

Lipopolysaccharide and Trovafloxacin Coexposure in Mice Causes Idiosyncrasy-Like Liver Injury Dependent on Tumor Necrosis Factor-Alpha

Patrick J. Shaw, Marie J. Hopfensperger, Patricia E. Ganey, and Robert A. Roth¹

Department of Pharmacology and Toxicology, National Food Safety and Toxicology Center, Center for Integrative Toxicology, Michigan State University, East Lansing, Michigan 48824

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Idiosyncratic adverse drug reactions (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 lipopolysaccharide (LPS) renders mice sensitive to trovafloxacin (TVX), a fluoroquinolone antibiotic linked to idiosyncratic hepatotoxicity in humans and if the cytokine tumor necrosis factor- α (TNF α) 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 levofloxacin (LVX), a fluoroquinolone without liability for causing IADRs in humans, was not hepatotoxic. Measurements of TNF α 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 TNF α transcription or etanercept to inhibit TNF α 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 TNF α .

Key Words: trovafloxacin; inflammation; liver toxicology; adverse drug reactions; cytokines; mechanisms of systems toxicology; idiosyncratic reactions.

Drug-induced liver injury is the leading cause of acute liver failure in the United States (Ostapowicz *et al.*, 2002). Some of these hepatotoxic responses are classified as idiosyncratic adverse drug reactions (IADRs). IADRs are usually unrelated

to the pharmacology of the drug and do not demonstrate obvious dose or time dependence. They occur in a small fraction of people and, therefore, are not seen in typical clinical trials with a limited number of people. The mechanisms of IADRs are unknown, and current preclinical testing does not predict which drugs will cause them.

Among drugs that cause IADRs is trovafloxacin (TVX). TVX is a broad-spectrum fluoroquinolone antibiotic with an extended half-life that allows for once-a-day dosing (Child *et al.*, 1995; Eliopoulos *et al.*, 1993; Melnik *et al.*, 1998). It was approved for use in the United States by the Food and Drug Administration in late 1997. From the time of the drug's launch in February 1998 to the initial reports of hepatotoxicity in 1999, over 2 million prescriptions were filled. In June 1999, the use of TVX was severely restricted due to liver toxicity. The limitations on TVX usage were in response to the strong association with TVX usage in 14 cases of hepatotoxicity, including six deaths and four patients who required liver transplantation (Ball *et al.*, 1999). Additionally, several less severe cases of hepatotoxicity were reported with TVX usage. The timing of the hepatotoxicity in relation to the duration of drug use was variable, and the toxicity was not associated with other fluoroquinolone antibiotics, such as levofloxacin (LVX) (De and De, 2001). The low incidence, sporadic occurrence, and toxicity unrelated to pharmacologic action classify TVX hepatotoxicity as an IADR.

It has been suggested that inflammatory stress might be a factor involved in IADRs in humans. Occurrences of mild systemic inflammatory episodes in people are commonplace and could play a role in lowering the threshold for toxicity of xenobiotic agents, thereby precipitating a toxic response (Ganey *et al.*, 2004). Such inflammation-drug interaction models in rats mimic human IADRs in that drugs that cause human IADRs are rendered toxic in rats by cotreatment with lipopolysaccharide (LPS), which induces inflammation. This has been demonstrated for several drugs, including ranitidine, chlorpromazine, diclofenac, and TVX (Buchweitz *et al.*, 2002; Deng *et al.*, 2006; Luyendyk *et al.*, 2003; Waring *et al.*, 2006).

¹ To whom the correspondence should be addressed at the Department of Pharmacology and Toxicology, National Food Safety and Toxicology Center, Center for Integrative Toxicology, 221, Food Safety and Toxicology Building, Michigan State University, East Lansing, MI 48824. Fax: (517) 432-2310. E-mail: rothr@msu.edu.

Inflammation-drug interaction leading to hepatotoxicity has not been demonstrated in mice. The development of such an IADR model in mice would have several benefits. It would show that this phenomenon is not specific to the rat. Additionally, cross-species comparison might identify common factors and mechanisms which could be extrapolated to humans. Thirdly, the availability of genetically modified mice provides avenues to explore mechanisms by which the responses occur.

