 was increased by TVX/LPS coexposure, and this was unaffected by treatment with VEGF antiserum (Fig. 7.7B). Hepatic fibrin deposition was increased by TVX/LPS coexposure (Fig. 7.7C). VEGF neutralization significantly attenuated the TVX/LPS-induced increase in fibrin deposition in the liver (Fig. 7.7C).

Fig. 7.7. The role of VEGF in TVX/LPS-induced hemostatic system activation. Mice were treated with TVX/LPS or Veh/Veh in addition to VEGF antiserum or control serum as described in Materials and Methods. They were killed 4.5 h after LPS administration. Plasma concentrations of (A) TAT dimers and (B) active PAI-1 were measured as described in Materials and Methods. (C) Frozen liver sections were stained immunohistochemically for fibrin and quantified as described in Materials and Methods. *n* = 4-6 animals/group. *significantly different from respective Veh/Veh group. #significantly different from TVX/LPS/control serum-treated group.


A
TAT dImers (µg/L)
600
500
400
300
200
100
0
Control
VEGF antiserum
*
*
Veh/Veh
TVX/LPS
B
active PAI-1 (ng/mL)
1200
1000
800
600
400
200
0
Control
VEGF antiserum
*
*
Veh/Veh
TVX/LPS
C
Sinusoidal Fibrin Deposition (fraction total area)
0.04
0.03
0.02
0.01
0.00
Control
VEGF antiserum
*
#
Veh/Veh
TVX/LPS

7.5 Discussion

VEGF is produced by endothelial cells, macrophages, activated T cells, and a variety of other cell types as a result of several stimuli, including hypoxia, IL-1β, IL-1α, IL-6, oncostatin M, TNFα and IL-8 (163-169). LPS caused an increase in plasma VEGF concentration at 1.5, 3, 4.5 and 6 hours. TVX treatment enhanced the LPS-induced increase in VEGF (Fig. 7.2). It is possible that this is a direct effect of TVX to enhance the stimulation of one or more cell types to produce and release VEGF in response to LPS. However, it is likely that the prolonged VEGF plasma concentration is a secondary effect. TVX enhanced the LPS-induced increase in TNFα (Fig. 2.4), which can induce VEGF expression in macrophages (91). Additionally, TVX/LPS coexposure causes hemostatic system dysregulation which leads hepatic fibrin deposition (Chapter 6). Fibrin deposition can alter blood flow and cause local tissue hypoxia, which induces VEGF expression (151). Therefore, it is possible that the prolongation of VEGF is a downstream effect in a cascade of inflammatory events.

To address whether VEGF is involved in the pathogenesis of liver injury, VEGF antiserum was used to neutralize it. VEGF neutralization decreased the TVX/LPS-induced increase in plasma concentration of VEGF. VEGF activity was not measured, and it is likely that VEGF antiserum reduced activity to a greater degree than the plasma concentration of VEGF. However VEGF neutralization attenuated TVX/LPS-induced liver injury (Fig. 7.2 and 7.3), suggesting that VEGF plays a critical role in the pathogenesis.

To determine possible mechanism(s) by which VEGF contributes to TVX/LPS-induced liver injury, plasma cytokine concentrations and hemostatic system parameters were measured. TVX enhanced the LPS induction of several proinflammatory cytokines, of which TNFα and IFNγ are known to be involved in the pathogenesis (Chapters 2, 3 and 5). To determine if VEGF plays a role in the induction of cytokines, mice were killed at a time near the onset of liver injury. VEGF neutralization did not alter the TVX/LPS-induced increase in IL-1β, IL-6, IL-18 or IFNγ. However, it attenuated the TVX/LPS-induced increase of TNFα and IL-10 (Fig. 7.4). The reduced IL-10 plasma concentration is likely due to the reduced plasma concentration of TNFα, inasmuch as IL-10 induction is primarily TNFα-dependent (282). The ability of VEGF to enhance TNFα concentration is consistent with a report in which VEGF neutralization reduced the plasma concentration of TNFα in a model of sepsis (179). It is possible that this effect is mediated by activation of early growth response-1, a transcription factor which can be activated by VEGF and which increases TNFα expression (92). TNFα neutralization attenuated the TVX/LPS-induced increase of VEGF (Fig. 4.2). It is therefore possible that VEGF and TNFα upregulate one another's production, creating a vicious proinflammatory cycle which could contribute to TVX/LPS-induced liver injury.

