The Role of Tumor Necrosis Factor Alpha in Lipopolysaccharide/ Ranitidine-Induced Inflammatory Liver Injury

Francis F. Tukov,*† James P. Luyendyk,*† Patricia E. Ganey,*† and Robert A. Roth*††

*Center for Integrative Toxicology and †Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824

Received March 20, 2007; accepted July 26, 2007

Exposure to a nontoxic dose of bacterial lipopolysaccharide (LPS) increases the hepatotoxicity of the histamine-2 (H2) receptor antagonist, ranitidine (RAN). Because some of the pathophysiologic effects associated with LPS are mediated through the expression and release of inflammatory mediators such as tumor necrosis factor alpha (TNF), this study was designed to gain insights into the role of TNF in LPS/RAN hepatotoxicity. To determine whether RAN affects LPS-induced TNF release at a time near the onset of liver injury, male Sprague-Dawley rats were treated with 2.5 × 10^6 endotoxin units (EU)/kg LPS or its saline vehicle (iv) and 2 h later with either 30 mg/kg RAN or sterile phosphate-buffered saline vehicle (iv). LPS administration caused an increase in circulating TNF concentration. RAN cotreatment enhanced the LPS-induced TNF increase before the onset of hepatocellular injury, an effect that was not produced by famotidine, a H2-receptor antagonist without idiosyncrasy liability. Similar effects were observed for serum interleukin (IL)-1β, IL-6, and IL-10. To determine if TNF plays a causal role in LPS/RAN-induced hepatotoxicity, rats were given either pentoxifylline (PTX; 100 mg/kg, iv) to inhibit the synthesis of TNF or etanercept (Etan; 8 mg/kg, sc) to impede the ability of TNF to reach cellular receptors, and then they were treated with LPS and RAN. Hepatocellular injury, the release of inflammatory mediators, hepatic neutrophil (PMN) accumulation, and biomarkers of coagulation and fibrinolysis were assessed. Pretreatment with either PTX or Etan resulted in the attenuation of liver injury and diminished circulating concentrations of TNF: IL-1β, IL-6, macrophage inflammatory protein-2, and coagulation/fibrinolysis biomarkers in LPS/RAN-cotreated animals. Neither PTX nor Etan pretreatments altered hepatic PMN accumulation. These results suggest that TNF contributes to LPS/RAN-induced liver injury by enhancing inflammatory cytokine production and hemostasis.

Key Words: tumor necrosis factor alpha; inflammation; liver injury; lipopolysaccharide; ranitidine; adverse drug reactions; coagulation; hemostasis; hepatotoxicity.

Previous studies in rats showed that modest inflammation triggered by bacterial lipopolysaccharide (LPS) decreases the threshold for xenobiotic hepatotoxicity (reviewed in Roth et al., 1997). One of the xenobiotics for which this is true is the histamine-2 (H2) receptor antagonist, ranitidine (RAN; Luyendyk et al., 2003). RAN has been associated with idiosyncratic adverse drug reactions (IADRs) in people, with the liver as a frequent target. Idiosyncratic hepatotoxicity occurs in less than 0.1% of people taking RAN (Vial et al., 1991), and most RAN-induced liver reactions are mild and reversible (Ribeiro et al., 2000). In contrast, another H2-receptor antagonist, famotidine (FAM), is not associated with idiosyncratic hepatotoxicity in humans: very few reports of FAM-associated hepatotoxicity have been published (Ament et al., 1994; Hashimoto et al., 1994; Jimenez-Saenz et al., 2000), and the contribution of FAM to liver injury in these cases has been questioned (Luyendyk et al., 2003).

Although it did not produce liver injury in healthy experimental animals, RAN (30 mg/kg) was rendered hepatotoxic in rats undergoing a mild inflammatory response induced by LPS (Luyendyk et al., 2003). By contrast, a dose of FAM that was equal in pharmacological efficacy (6 mg/kg) did not interact with LPS to produce liver injury. LPS/RAN-treated animals developed midzonal hepatocellular necrosis and a liver-related clinical chemistry pattern resembling human cases of RAN idiosyncrasy. Hepatocellular oncocytic necrosis in LPS/RAN-treated rats was preceded by hepatic neutrophil (PMN) accumulation, and depletion of circulating PMNs attenuated LPS/RAN-induced liver injury, suggesting a critical role for these cells in this response (Luyendyk et al., 2005). In other studies, activation of the coagulation system was shown to be crucial to the pathogenesis of LPS/RAN hepatotoxicity (Luyendyk et al., 2004b).

These results support the hypothesis that inflammation and coagulation initiated by exposure to small amounts of LPS are involved in the mechanism of liver injury from LPS/RAN cotreatment. Evidence from both in vivo and in vitro studies suggests that tumor necrosis factor alpha (TNF) is a critical and proximal mediator of the inflammatory and hemostatic pathways stimulated by LPS (Aderka et al., 1992; Beutler and Cerami, 1988; van der Poll et al., 1997). Furthermore, at a
relatively larger dose of LPS, liver injury is caused by complex mechanisms involving the interaction of numerous soluble mediators such as TNF and inflammatory cells, including Kupffer cells (KCs), which are thought to be the major source of TNF in liver (Ganey and Roth, 2001; Hewett et al., 1992, 1993). These findings raise the possibility that TNF contributes to liver damage caused by cotreatment with LPS and RAN.