One mediator of inflammation is tumor necrosis factor- α (TNF α), the production and release of which can be stimulated by LPS and other bacterial products. It is involved as a critical factor in various models of liver injury, including ischemia/reperfusion and endotoxemia (Colletti *et al.*, 1990; Zhang, 1990). In this study, we developed a model of TVX/LPS-induced liver injury in the mouse and compared TVX/LPS-induced liver injury to previous results obtained in the rat. We also tested the hypothesis that TNF α is critically involved in the development of TVX/LPS-induced liver injury.

MATERIALS AND METHODS

Materials. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St Louis, MO). LPS derived from *Escherichia coli* serotype O55:B5 was used for these studies. Lot 024K4067 with activity of 9.2×10^6 EU/mg was used for the experiments represented in Figures 1–3. Lot 075K4038 with an activity of 3.3×10^6 EU/mg was used for the experiments represented in Figures 4–8. The activity was determined using a colorimetric, kinetic *Limulus* amoebocyte 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 Alcnine aminotransferase (ALT) reagent was purchased from Thermo Electron Corp. (Louisville, CO).

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 Michigan state university Committee on Animal Use and Care.

Experimental protocols. 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 67×10^6 EU/kg or 2.0×10^6 EU/kg (lots 024K4067 or 075K4038, respectively) by ip 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, ip) at various times, and blood was drawn from the vena cava into a syringe containing sodium citrate (final concentration, 0.9%) 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 (PTX) (200 mg/kg) or sterile saline by ip injection 1 h before LPS injection. In other studies, mice were treated with etanercept (8 mg/kg) or sterile water by ip injection either 1 h before LPS injection or 1.5 h after LPS

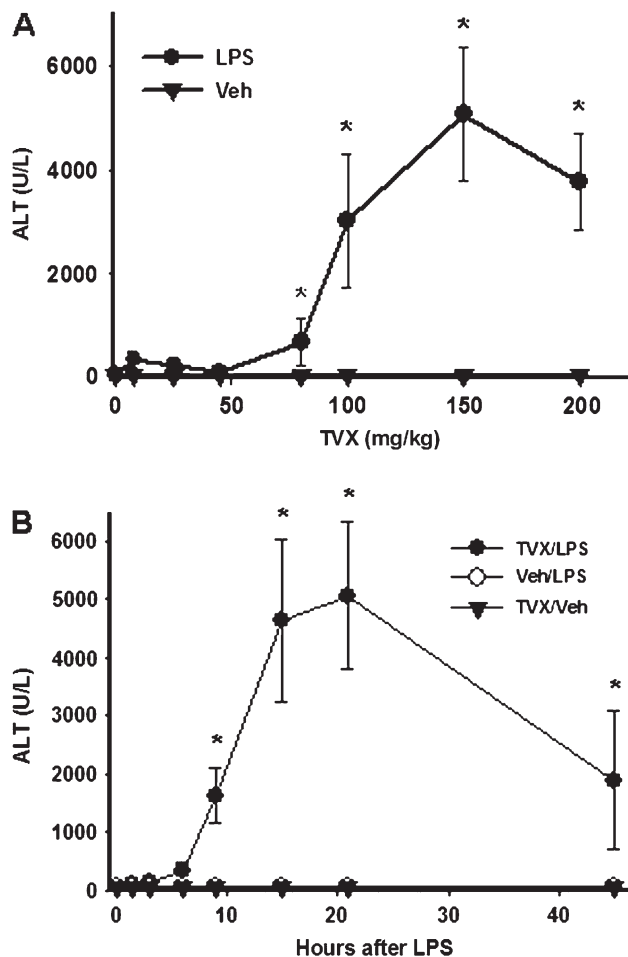


FIG. 1. Dose response and development of liver injury from TVX/LPS cotreatment in mice. (A) Mice were given TVX at various doses (8, 25.3, 45, 80, 100, 150, or 200 mg/kg; po) and then 3 h later LPS (67×10^6 EU/kg; ip) or Veh (sterile saline). Hepatic parenchymal cell injury was estimated 15 h after LPS administration from increases in plasma ALT activity. $n = 4-9$ animals per group. *Significantly different from Veh-treated group. (B) Mice were treated with TVX (150 mg/kg; po) or Veh and then 3 h later with LPS (67×10^6 EU/kg; ip) or Veh. Plasma ALT activity at various times after LPS dosing is depicted. $n = 4-6$ animals per group. *Significantly different from 0 h group.

dosing. Etanercept (Enbrel, Amgen Pharmaceuticals Thousand oaks, CA) was purchased from the Michigan State University Pharmacy (East Lansing, MI).