TVX/LPS coexposure induced increases in the plasma concentrations of several chemokines, KC, MCP-1 and MIP-1α, and these were unchanged by VEGF neutralization (Fig. 7.5). This result was surprising, inasmuch as VEGF neutralization reduced TNFα concentration and TNFα neutralization attenuated

the TVX/LPS-induced increase in these chemokines (Fig. 4.2 and 4.3). However, it is possible that VEGF neutralization did not reduce the concentration of TNFα to a great enough extent to see a difference in chemokine induction.

The TVX/LPS-induced increase in KC, MCP-1 and MIP-1α appears to be VEGF-independent, but it was possible that VEGF played a role in neutrophil accumulation independent of these chemokines. Accordingly, livers were stained immunohistochemically for PMNs. VEGF neutralization did not affect TVX/LPS-induced neutrophil accumulation in the livers of treated mice (Fig. 7.6). This result is in contrast to a study which found that VEGF neutralization significantly attenuated MCP-1 production and hepatic neutrophil accumulation induced by ischemia/reperfusion (180). Thus, it is likely that the mechanism of MCP-1 induction and hepatic PMN accumulation between TVX/LPS coexposure and ischemia/reperfusion is different. It is possible that VEGF interacted with neutrophils to enhance activation, which is critical for TVX/LPS-induced hepatotoxicity.

TVX/LPS coexposure caused hemostatic system dysregulation, which is involved in the pathogenesis (see Chapter 6). VEGF can potentially interact with the hemostatic system in several ways. For example, VEGF induces tissue factor, which causes coagulation system activation (183), and PAI-1, which downregulates fibrinolysis (184). VEGF neutralization caused trends toward attenuated plasma concentrations of TAT dimers and PAI-1. The TVX/LPS-induced increase in PAI-1 and hepatic fibrin deposition was TNFα-dependent (Fig. 4.5); therefore, it is possible that the reduction by VEGF neutralization is

secondary to its effect on TNFα. The trends toward a decrease in concentrations of TAT dimers and PAI-1 may have resulted in reduced hepatic fibrin deposition after TVX/LPS coexposure (Fig. 7.7). The ability of VEGF neutralization to reduce hepatic fibrin deposition is a novel finding and might be involved in other models of inflammatory tissue injury. TVX/LPS-induced hepatic fibrin deposition might be involved in the pathogenesies. Additionally, fibrin deposition could alter blood flow and cause local tissue hypoxia, which can drive VEGF expression (151). Inasmuch as VEGF neutralization reduced fibrin deposition, it is possible that fibrin deposition and VEGF enhance one another to create a feedforward cycle involved in pathogenesis.

In summary, TVX prolonged the LPS-induced increase of VEGF and caused hepatotoxicity. VEGF neutralization attenuated liver injury caused by TVX/LPS coexposure. Additionally, VEGF neutralization reduced the TVX/LPS-induced increase in TNFα and hepatic fibrin deposition. It is possible that VEGF is involved in dysregulated feedforward cycles with both TNFα and the hemostatic system, which could drive the development of liver injury.

CHAPTER 8

Summary and conclusions

8.1 Summary of research

At the outset, the hypothesis was tested that TVX interacts with an inflammatory stress to cause idiosyncrasy-like liver injury in mice. Initial dose response studies were conducted to find nontoxic doses of TVX (Fig. 2.1A), LPS and PGN-LTA. TVX, up to 1000 mg/kg, was administered orally to mice without finding a hepatotoxic dose. TVX administered 3 h before a nonhepatotoxic dose of LPS, which activates TLR4, caused hepatocellular injury. Preliminary dose-response studies revealed that mice treated with 150 mg/kg TVX before LPS developed significant liver injury with a mortality rate less than 10% (Fig. 2.1A); therefore, 150 mg/kg TVX was used for all subsequent studies. Additionally, TVX interacted with an inflammatory stress induced by PGN-LTA coexposure, which activates TLR2, to cause liver injury (Fig. 3.1). A timecourse study of TVX/LPS- and TVX/PGN-LTA-induced liver injury found that plasma ALT activity was increased by 4.5 h and progressed to a maximum by 15h (Fig. 2.1B, 3.1). The primary histopathologic finding in TVX/LPS-treated mice was lesions of hepatocellular necrosis and apoptosis primarily in midzonal regions (Fig. 2.3). TVX-PGN-LTA-treated mice had similar lesions, but these were primarily located in centrilobular regions of the liver (Fig. 3.2). Overall, these results showed that TVX interacts with an inflammatory stress, induced by LPS or PGN-LTA, to cause hepatotoxicity in mice.