The purpose of this study was to characterize the expression of inflammatory mediators during LPS/RAN interaction in vivo and in vitro and to determine the role of TNF in the ability of LPS to render a nontoxic dose of RAN hepatotoxic. Toward these ends, the effects of RAN and LPS on production of TNF and other inflammatory factors in rats and in KC/hepatocyte (KC/HPC) cocultures were determined and compared with those elicited by FAM. Finally, by neutralizing TNF, the hypothesis that TNF is a crucial mediator in LPS/RAN-induced hepatotoxicity was tested.

MATERIALS AND METHODS

Materials. LPS derived from Escherichia coli serotype O55:B5 (Sigma Chemical Co., St Louis, MO) with an activity of $1 \times 10^{8}$ endotoxin units (EU)/mg was used for these studies. This activity was determined using a limulus amebocyte lysate colorimetric end-point assay (kit 50-6480) purchased from Cambrex Corp. (East Rutherford, NJ). Pentoxifylline (PTX), and fetal bovine serum (FBS) were obtained from Sigma Chemical Co. Etoanercept (Etan; Enbrel; Immunex Corporation, Thousand Oaks, CA) was obtained from the Michigan State University Clinical Center Pharmacy. Antibiotic/antimycotic solution, gentamicin and RPMI 1640 (supplemented with 2mM l-glutamine) were purchased from Gibco BRL (Rockville, MD). The WEHI-13VAR mouse fibroblast cell line was obtained from ATCC (Manassas, VA). Unless otherwise stated, all other materials were purchased from Sigma Chemical Co.

Animals. Male Sprague-Dawley rats (Crl:CD [SD]IGS BR; Charles River, Portage, MI) weighing 250–350 g were used for these studies. Animals were fed standard chow (Rodent ChowTek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum. They were allowed to acclimate for 1 week in a 12-h light/dark cycle before use. All procedures on animals followed the guidelines for humane treatment set by the American Association of Laboratory Animal Sciences and the University Laboratory Animal Research Unit at Michigan State University.

Assessment of the effects of RAN on LPS-induced TNF release in vivo. Rats fasted for 24 h were given $2.5 \times 10^{8}$ EU/kg LPS or its saline vehicle, iv via a tail vein. Two hours later, 30 mg RAN/kg or sterile phosphate-buffered saline (PBS) vehicle was administered through a tail vein. At 0, 2, 3, 6, or 12 h after RAN administration, rats were anesthetized with sodium pentobarbital (50 mg/kg, ip) and killed by exsanguination. For the collection of plasma, blood was collected into vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ, USA) containing sodium citrate (final concentration, 3.8%). Remaining blood was allowed to clot at room temperature, and serum was collected and stored at −80°C until use. TNF concentration was determined by enzyme-linked immunosorbent assay (ELISA; see below). In a separate study to examine inflammatory mediator release before the onset of injury, rats were treated as above except that a group given 6 mg/kg FAM instead of RAN was included, and the animals were killed 1, 2, or 3 h later. For these studies, RAN and FAM were administered at doses that have equal pharmacological efficacy in humans (Lin, 1991; Scarpignato et al., 1987) to simulate the ratio of doses taken by people. As a result, the dose of RAN was fivefold greater than the dose of FAM. We have shown previously that cotreatment of rats with LPS and a dose of FAM equimolar or equally efficacious to the dose of RAN used in these studies does not result in liver injury (Luyendyk et al., 2003).

Determination of PTX efficacy. PTX, a xanthine oxidase inhibitor, has been used as a tool for exploring the role of TNF in xenobiotox-induced hepatotoxicity (Sneed et al., 2000; Yee et al., 2003). PTX inhibits transcription of TNF mRNA through a mechanism that involves phosphodiesterase inhibition and the elevation of intracellular cyclic adenosine monophosphate (Doherty et al., 1991; Schandene et al., 1992). Rats fasted for 24 h received PTX (100 mg/kg) or its saline vehicle intravenously 1 h before treatment with LPS ($2.5 \times 10^{6}$ EU/kg) or its saline vehicle, iv. This treatment protocol for PTX has been shown to prevent the LPS-induced rise in plasma TNF concentration (Hewett et al., 1993). Two hours after LPS or vehicle exposure, rats were anesthetized with sodium pentobarbital and killed by exsanguination. Blood was allowed to clot at room temperature, and serum was collected and stored at −80°C until use. TNF concentration was determined using ELISA.