Histopathology. Formalin-fixed left lateral liver lobes were embedded in paraffin, sectioned at 5 μ m, stained with hematoxylin and 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.

TNF α analysis. The plasma concentrations of TNF α 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).

Statistical analyses. Results are presented as mean \pm SEM. A 1-, 2-, or 3-way ANOVA was used as appropriate after data normalization. For the TVX/LPS time course (Fig. 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.

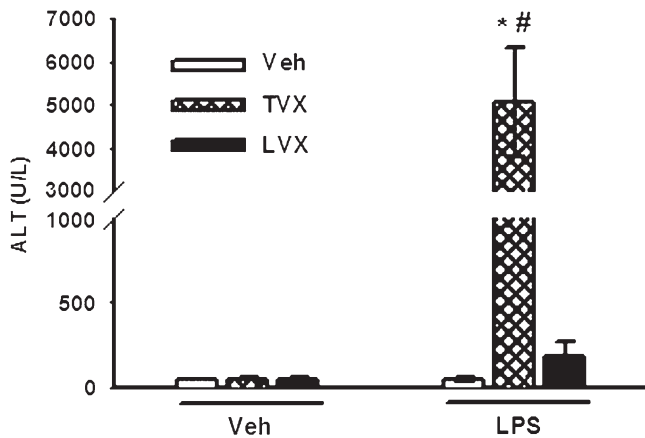


FIG. 2. Comparison of TVX/LPS with LVX/LPS coadministration. TVX (150 mg/kg), LVX (375 mg/kg), or Veh (saline) was administered by oral gavage and 3 h later LPS (67×10^6 EU/kg; ip) or Veh was administered. Mice were anesthetized and sacrificed 15 h after LPS administration. Hepatic parenchymal cell injury was estimated as increases in plasma ALT activity. $n = 4-6$ animals per group. *Significantly different from Veh/Veh. #Significantly different from Veh/LPS.

RESULTS

Dose Response and Time Course of Liver Injury

Administration of LPS after TVX caused a significant increase in plasma ALT activity in a TVX dose-dependent manner (Fig. 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. 1B).

Comparison of TVX and LVX

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 (Girard *et al.*, 1995; Sokol *et al.*, 2002) and the same is true for LVX (Croom and Goa, 2003; Onyeji *et al.*, 1999). We chose a dose of LVX (375 mg/kg) to keep the dose ratio of TVX/LVX similar to the ratio of doses used clinically in humans (Lubasch *et al.*, 2000). LVX, TVX, or Veh were 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,