In a subsequent study, an equiefficacious dose of LVX did not interact with LPS coexposure to cause liver injury, whereas TVX/LPS coexposure did (Fig.

2.2). Accordingly, the propensity of the fluoroquinolones to cause idiosyncratic hepatotoxicity in people tracked with the potential of each to interact with LPS coexposure to cause hepatotoxicity in mice. Inasmuch as LVX/LPS did not cause liver injury, type II topoisiomerase inhibition alone by fluoroquinolones is not sufficient to interact with LPS to cause liver injury.

Neutrophils are critical to the pathogenesis of several models of liver injury. Both LPS and PGN-LTA induced accumulation of neutrophils within the liver which was unaffected by TVX pretreatment (Fig. 3.5). Prevention of neutrophil activation by CD18 antiserum attenuated both TVX/LPS- and TVX/PGN-LTA-induced liver injury (Fig. 3.6). However, CD18 antiserum did not affect TVX/LPS-induced increases in plasma concentrations of cytokines (Fig. 3.8). These results suggest that neutrophil activation plays a role which is independent of the induction of proinflammatory cytokines in TVX/LPS-induced liver injury.

TNFα is critical to several models of liver injury, therefore we hypothesized that this cytokine plays a role in TVX/LPS-induced hepatotoxicity via interactions with other factors known to be important in inflammatory liver injury. These included PMNs, IFNγ, VEGF and the hemostatic system. TVX enhanced the LPS-induced increase in plasma TNFα (Fig. 2.4). In contrast, LVX did not affect this. TNFα neutralization, via etanercept administration, attenuated TVX/LPS-induced liver injury (Fig. 2.7 and 2.8). Additionally, $p55^{-/-}$ and $p75^{-/-}$ mice were protected from TVX/LPS-induced liver injury (Fig. 4.1), suggesting that both TNF receptors play a role. To explore further the role of TNFα in the pathogenesis, a nontoxic dose of TNFα was administered in place of LPS. TVX/TNFα-

coexposure caused hepatotoxicity (Fig. 4.6), suggesting that TVX acts, at least in part, at a point downstream of TNFα production.

TNFα has the potential to be involved in TVX/LPS-induced hepatotoxicity in several ways. To explore possible interactions with PMNs, IFNγ, VEGF and/or the hemostatic system, mice were killed at a time near the onset of liver injury. TNFα neutralization attenuated TVX/LPS-induced increases in the plasma concentrations of IFNγ, IL-6, MCP-1, VEGF, MIP-2, KC and MIP-1α (Fig. 4.2 and 4.3). However, despite a reduction in plasma concentrations of chemokines, hepatic PMN accumulation induced by TVX/LPS coexposure was unaffected by TNFα neutralization (Fig. 4.4). Dysregulation of the hemostatic system by TVX/LPS coexposure was also lessened by etanercept treatment. The TVX/LPS-induced increase in the plasma concentration of PAI-1 was attenuated by TNFα neutralization (Fig. 4.5A), whereas the plasma concentration of TAT dimers was unaffected (Fig. 4.5B). However, TNFα neutralization reduced TVX/LPS-induced hepatic fibrin deposition (Fig. 4.5C). Overall, the TVX/LPS-induced increases in plasma concentrations of IFNγ and VEGF and dysregulation of the hemostatic system were TNFα-dependent.