Determination of Etaan efficacy. Rats fasted for 24 h received 8 mg Etaan/kg or its sterile water vehicle sc 1 h before LPS ($2.5 \times 10^{6}$ EU/kg) or its saline vehicle. Two hours later, they were anesthetized and bled as described above. This Etaan treatment has been shown to inactivate TNF activity (Geier et al., 2003). A cytolytic cell assay (WEHI assay) was used to measure TNF activity as described previously (Eskandari et al., 1990; Espevik and Nissen-Meyer, 1986; Hewett et al., 1993; Sneed et al., 2000) with slight modifications. Briefly, dilutions of serum samples from rats previously exposed to either veh/ LPS or Etaan/LPS were assayed in triplicate for cytotoxic activity in WEHI-13VAR mouse fibroblast cells seeded at a density of $5 \times 10^{4}$ cells/well in RPMI 1640 medium supplemented with 10% FBS, 1% antibiotic/antimycotic solution, and 50 μg/ml gentamicin. The extent of cell death was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide using a Bio-Tek plate reader. Absorbance was read at 570 nm. A recombinant rat TNF (BD Biosciences, San Diego, CA) was used as standard to calculate TNF activity in the samples.

Assessment of LPS/RAN-induced hepatotoxicity in rats after PTX or Etaan pretreatment. Rats were fasted for 24 h before experiments. One hour before administration of LPS ($2.5 \times 10^{6}$ EU/kg) or its saline vehicle, rats received either PTX (100 mg/kg) or its saline vehicle intravenously or Etaan (8 mg/kg) or its water vehicle sc. Two hours after LPS treatment, they were treated with either 30 mg RAN/kg or its vehicle, and 6 h later they were anesthetized, bled, and killed as described above. Blood was allowed to clot at room temperature, and serum was collected by centrifugation and assayed for alanine aminotransferase (ALT) activity using a diagnostic kit (ALT GPT; Ref: TR7 111-125, Infinity; Thermo Electron, Melbourne, Australia).

Histopathology. Livers were fixed by immersion in 10% neutral-buffered formalin for at least 3 days before being processed for histologic analysis. Formalin-fixed liver samples from the left lateral liver lobe (3 samples/rat) were embedded in paraffin, sectioned at 5 μm, stained for hematoxylin and eosin, and examined by light microscopy for lesion size as described previously by Luyendyk et al., 2003). All tissues were examined without knowledge of treatment. The nature of lesions did not differ qualitatively among groups, and each was assigned a score of 0 (no injury) to 6 based on increasing size of the lesions examined at 100× magnification.

Concentrations of cytokines, plasminogen activator inhibitor-1, and thrombin-antithrombin dimers. Serum TNF concentration was measured using a commercial kit (Rat TNF ELISA Kit II, BD OptEIA; BD Biosciences) with recombinant rat TNF as standard. Measurements were performed in duplicate. The concentrations of interleukin (IL)-1β, IL-6, IL-10, interferon-γ, and macrophage inflammatory protein-2 (MIP-2) in serum were determined using enzyme immunoassay (EIA) kits obtained from BioSource International, Inc. (Camarillo, CA). The assays were performed according to the manufacturer’s instructions, and all samples were assayed in duplicate. The concentration of plasminogen activator inhibitor-1 (PAI-1) in serum was evaluated using an ELISA purchased from American Diagnostica, Inc.
(Greenwich, CT). This ELISA measures total PAI-1 including active, inactive, and tissue plasminogen activator–complexed forms. Plasma thrombin-antithrombin (TAT) concentration was determined using an EIA kit (Enzygnost TAT micro; #OWMG15) from Dade Behring Inc. (Deerfield, IL). Serum concentrations of prostaglandin E$_2$ (PGE$_2$) were determined using a commercially available EIA kit (PGE$_2$ Express EIA Kit; Cayman Chemical, Ann Arbor, MI). EIA was performed according to the manufacturer’s instructions, and all samples were assayed in duplicate.

**Detection of hepatic fibrin.** Fibrin immunohistochemistry and quantification were performed as described previously (Copple et al., 2002; Luyendyk et al., 2004a). Eight micrometer thick sections of frozen liver tissue were fixed in 10% buffered formalin containing 2% acetic acid for 30 min at room temperature. This fixation protocol solubilizes all fibrinogen and fibrin species except for cross-linked fibrin. Sections were blocked with PBS containing 10% horse serum (i.e., blocking solution; Vector Laboratories, Burlingame, CA) for 30 min, and this was followed by incubation overnight at 4°C with goat antirat fibrinogen diluted (1:1000; ICN Pharmaceuticals, Aurora, OH) in blocking solution. Next, sections were incubated for 3 h with donkey anti-goat secondary antibody conjugated to Alexa 594 (1:1000; Molecular Probes, Eugene, OR) in blocking solution for 3 h. Sections were washed three times, 5 min each, with PBS and visualized using an Olympus AX-70T fluorescence microscope (Olympus, Lake Success, NY). Ten randomly chosen digital images (100x magnification) were captured using a SPOT camera and SPOT advanced software (Diagnostic Instruments, Sterling Heights, MI). Samples were coded such that the evaluator was not aware of treatment. Each digital image encompassed a total area of 1.4 mm$^2$ and contained several centrilobular and perportal regions. Quantification of immunostaining was performed with Scion Image Beta 4.0.2 (Scion Corporation, Frederick, MD) using the method described by Copple et al. (2002). Results from 10 random fields analyzed per liver section were averaged and counted as a replicate, that is, each replicate represents a different rat.