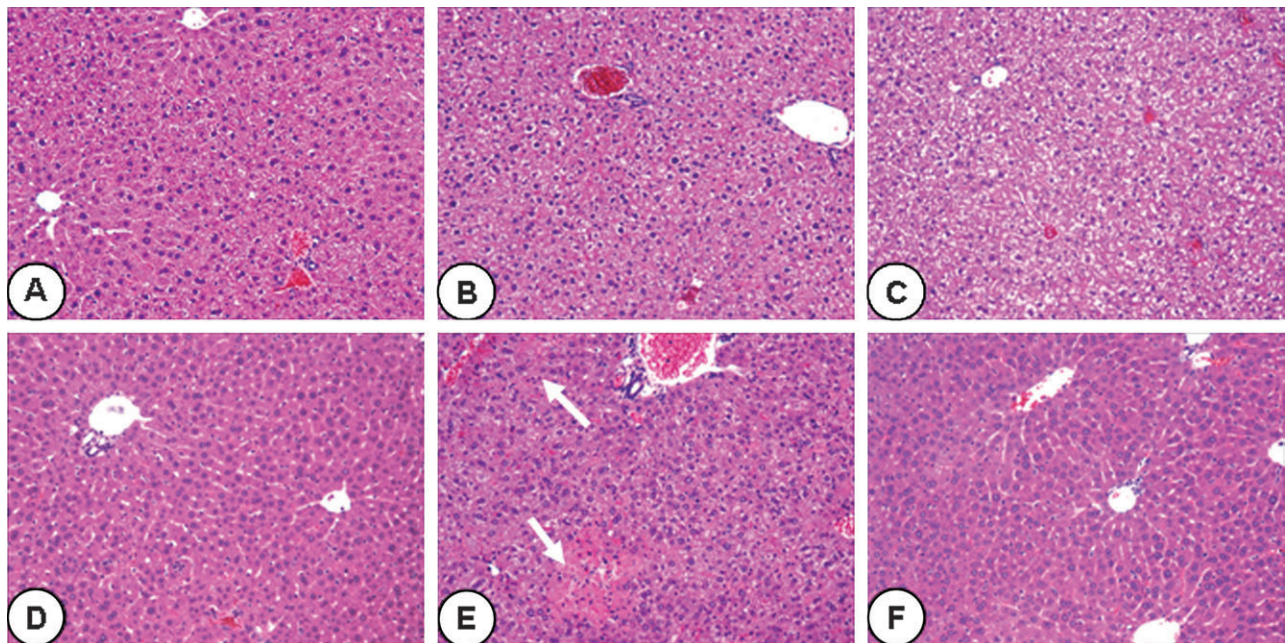


FIG. 3. Liver histopathology in mice cotreated with LPS and either TVX or LVX. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg), or Veh (po) and then dosed with LPS (67×10^6 EU/kg; ip) or Veh. Liver sections are from mice treated with Veh/Veh (A), TVX/Veh (B), LVX/Veh (C), Veh/LPS (D), TVX/LPS (E), and LVX/LPS (F). 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.

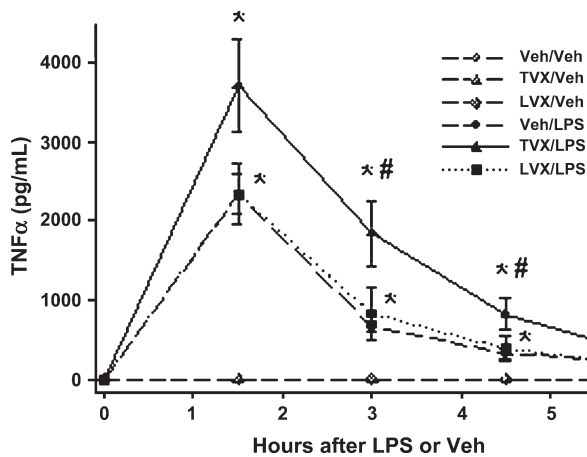


FIG. 4. Time course of TNF α concentration in the plasma of treated mice. Mice were treated with TVX (150 mg/kg), LVX (375 mg/kg), or Veh (po) and then dosed with LPS (2×10^6 EU/kg; ip) or Veh 3 h later. $n = 3$ –10 animals per group. *Significantly different from the same treatment group at 0 h. #Significantly different from Veh/LPS-treated mice at the same time.

LVX, or LPS were all nontoxic when administered alone (Fig. 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 (Figs. 3A–C, respectively). Histopathological examination of livers from TVX/LPS-cotreated mice (Fig. 3E) revealed hepatocellular necrosis, which was not seen in Veh/LPS- (Fig. 3D) or LVX/LPS-treated mice (Fig. 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 time course as was seen for ALT activity in the plasma (data not shown).

Time course of TNF α 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 1–3. The dose used for these studies was 2×10^6 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 h after LPS (data not shown). LPS-treated groups showed a significant increase in plasma TNF α concentration at all times measured (Fig. 4). TVX administered prior to LPS caused greater elevation of TNF α 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 TNF α concentration.