Hepatic gene expression analysis at a time before the onset of liver injury distinguished TVX/LPS-treated mice from those treated with TVX or LPS alone (Fig. 5.2). Further analysis of genes selectively changed by TVX/LPS compared to all other treatment groups suggested a role for interferon signaling, inasmuch as 18% of the genes were increased and regulated by interferons (Table 5.2). Therefore, the hypothesis was tested that IFNγ plays a role in the progression of

liver injury induced by TVX/LPS coexposure. TVX enhanced the LPS-induced increase in the plasma concentrations of IFNγ and IL-18, which stimulates cells to release IFNγ (Fig. 5.4). Both IL-18$^{-/-}$ and IFNγ$^{-/-}$ mice were protected from TVX/LPS-induced hepatotoxicity (Fig. 5.5 and 5.6). These results suggest that IFNγ is a critical mediator involved in the pathogenesis.

The role which IFNγ plays in the TVX/LPS-induced increase of plasma concentrations of TNFα, VEGF and hepatic neutrophil accumulation were explored. After TVX/LPS coexposure, IFNγ$^{-/-}$ mice had reduced plasma concentrations of TNFα, IL-18 and IL-1β, whereas VEGF was unchanged compared to wild-type mice (Fig. 5.9). TVX/LPS-induced hepatic neutrophil accumulation was unchanged in IFNγ$^{-/-}$ mice (Fig. 5.7). These results suggest that TVX/LPS-induced increases in plasma VEGF and hepatic neutrophil accumulation are independent of IFNγ, but IFNγ plays a role in the increased plasma concentrations of TNFα and IL-18.

Hemostatic system dysregulation can result in hepatic fibrin deposition and has the potential to be involved in the progression of liver injury through several mechanisms. Therefore, the hypothesis tested was that an imbalance in the hemostatic system plays a role in the pathogenesis of TVX/LPS-induced liver injury. At the onset of liver injury, coexposure to TVX/LPS, but not exposure to TVX, LVX, LPS or LVX/LPS caused coagulation system activation as measured by increased plasma concentration of thrombin-antithrombin dimers and decreased plasma circulating fibrinogen (Fig. 6.1). LPS treatment induced a small increase in plasma PAI-1 concentration, and TVX pretreatment enhanced

this effect (Fig. 6.2). This imbalance in coagulation and fibrinolysis induced by TVX/LPS coexposure was associated with hepatic fibrin deposition (Fig. 6.3). Anticoagulant heparin administration reduced TVX/LPS-induced hepatic fibrin deposition and liver injury (Fig. 6.4). PAI-$1^{-/-}$ mice treated with TVX/LPS exhibited similar fibrin deposition to wild-type mice yet had less hepatocellular injury (Fig. 6.6). These results suggest that TVX/LPS coexposure caused an imbalance in the hemostatic system, resulting in increased thrombin activation, plasma concentrations of PAI-1 and hepatic fibrin deposition. Furthermore, both thrombin activation and PAI-1 play a critical role in the progression of TVX/LPS-induced liver injury, but through different modes of action.

To examine the mechanisms by which thrombin inhibition and PAI-$1^{-/-}$ mice are protected from TVX/LPS-induced liver injury, plasma concentrations of several cytokines were measured at a time near the onset of liver injury. After TVX/LPS coexposure, PAI-$1^{-/-}$ mice had reduced plasma concentrations of several cytokines including IFNγ, but not TNFα or VEGF compared to wild-type controls (Fig. 6.7). In contrast, heparin treatment did not attenuate the TVX/LPS-induced increase in the plasma concentrations of any cytokines measured (Fig. 6.7). These results suggest that PAI-1, but not thrombin, has a proinflammatory role during TVX/LPS coexposure.