**Evaluation of hepatic PMN accumulation.** PMN immunohistochemistry was performed on formalin-fixed liver sections as described previously (Luyendyk et al., 2004a; Yee et al., 2003). Briefly, the paraffin-embedded liver tissue was cut into 6 μm thick slices (three serial liver sections per slide). Paraffin was removed from the liver tissues with xylene before staining. PMNs within liver sections were stained with a rabbit anti-PMN Ig isolated from serum of rabbits immunized with rat PMNs as described by Hewett et al. (1992). After incubation with the primary antibody, tissue sections were incubated with biotinylated goat antirabbit IgG, avidin-conjugated alkaline phosphatase, and Vector Red substrate to stain PMNs. The number of PMNs in 10–20 randomly selected, high-power fields (400x) was counted for each liver. The average of these numbers was calculated, and hepatic PMN accumulation is presented as the mean for each treatment group.

**Isolation and culture of rat HPCs and KCs.** HPCs and KCs were isolated from the same rat liver as described by Tukov et al. (2006). After isolation of HPCs, cell viability was assessed by trypan blue exclusion. If cells were greater than 80% viable, they were kept on ice for later use, at which time the HPCs were plated in 24-well culture plates at a density of 2.5 × 10$^6$ cells/well. Similarly, the viability of the isolated KCs was determined by trypan blue exclusion and was usually >90%. The cell concentration was adjusted to 1 × 10$^6$ viable cells/ml, and the KCs (1 × 10$^6$ cells/well) were plated in 24-well plastic culture plates (Costar, Cambridge, MA) for 20 min at 37°C in a humidified incubator (95% air/5% CO$_2$). After this time, nonadherent cells were removed by replacing the culture medium with fresh complete medium (RPMI 1640 supplemented with 10% FBS, glutamine, 0.1% gentamicin, and 1% antibiotic/antimycotic solution). Isolated KCs were identified after staining using ED1 antibody (mouse anti-rat CD68, Serotec; 1:500 in blocking serum), and a secondary antibody coupled to fluorescein isothiocyanate (Alexa Fluor 594 goat anti-mouse IgM [microchain]; Molecular Probes) as described previously (Tukov et al., 2006). These techniques revealed a purity of ≥90% KCs after differential plating. In experiments using cocultures, HPCs (2.5 × 10$^5$/well) were added after differential plating of KCs, and the plates were returned to the incubator.

**Exposure of KC/HPC cocultures and KC monocultures to test agents.** After the cells were plated as described above, culture medium in KC/HPC cocultures or KC monocultures was replaced with fresh, complete RPMI medium and allowed to incubate overnight at 37°C. The medium was then removed and replaced with fresh medium prior to treatment. Cell cultures were treated with LPS, RAN, FAM, vehicle (culture medium), or a combination of LPS/RAN or LPS/FAM and returned to the incubator for an additional 6 h, after which medium from each well was collected and centrifuged at 9000 × g for 5 min. A portion of the medium was used for the determination of activity of ALT, and the remainder was stored at −80°C for future use.

**Assessment of HPC toxicity in vitro.** ALT activity in culture supernatants was assayed as a measure of HPC injury using a diagnostic kit (ALT (GPT); Ref: TR7 111-125, Infinity; Thermo Electron). Enzyme leakage into the medium was expressed as a percentage of total intracellular ALT content, which was determined in vehicle-treated control cells lysed with Triton X. Experiments were considered valid only if the ALT activity in the medium of vehicle-treated cells was less than 25% of total activity. TNF and PGE$_2$ in culture medium were determined as described above for serum.

**Statistical analysis.** Results are presented as means ± SEM. For these studies, if the variances were homogenous, data were analyzed by Student’s t-test or one-way or two-way ANOVA as appropriate. For data sets with nonhomogenous variances, Kruskal-Wallis nonparametric ANOVA was used or data were log-transformed prior to analysis using Student’s t-test or ANOVA. Data points identified as statistical outliers using Grubb’s test were not included in statistical analyses. For ANOVA, group means were compared using Student-Newman-Keul post hoc test. Histopathology scores were compared using Mann-Whitney rank sum test. For studies in vitro, two-way repeated measures ANOVA was applied after appropriate data transformation, and group comparisons were made using the Student Newman Keulh post hoc test. The criterion for significance was p < 0.05 for all studies.