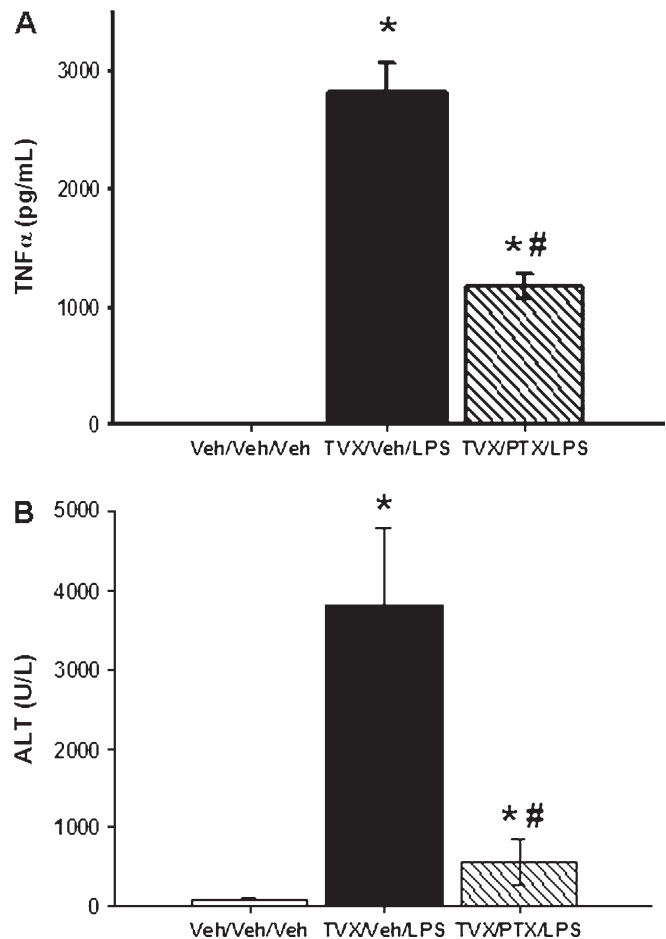


FIG. 5. The effect of PTX on LPS-induced TNF α expression and TVX/LPS-induced liver injury. (A) Mice were treated with PTX (200 mg/kg; ip) or Veh (saline) 1 h prior to LPS. Mice were sacrificed 1.5 h after LPS treatment, and plasma TNF α concentration was measured. (B) TVX (150 mg/kg) or Veh was administered by oral gavage, followed by PTX or Veh 2 h later. LPS (2×10^6 EU/kg; ip) 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 per group. *Significantly different from Veh/Veh/Veh control. #Significantly different from TVX/Veh/LPS treatment group.

PTX Study

As mentioned above, ALT activity was increased in TVX/LPS-cotreated mice 4.5 h after LPS administration, and plasma TNF α 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. PTX is a nonspecific phosphodiesterase inhibitor that inhibits LPS-induced TNF α production by increasing cyclic adenosine 3',5'-monophosphate (cAMP) in monocytes/macrophages. The increase in cAMP inhibits the translocation and activation of Nf κ B, which controls TNF α expression (Witkamp and Monshouwer, 2000). A dose of PTX 200 mg/kg (ip) given 1 h before LPS administration significantly decreased plasma TNF α concentration 1.5 h after LPS treatment (Fig. 5A). This dose of PTX significantly reduced TVX/LPS-induced liver injury, as estimated