VEGF is a cytokine with proinflammatory properties and has the potential to participate in several pathways involved in the pathogenesis. TVX prolonged the LPS-induced increase in plasma concentration of VEGF (Fig. 7.1). VEGF neutralization protected mice from TVX/LPS-induced liver injury, as reflected by

both plasma ALT activity and liver histopathology (Fig. 7.2 and 7.3). VEGF neutralization did not affect the TVX/LPS-induced increases in plasma concentrations of IL-1β, IL-6, IL-18, IFNγ, MCP-1, KC or MIP-1α (Fig. 7.4 and 7.5). However, it reduced the increase in plasma TNFα induced by TVX/LPS coexposure (Fig. 7.4). Hepatic neutrophil accumulation was unaffected by VEGF neutralization (Fig. 7.6), but it caused a trend toward a reduction in TVX/LPS-induced plasma concentrations of TAT dimers and PAI-1 (Fig. 7.7).This was associated with significantly decreased hepatic fibrin deposition (Fig. 7.7). These results suggest that VEGF plays a critical role in the progression of TVX/LPS-induced liver injury, perhaps by promoting increased TNFα and hepatic fibrin deposition.

In summary, TVX interacts with an inflammatory stress to cause liver injury in mice which is dependent on PMN activation, TNFα, IFNγ, thrombin, PAI-1 and VEGF. Furthermore, TNFα, IFNγ and VEGF interact with one another to create possible cycles of inflammation. These proinflammatory cycles and the proposed pathways of TVX/LPS-induced liver injury are outlined in Section 8.2.

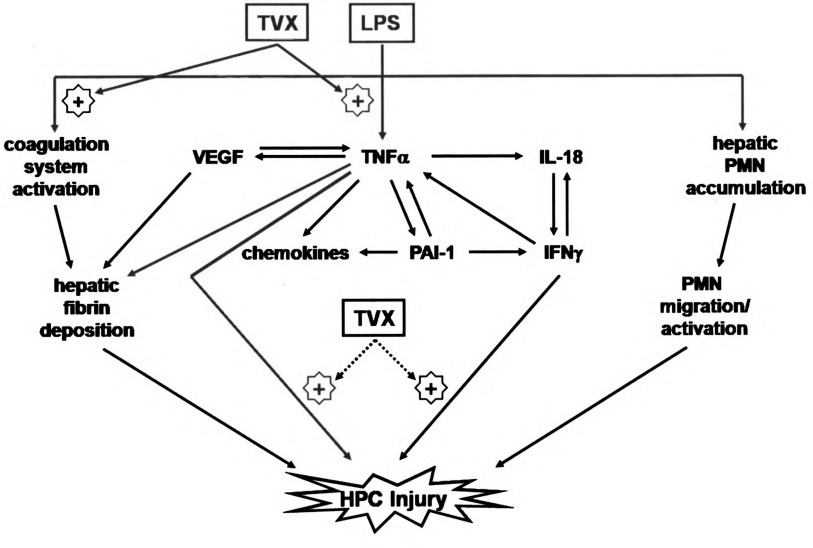
8.2 Proposed pathways of TVX/LPS-induced liver injury

Figure 8.2 illustrates proposed pathways to TVX/LPS-induced liver injury. These pathways are based on the results presented in this dissertation and are likely to be modified as we learn more about this model. LPS caused coagulation system activation, hepatic PMN accumulation and increased the plasma concentration of TNFα. TVX treatment enhanced the LPS-induced increase in coagulation system activation (Fig. 6.1) and TNFα (Fig. 2.4).

LPS-induced hepatic neutrophil accumulation was unaffected by TVX. Additionally, PMN accumulation was not reduced by neutralizing or removing TNFα (Fig. 4.4), IFNγ (Fig. 5.7) or VEGF (Fig. 7.6). Neutrophil activation is critical to TVX/LPS-induced liver injury (Fig. 3.6), but is not required for the induction of cytokines by TVX/LPS coexposure (Fig. 3.9). Therefore, hepatic PMN accumulation and activation appear to be a mechanism required for TVX/LPS-induced hepatotoxicity which does not interact with any other pathways explored. However, this interpretation is subject to change, as some of the cytokines involved in TVX/LPS-induced liver injury might affect PMN activation.

Coagulation system activation induced by LPS was enhanced by TVX (Fig. 6.1); and along with TNFα (Fig. 4.5) and VEGF (Fig. 7.7) played a critical role in TVX/LPS-induced hepatic fibrin deposition (Fig. 6.3). However, TNFα and VEGF contribute to hepatic fibrin deposition by a mechanism independent of coagulation system activation, since neutralization of either did not affect plasma concentrations of TAT dimers (Fig. 4.5 and 7.7). However, coagulation system

Fig. 8.1. Proposed pathways to TVX/LPS-induced liver injury. See Section 8.2 for a detailed explanation.