**FIG. 1.** Time-dependent effect of LPS/RAN cotreatment on serum TNF-α concentration. Rats fasted for 24 h were given 2.5 × 10$^6$ EU/kg LPS or its saline vehicle iv. Two hours after LPS, either 30 mg RAN/kg or its vehicle was administered iv. At 2, 3, 6, or 12 h after RAN administration, rats were killed, and serum TNF-α concentration was determined using ELISA. Data are expressed as mean ± SEM, n = 4–13 rats/group. a, Significantly different from LPS/Veh group at the same time; b, significantly different from Veh/Veh group at the same time; c, significantly different from Veh/RAN group at the same time.
FIG. 2. Early liver injury and inflammatory mediator production after LPS/RAN cotreatment. Rats fasted for 24 h were given 2.5 × 10^6 EU/kg LPS or its saline vehicle iv. Two hours after LPS, 30 mg RAN/kg, a pharmacologically equally efficacious dose of FAM (6 mg/kg) or its vehicle was administered, iv, and rats were killed 1, 2, or 3 h later. Liver injury was assessed from the activity of ALT in serum (A). The concentrations of TNF-α (B), IL-1 beta (C), IL-6 (D), IL-10 (E), PAI-1 (F), interferon-gamma (IFN) (G), and PGE_2 (H) were determined as described in “Materials and Methods” section. Data are expressed as mean ± SEM. n = 4–9 rats/group. a, Significantly different from respective group in the absence of LPS; b, significantly different from respective group in the absence of drug; c, significantly different from respective group in the presence of FAM.
RESULTS

Time-Course of Serum TNF Concentration in LPS/RAN-Cotreated Rats

At the time of RAN administration, the concentrations of TNF in serum of rats treated 2 h earlier with LPS or saline were $32 \pm 1$ and $0.14 \pm 0.05$ ng/ml, respectively. This large LPS-induced increase in TNF is consistent with previous reports (Hewett et al., 1993). Neither vehicle nor RAN alone caused a significant increase in serum TNF concentration over the 12-h examination period (Fig. 1). Serum TNF concentration was greater at all times in LPS-treated rats compared with vehicle-treated rats. RAN cotreatment maintained plasma TNF concentration at significantly greater values compared with rats given LPS alone (Fig. 1). Serum TNF concentrations were approximately twofold greater in LPS/RAN-cotreated animals compared with LPS/veh-treated animals at 2 and 3 h after RAN administration.

Early Inflammatory Mediator Release and Comparison to FAM

Results from the experiment above suggested that RAN enhanced the LPS-induced elevation of serum TNF around the time that hepatocellular injury began to occur (i.e., 2–3 h [Luyendyk et al., 2003, 2004b]). An additional experiment was designed to (1) explore the effect of RAN on LPS-induced TNF release before the onset of hepatocellular injury, (2) compare the TNF-enhancing effects of RAN to those of FAM, which does not interact with LPS to produce liver injury in rats and does not have IADR liability in humans (Luyendyk et al., 2006), and (3) elucidate the effects of RAN on other inflammatory mediators. Rats were treated with a nontoxic dose of LPS or its vehicle followed 2 h later by a non-hepatotoxic dose of RAN, its vehicle, or a dose of FAM that is pharmacologically equally efficacious to that of RAN (Luyendyk et al., 2003). Times were chosen to encompass those before (1 h) and during (2–3 h) the onset of hepatocellular injury. Of the six treatments, only LPS/RAN was hepatotoxic, confirming previous results (Luyendyk et al., 2003, 2006). In this group, serum ALT activity was unchanged 1 h after RAN treatment, slightly elevated at 2 h, and more markedly elevated by 3 h (Fig. 2A). Serum TNF concentration was not affected by either RAN or FAM given alone (Fig. 2B). LPS exposure elevated TNF concentration relative to control at all three of these early times; RAN cotreatment enhanced this LPS effect at all times, whereas FAM cotreatment did not influence it (Fig. 2B). Similar effects of LPS-drug treatment were observed for serum IL-1β, interferon-gamma, IL-6, IL-10, and PAI-1 (Figs. 2C–G).

PGE2 is a product of cyclooxygenase-2 (COX-2), which is expressed by cells during inflammatory responses. A previous study indicated that COX-2 mRNA was upregulated in livers of LPS/RAN-cotreated rats (Luyendyk et al., 2006). Furthermore, it has been reported that TNF increases production of PGE2 and that PGE2 diminishes LPS-stimulated production of TNF (Kreydiyyeh et al., 2007; Scales et al., 1989). Serum PGE2 concentration was elevated slightly by either RAN or FAM as well as by LPS (Fig. 2H). In LPS-treated rats, cotreatment with either RAN or FAM enhanced serum PGE2 concentration compared to LPS treatment alone at all three times.

Effect of PTX and Etan on LPS/RAN-Induced Liver Injury

In a preliminary efficacy study, the concentration of circulating TNF was determined 2 h after administration of LPS to rats pretreated with PTX or Etan. The LPS-induced increase in TNF was markedly attenuated by pretreatment with PTX (Fig. 3A). Similarly, Etan pretreatment significantly reduced the increase in circulating TNF activity (Fig. 3B).
LPS/RAN treatment increased serum ALT activity at 6 h (Figs. 4A and 4B). Serum ALT activity was significantly less in animals given either PTX (100 mg/kg, iv) or its vehicle or Etan (8 mg/kg, sc) or its vehicle 1 h before treatment with LPS (2.5 × 10^6 EU/kg, iv) or its vehicle. Two hours after LPS, either RAN (30 mg/kg, iv) or its vehicle was administered. Hepatocellular injury was assessed 6 h after RAN as increased serum ALT activity. Data are expressed as mean ± SEM; n = 4–6 rats/group; a, significantly different from the veh/veh/veh group; b, significantly different from the veh/LPS/RAN group.