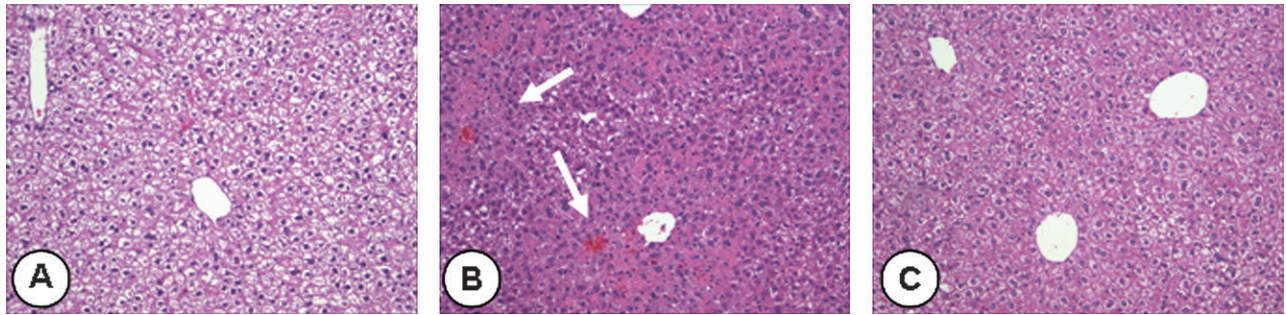


FIG. 6. Effect of PTX on TVX/LPS-induced liver pathology. Mice were treated as described in Figure 5 and sacrificed 15 h after LPS. Liver sections from mice treated with Veh/Veh/Veh (A), TVX/Veh/LPS (B), and TVX/PTX/LPS (C) were examined. The arrows indicate randomly distributed, variably sized foci 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).

by plasma ALT activity 15 h after LPS dosing (Fig. 5B). TVX/Veh/LPS-treated livers had much less glycogen deposition and had foci of midzonal hepatocellular necrosis compared to vehicle-treated control mice (Figs. 6A and 6B, 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. 6C).

Etanercept Inhibition of TNF α Activity

Etanercept is a recombinant, human soluble TNF α receptor that inhibits TNF α activity. An etanercept dose of 8 mg/kg (ip) caused a significant decrease in plasma TNF α concentration in TVX/LPS-treated mice at 4.5 h after LPS administration (Fig. 7A). This dose of etanercept administered 1 h before LPS completely protected mice from the TVX/LPS-induced increase in plasma ALT activity (Fig. 7B) and from hepatocellular necrosis (Fig. 8). The TVX/LPS-treated mice consistently had midzonal and centrilobular foci of coagulative necrosis which were not observed when etanercept was administered (Figs. 8B and 8C, respectively).

In an attempt to determine if the prolongation of the LPS-induced plasma TNF α peak by TVX pretreatment (Fig. 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 TNF α concentration had peaked). Etanercept administration at this time provided significant reduction in TVX/LPS-induced liver injury (Fig. 9).

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 (Utrecht, 2003). 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