TVX
LPS
coagulation system activation
VEGF
TNFα
IL-18
hepatic PMN accumulation
chemokines
PAI-1
IFNγ
hepatic fibrin deposition
TVX
PMN migration/ activation
HPC Injury

activation did not increase plasma concentrations of any of the cytokines examined (Fig. 6.7). Thrombin activation was critical to the progression of TVX/LPS-induced liver injury (Fig. 6.3), likely at least in part, via hepatic fibrin deposition.

TVX enhanced the LPS-induced increase in the plasma concentration of TNFα, which appears to be a central player in TVX/LPS-induced liver injury. TVX/LPS-induced increases in the plasma concentrations of VEGF (Fig. 4.2), chemokines (Fig. 4.3), PAI-1 (Fig. 4.5), IL-18 (data not shown) and IFNγ (Fig. 4.2) were TNFα-dependent. Due to their TNFα-dependence and because TNFα was enhanced before these factors, they are likely downstream of TNFα. Furthermore, TNFα and IFNγ could have directly caused hepatocellular death which might be enhanced by TVX.

Likely proinflammatory cycles were identified, inasmuch as several factors were found which upregulate one another. The removal or inhibition of VEGF (Fig. 7.4), PAI-1 (Fig. 6.7) or IFNγ (Fig. 5.8) attenuated the TVX/LPS-induced increase in plasma concentration of TNFα. Conversely, TNFα neutralization reduced the appearance of each of these. Similarly, IFNγ$^{-/-}$ mice had a reduced plasma concentration of IL-18 (Fig. 5.8) following TVX/LPS coexposure, and IL-18 is a known inducer of IFNγ expression. PAI-1 also had other proinflammatory properties beyond upregulating TNFα, such as increasing plasma concentrations of chemokines and IFNγ (Fig. 6.7) after TVX/LPS administration. These vicious proinflammatory cycles are summarized in Fig. 8.2. Several of these properties

found were novel and the inflammatory cycles of upregulation have the potential to be involved in the pathogenesis.

Fig. 8.2. Possible proinflammatory cycles induced by TVX/LPS coexposure. Based on the results obtained, there exist several possible loops of uncontrolled upregulation. These vicious cycles of inflammation could be involved in TVX/LPS-induced liver injury. See Section 8.2 for additional information.

IL-18
IFNγ
TNFα
PAI-1
VEGF

Section 8.3 Major findings and implications

1. TVX, but not LVX interacts with an inflammatory stress to cause liver injury in mice. This observation had been reported in rats (58). However, the development of hepatotoxic TVX-inflammation interaction in mice demonstrates that the phenomenon is not species-specific and might be extrapolated to TVX IADRs in humans. Furthermore, the degree of TVX/LPS-induced liver injury was much greater in mice (Fig. 2.1) compared to rats (58). Both moderate and severe hepatotoxic responses have been reported in people who took TVX (206). The robustness of the murine model of liver injury resembles the severe hepatotoxicity caused by TVX in humans more so than the rat model. Additionally, that TVX, but not LVX interacts with an inflammatory stress to cause hepatotoxicity suggests that this model could distinguish between fluroquinolones based on their propensity to cause IADRs. This suggests that the drug/inflammation animal model could potentially be used as a preclinical tool to help select a drug candidate based on its potential IADR liability.

2. TVX interacted with an inflammatory stress induced by either gram-negative or gram-positive stimuli. That TVX interacted with either TLR2- or TLR4-activating ligands to cause liver injury proves that TVX/inflammation-induced liver injury is not specific to TLR4 activation. Indeed, it suggests that TVX interacts with an inflammatory stress,

regardless of its source, to precipitate liver injury. The result suggests that inflammatory stress induced by either gram-positive or gram-negative bacteria might play a role in TVX hepatotoxicity. This is of particular importance inasmuch as TVX was approved for treating both gram-positive and gram-negative bacterial infections by the FDA.