LPS/RAN treatment increased serum ALT activity at 6 h (Figs. 4A and 4B). Serum ALT activity was significantly less in animals given either PTX (Fig. 4A) or Etan (Fig. 4B) prior to LPS/RAN treatment. In preliminary studies, ALT activity was not altered in Etan/veh/veh/LPS/RAN-treated animals compared with veh/veh/veh/LPS/RAN-treated rats (data not shown). Similarly, we demonstrated previously that the dose of PTX used for these studies was not hepatotoxic in rats (Hewett et al., 1993). These findings were supported by histologic examination of liver sections. Liver from veh/veh/veh/LPS/RAN-treated animals appeared normal (Fig. 5A). Sections from LPS/RAN-cotreated rats taken 6 h after administration of RAN had acute, multifocal, midzonal hepatocellular necrosis (Fig. 5B). These lesions have been described in detail previously (Luyendyk et al., 2003). In livers from animals that received PTX (Fig. 5C) or Etan (Fig. 5D) prior to LPS/RAN cotreatment, the size and frequency of the necrotic foci were significantly reduced compared with the LPS/RAN-treated rats.

**Effect of PTX or Etan on LPS/RAN-Induced Increases in Serum IL-1 β and IL-6 Concentrations**

Cotreatment of rats with LPS/RAN significantly increased circulating concentrations of IL-1 β and IL-6 compared with vehicle-treated rats (Fig. 6). Pretreatment with PTX reduced the LPS/RAN-induced increase in IL-1 β and IL-6 (Figs. 6A and 6B). Etan reduced the LPS/RAN-induced increase in circulating IL-1 β (Fig. 6C) but did not significantly affect IL-6 concentration, although there was a trend toward reduced concentration (Fig. 6D).

**Effect of PTX or Etan on LPS/RAN-Induced Changes in the Hemostatic System**

Increased TAT concentration in plasma is a biomarker of coagulation system activation. PAI-1 is the major endogenous inhibitor of fibrinolysis, thus an increase in its concentration in plasma suggests impaired fibrinolysis. Previous studies demonstrated that both TAT and PAI-1 concentrations were enhanced early after LPS/RAN treatment (Fig. 2F; Luyendyk et al., 2004a,b). Confirming these results, LPS/RAN treatment increased plasma TAT concentration and total PAI-1 concentration in serum (Fig. 7). These increases were significantly attenuated by either PTX or Etan pretreatment (Fig. 7).

![FIG. 4. Effect of PTX (A) or Etan (B) on the LPS/RAN-induced elevation in serum ALT activity. Rats fasted for 24 h received either PTX (100 mg/kg, iv) or its vehicle or Etan (8 mg/kg, sc) or its vehicle 1 h before treatment with LPS (2.5 × 10^6 EU/kg, iv) or its vehicle. Two hours after LPS, either RAN (30 mg/kg, iv) or its vehicle was administered. Hepatocellular injury was assessed 6 h after RAN as increased serum ALT activity. Data are expressed as mean ± SEM; n = 4–6 rats/group; a, significantly different from the veh/veh/veh group; b, significantly different from the veh/LPS/RAN group.](https://academic.oup.com/toxsci/article-abstract/100/1/267/1625687/272-TUKOV-ET-AL)

![FIG. 5. Representative photomicrographs of livers after LPS/RAN cotreatment. Rats were treated as described in the legend to Fig. 4. At 6 h after LPS/RAN administration, rats were killed and livers were removed and fixed in 10% neutral-buffered formalin, stained with H&E, and examined by light microscopy. (A) veh/veh/veh, (B) veh/LPS/RAN, (C) PTX/LPS/RAN, and (D) Etan/LPS/RAN-treated rats. The photomicrographs were taken at 100× magnification. Acute, multifocal, midzonal hepatocellular necrosis (arrows) developed in LPS/RAN-cotreated rats. Reduction in the size and frequency of necrotic lesions was observed in PTX- and Etan-pretreated rats.](https://academic.oup.com/toxsci/article-abstract/100/1/267/1625687/272-TUKOV-ET-AL)
Liver sections from veh/veh/veh-treated animals showed very little fibrin deposition, with some staining of the larger vessels due to fibrin deposition during animal sacrifice (Luyendyk et al., 2003) (Fig. 8A). Fibrin deposition in liver tissues from veh/LPS/RAN-treated rats (Fig. 8B) was significantly increased compared with control. Pretreatment with either PTX (Fig. 8C) or Etan (Fig. 8D) attenuated the increase in fibrin deposition caused by veh/LPS/RAN treatment. Morphometric analysis confirmed this result (Figs. 8E and 8F).