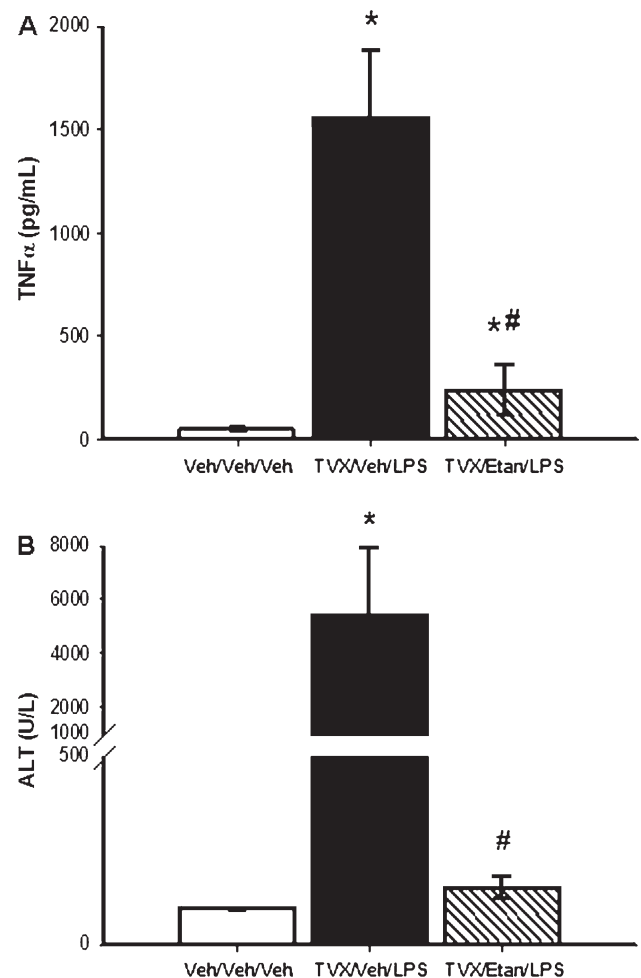


FIG. 7. The effect of etanercept on TVX/LPS-induced TNF α expression and liver injury. (A) TVX (150 mg/kg) or Veh was administered by oral gavage followed by etanercept (8 mg/kg; ip) or Veh 2 h later. LPS (2×10^6 EU/kg; ip) was given 1 h after etanercept dosing. Mice were sacrificed 4.5 h after LPS treatment, and plasma TNF α 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 per group. *Significantly different from Veh/Veh/Veh control group. #Significantly different from TVX/Veh/LPS treatment group.

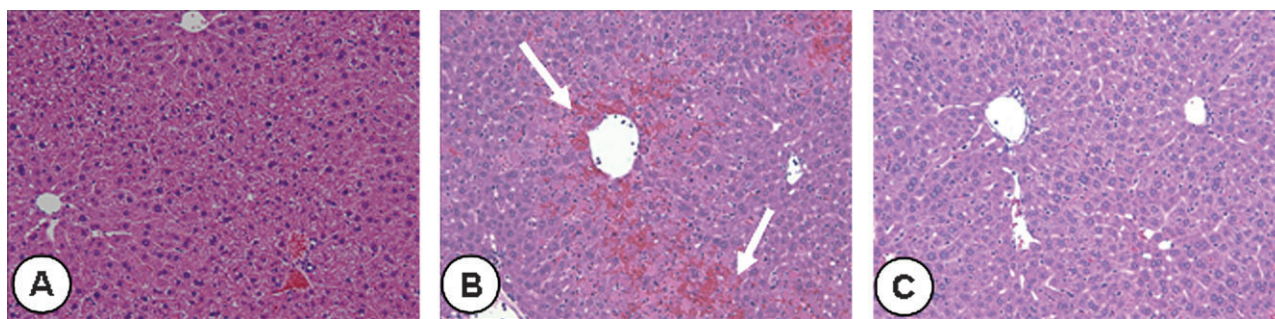


FIG. 8. Effect of etanercept on TVX/LPS-induced liver pathology. Mice were treated as described in Figure 7 and sacrificed 15 h after LPS. Liver sections from mice treated with Veh/Veh/Veh (A), TVX/Veh/LPS (B), and TVX/etanercept/LPS (C) were examined. The arrows indicate randomly distributed, variably sized foci of coagulative necrosis and hemorrhage seen only in TVX/Veh/LPS-treated mice.

from human hepatocytes that TVX can cause oxidative stress and mitochondrial damage (Liguori *et al.*, 2005). 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* (Waring *et al.*, 2006). Thus, mechanisms of TVX-mediated liver injury remain unknown.

In rats, the TVX/LPS interaction was found to precipitate a hepatotoxic response (Waring *et al.*, 2006). 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 *iv* 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 *iv* 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 (Ng *et al.*, 1999; Teng *et al.*, 1996). 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 compared to rats (Waring *et al.*, 2006). 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 (Nightingale, 1999). 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 (Ng *et al.*, 1999; Teng *et al.*, 1995). TVX binding to serum proteins is greater in rats compared to humans, 92 versus 70%, respectively (Teng *et al.*, 1995, 1996). 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 and histopathological examination. 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 (Ernst *et al.*, 1997). Another possible explanation for the selective hepatotoxicity with TVX/LPS coexposure is that TVX is more potent against gastrointestinal (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 TNF α ;