3. TVX/LPS-coexposure resulted in unique gene expression changes in mice prior to the onset of liver injury. These results suggest that global gene expression change is an earlier marker of liver toxicity than plasma ALT activity in this model. It is possible that gene expression analysis could be used to identify drugs with IADR liability when coadministered with LPS, even if the coexposure does not result in hepatotoxicity. It is possible that in the drug/inflammation model gene expression analysis offers a more sensitive endpoint to filter out than liver injury to identify drug candidates with IADR liability.

4. TVX/LPS-induced liver injury was dependent on PMN activation, TNFα, IFNγ, thrombin activation, PAI-1 and VEGF. It is possible that these mediators of inflammatory liver injury are involved in the pathogenesis of TVX-induced hepatotoxicity in people.

5. TVX/LPS-induced liver injury is dependent on both TNF receptors. The p55 receptor has been well studied, but the role of the p75 receptor in inflammatory liver injury is unclear. The p75 receptor is not involved in some models of liver injury that are dependent on TNFα (96, 240) but is involved in others (234-236). Indeed, $p75^{-/-}$ mice were protected to a greater degree than $p55^{-/-}$ mice from TVX/LPS-induced liver injury. Therefore, TVX/LPS coexposure would be an ideal model to use for further studies examining the role of the p75 receptor in drug-induced liver injury.

6. The comprehensive studies exploring the roles of TNFα, IFNγ, PAI-1 and VEGF following TVX/LPS coexposure led to the finding that several proinflammatory cycles appear to be involved in the pathogenesis. It is possible that they result in an unregulated inflammatory response resulting in host tissue injury. These proinflammatory cycles could be involved in numerous models of inflammatory tissue injury and might not be specific to the liver or to TVX/LPS coexposure.

Section 8.4 Knowledge gaps and future studies

Several important findings were identified by this research including the development of an animal model of TVX-induced hepatotoxicity. It is possible that the drug/inflammation model could be used preclinically to identify drugs with the propensity to cause IADRs. Further studies with additional fluoroquinolones would provide more positive and negative comparators and would add merit to the model. Additionally, extensive studies with other drugs linked with IADRs in people would be needed to validate the model. It is essential to determine the rate of false negatives and false positives identified by the model before any widescale use in drug development.

The findings presented elucidated some of the mechanisms involved in TVX/LPS-induced liver injury. However, the mechanism by which TVX interacts with TLR2 and TLR4 activation to cause liver injury is still unknown. Future studies will examine signaling pathways initiated by TLR activation to determine if TVX pretreatment alters the initial signaling pathways. The identification of specific pathways might lead to the finding of a specific enzyme or adapter protein which TVX interacts with.

Whether TVX affects hepatocytes directly was not examined. It is possible that TVX directly sensitizes to insults such as PMN proteases, hypoxia, TNFα or IFNγ. A direct sensitization of hepatocytes by TVX might account for the extensive liver injury caused by TVX/LPS coexposure.

TVX treatment prior to a nonhepatotoxic dose of recombinant murine TNFα resulted in significant liver injury. Other studies have shown that TNFα by itself does not cause liver injury in mice but can when administered with galactosamine or a DNA synthesis inhibitor (249, 250). Indeed, the hepatocellular lesions in TVX/TNFα-treated mice appear similar to those seen after galactosamine/TNFα coexposure, suggesting commonalities in mechanisms (251). This raises the possibility that TVX affects DNA synthesis of eukaryotic cells, rendering them sensitive to inflammatory stress. Additional studies need to be done to determine if TVX interacts with eukaryotic cells to affect DNA synthesis to a greater degree than other fluoroquinolones. This could provide understanding of the mechanism by which TVX causes liver injury in people, whereas other fluoroquinolones do not.

Another focus which requires further research is examination of specific cell types. Neutrophil activation was not measured. Furthermore, it is unknown whether any of the cytokines involved in the pathogenesis affect PMN activation. It is possible that they cause liver injury, at least in part, by enhancing PMN activation. Additionally, it is unknown whether cell types other than PMNs are involved in TVX/LPS-induced hepatotoxicity. It is possible that T cells, NK cells, platelets and Kupffer cells are involved in the pathogenesis, and requires further examination.

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