**Effect of PTX or Etan on LPS/RAN-Induced Hepatic PMN Accumulation and Serum MIP-2 Concentration**

Previous results suggested that PMNs play a causal role in LPS/RAN-induced hepatocellular injury (Luyendyk et al., 2005). To test the possibility that TNF mediates liver injury after LPS/RAN exposure by promoting PMN accumulation in the liver, the effects of PTX or Etan pretreatment on the LPS/RAN-induced increase in liver PMNs were evaluated. Quantitative analysis of liver sections immunostained for PMNs revealed that PMN accumulation occurred within 6 h in livers from animals treated with LPS/RAN. PMN numbers in the livers of LPS/RAN-treated animals were unaffected by either PTX or Etan (Figs. 9A and 9C). PMN distribution was panlobular in livers of LPS/RAN-cotreated rats, although foci of PMNs were noted in lesioned areas.

MIP-2 is a chemokine that can cause transmigration of PMNs from vessels and PMN activation. The serum concentration of MIP-2 was significantly increased in rats cotreated with LPS and RAN. This increase was attenuated by pretreatment with either PTX or Etan (Figs. 9B and 9D).

**Mediator Production In Vivo**

KC are major producers of the inflammatory mediators altered by LPS/RAN cotreatment in vivo. Accordingly, release of mediators was evaluated in vitro in KC/HPC cocultures at noncytotoxic drug and LPS concentrations. Concentrations of RAN were chosen to approximate the maximum concentration likely to occur in plasma in the study in vivo above (i.e., approximately 100 μM) and one larger, but noncytotoxic concentration (i.e., 500 μM). Neither LPS, RAN, FAM nor combinations of LPS and the drugs caused release of ALT in KC/HPC cocultures within 6 h of incubation (Figs. 10A and 10B). LPS increased TNF release (Figs. 10C and 10D). RAN enhanced slightly the LPS-induced TNF release, but only at the larger RAN concentration, whereas both FAM concentrations led to a modest enhancement. Unlike the results in KC/HPC cocultures, RAN was without effect on LPS-stimulated TNF release when it was added to KCs cultured by themselves (Fig. 11A), suggesting the presence of HPCs modified the ability of RAN to alter this response. At the larger...
concentration, FAM by itself elicited TNF release in KC cultures (Fig. 11B).

MIP-2, the release of which was selectively enhanced by RAN in LPS-cotreated rats (Luyendyk et al., 2006), was unaffected by RAN or FAM cotreatment in KC/HPC cocultures (Figs. 12A and 12B). Interestingly, PGE2 release in vitro was largely unaffected by LPS but enhanced markedly by RAN (Fig. 12C). In contrast, FAM caused only a slight enhancement (Fig. 12D).

**DISCUSSION**

TNF is an important proinflammatory cytokine involved in normal physiological immune and inflammatory processes. A causative role for TNF in promoting liver injury has been identified for inflammation interaction with a number of xenobiotic agents (Barton et al., 2001; Endo et al., 1999; Yee et al., 2002) as well as for large, hepatotoxic doses of LPS (Hewett et al., 1993). The present work explored the role of TNF in an animal model of RAN-induced idiosyncratic liver injury. LPS exposure induces a large and rapid increase in plasma TNF concentration (Hewett et al., 1993). In the current study, despite increased plasma TNF concentration, rats given a small dose of LPS did not develop liver injury by 3 h. This indicates that an increase in TNF of this magnitude and duration is not sufficient to cause hepatocellular damage. Despite this insufficiency, this large increase in TNF concentration could be critical for the genesis of hepatocellular injury when combined with other, independent effects of RAN cotreatment. Indeed, RAN-cotreated rats sustained greater plasma TNF levels compared with rats given LPS alone and developed hepatotoxicity. In contrast, FAM at an equally efficacious dose neither enhanced TNF production nor synergized with LPS to produce liver injury. Thus, the increase in LPS-stimulated TNF production could contribute to hepatotoxicity in LPS/RAN-treated rats.

To determine the role of TNF in LPS/RAN-induced hepatotoxicity, we used two approaches. PTX is a methylxanthine that inhibits the synthesis of TNF (Barton et al., 2001; Dezube et al., 1993; Yee et al., 2003). However, PTX also has several other pharmacological effects (Bañi et al., 2004). Accordingly, we also used Etan, a dimeric fusion protein that contains a soluble TNF receptor capable of selectively neutralizing TNF. PTX and Etan significantly reduced serum TNF concentration and activity, respectively, and were both effective in reducing hepatocellular
injury in LPS/RAN-cotreated rats. These results indicate that TNF is involved in LPS/RAN-induced liver injury.