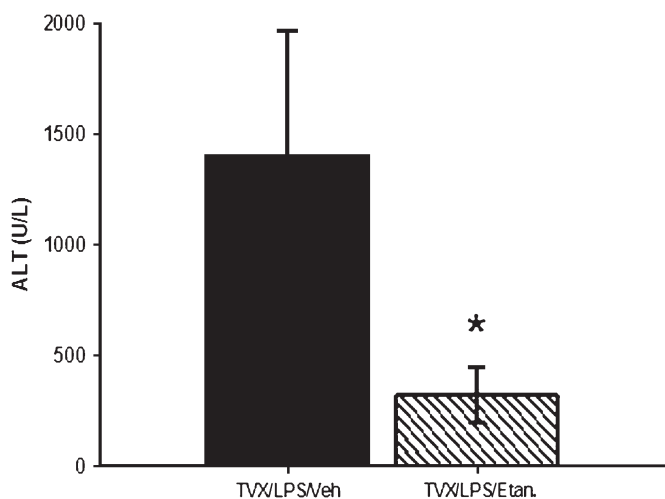


FIG. 9. Effect of etanercept treatment given at the peak of plasma TNF α on TVX/LPS-induced liver injury. Mice were treated with TVX (150 mg/kg; *po*) 3 h before LPS (2×10^6 EU/kg; *ip*). Mice were then treated with etanercept (8 mg/kg; *ip*) 1.5 h after LPS dosing and sacrificed 15 h after LPS; ALT activity was measured in the plasma. $n = 5$ animals per group. *Significantly different from TVX/LPS/Veh-treated group.

however, neither of the drugs alone caused such an increase. Moreover, LVX failed to enhance the TNF α response to LPS administration 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 TNF α concentration.

TVX pretreatment selectively prolonged the LPS-induced plasma TNF α peak before and during the onset of liver injury. TNF α is critically involved in several models of liver injury including ischemia/reperfusion and endotoxemia (Colletti *et al.*, 1990; Tiegs *et al.*, 1989). To explore the role of TNF α , both PTX and etanercept were used to inhibit TNF α activity. PTX pretreatment provided significant protection from TVX/LPS-induced liver injury. In addition to inhibiting TNF α , PTX has several other effects including preventing platelet aggregation, decreasing other proinflammatory cytokines, and inhibiting hepatic fibrogenesis (Windmeier and Gressner, 1997). Accordingly, a more selective inhibitor was also used.

Etanercept is a recombinant human soluble TNF α receptor which specifically neutralizes the activity of TNF α . Pretreatment with etanercept completely protected mice from TVX/LPS-induced liver injury. Additionally, etanercept administration at a later time to eliminate the prolongation by TVX of the LPS-induced plasma TNF α peak also provided protection. Thus, the prolonged TNF α 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 TNF α (0–1.5 h after LPS). Whether TNF α directly causes hepatotoxicity or acts indirectly through other mediators will require further investigation.

The cellular sources of TNF α in this model have not been explored. In the liver, Kupffer cells can be stimulated by LPS to release TNF α and other cytokines. These cells are an important source of TNF α in several models of liver injury (Kiemer *et al.*, 2002; Tsukada *et al.*, 2003) 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 (Khan *et al.*, 2000). 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 (as was done by Khan *et al.*, 2000), 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 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 TNF α concentrations induced by LPS in mice (Khan *et al.*, 2000; Purswani *et al.*, 2000). These results contrast with data presented in Figure 4. The difference in results could be due to different strains of mice, doses of LPS (lethal vs. non-hepatotoxic), or different treatment protocols, in which TVX was given 1 h (Khan *et al.*, 2000) or 3 h (data presented here) before LPS. In that study, TVX alone increased plasma TNF α concentration, an effect not observed in our study. The plasma concentration of TNF α in control mice was reported to be 1.4 ± 0.5 ng/ml (Khan *et al.*, 2000), a value that is extremely high for normal mice and might reflect an ongoing inflammatory response in their control animal.

It has also been reported that alatrofloxacin, a prodrug of TVX, decreased LPS-stimulated expression of TNF α mRNA and protein *in vitro* in human peripheral blood mononuclear cells (PBMCs) (Purswani *et al.*, 2000), a result that contrasts with our findings. Interestingly, in rats cotreated with TVX and LPS, mRNA for TNF α in liver was not increased, but mRNA for TNF-induced protein was elevated (Waring *et al.*, 2006), suggesting a transcription-independent mechanism for increasing TNF α protein. It is also possible that Kupffer cells, a major source of TNF α in the liver, respond differently to the interaction of TVX with LPS than human PBMCs with respect to TNF α 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. Our treatment regimen 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 TNF α in the plasma. The increase in TNF α 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.

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