Although this enhancement of TNF concentration by RAN was small compared with the pronounced increase caused by LPS exposure, the prolongation of TNF levels above a particular threshold could have pathogenic importance since it occurred prior to injury in LPS/RAN-treated rats. The importance of the RAN-induced potentiation of LPS-induced TNF release is difficult to test. We have shown previously that LPS given alone at the dose used in these studies does not cause liver injury up to 24 h (Luyendyk et al., 2003). Accordingly, LPS-induced TNF is insufficient to cause liver damage. TNF induces the expression of several cytokines including IL-1β, IL-6, IL-8, MIP-2, as well as other gene products capable of damaging the liver directly or in concert with TNF (Aggarwal and Natarajan, 1996; Locksley et al., 2001). For example, IL-1β and IL-6 display partially overlapping activities with TNF (Eigler et al., 1997). TNF and

---

**FIG. 8.** Effects of PTX or Etan on hepatic fibrin deposition after LPS/RAN cotreatment. Rats were treated as described in the legend to Fig. 4. Livers were removed 6 h after RAN administration and processed for fibrin immunohistochemistry as described in “Materials and Methods” section. Fibrin appears as white on a gray background. Representative liver section from (A) a veh/veh/veh-treated rat showing minimal fibrin staining, (B) a veh/LPS/RAN-treated rat with sinusoidal fibrin deposits in both periportal and centrilobular regions and (C) a PTX/LPS/RAN-treated animal, and (D) an Etan/LPS/RAN-treated animals showing reductions in fibrin staining compared with those from veh/LPS/RAN-treated rats. Fibrin deposition was quantified as described in “Materials and Methods” section. (E and F). Data are expressed as mean ± SEM; n = 5–6 rats/group. a, significantly different from the veh/veh/veh group; b, significantly different from the veh/LPS/RAN group.
IL-1β activate PMNs (Klebanoff et al., 1986), induce their own production, stimulate production of other inflammatory mediators, and antagonize anti-inflammatory cytokines such as IL-10 (Ji et al., 2004). In the present study, LPS induction of IL-1β and IL-6 was enhanced by RAN cotreatment but not by cotreatment with FAM. The LPS/RAN-induced increase in these cytokines was inhibited by TNF neutralization, suggesting that the RAN-dependent increase in TNF is required for enhanced expression of inflammatory genes in LPS/RAN-treated rats (Figs. 2C and 2D and 6B and 6C).

In addition to enhancing cytokine expression, TNF-dependent gene expression might contribute to LPS/RAN-induced liver injury by enhancing coagulation. RAN cotreatment enhanced LPS-induced coagulation prior to liver injury, and anticoagulants reduced liver damage in LPS/RAN-treated rats (Luyendyk et al., 2004a). TNF and other cytokines, including IL-1β and IL-6, induce tissue factor expression on endothelial cells and monocytes/macrophages in vitro (Neumann et al., 1997). Tissue factor is generally accepted as the pivotal initiator of coagulation system activation in endotoxemia and bacteremia (de Jonge et al., 2000; Levi et al., 1994; Pawlinski et al., 2004; Taylor et al., 1991). In addition, in the presence of PMNs, TNF causes sinusoidal endothelial cell (SEC) damage in vitro (Smedly et al., 1986; Takei et al., 1995), which can activate the coagulation system (Colman et al., 1994). Indeed, PTX and Etan inhibited coagulation activation in LPS/RAN-treated rats, as marked by reduced plasma TAT concentration (Fig. 7). This result indicates that TNF contributes to LPS/RAN-induced liver injury at least in part by enhancing activation of the coagulation system.

Activation of the coagulation system in LPS/RAN-treated rats results in the formation of fibrin clots in liver sinusoids, and prevention of fibrin deposition was associated with reduced hepatocellular injury (Luyendyk et al., 2004a). Neutralization of TNF reduced LPS/RAN-induced liver fibrin deposition (Fig. 8). This reduction could be a consequence of reduced coagulation and/or increased fibrinolysis. TNF has been shown to play a role in the induction of PAI-1 in the liver and plasma of LPS-treated mice (Fears and Loskutoff, 1997). Like TNF, the concentration of PAI-1 in the plasma was enhanced by RAN cotreatment (Fig. 2F, Luyendyk et al., 2006), and TNF neutralization inhibited the induction of PAI-1 in LPS/RAN-treated rats (Figs. 7B and 7D). The cellular source and mechanism of enhanced PAI-1 expression in livers of LPS/RAN-cotreated rats is not known. TNF and IL-1 can stimulate the expression and release of PAI-1 by endothelial cells (Schleef et al., 1988). Indeed, RAN cotreatment enhanced the LPS-induced alteration of SEC function (Luyendyk et al., 2004a and b). Taken together, these results suggest that TNF enhancement of PAI-1 production promotes fibrin deposition and injury in this animal model.

**FIG. 9.** Effect of PTX or Etan on serum MIP-2 concentration and the accumulation of PMNs in liver after LPS/RAN cotreatment. Rats were treated as described in the legend to Fig. 4. Serum MIP-2 concentration (B and D) was evaluated 6 h after RAN treatment. Liver PMN accumulation (A and C) was evaluated in 10–20 randomly selected 400× fields 6 h after RAN administration as described under “Materials and Methods” section. Data are expressed as mean ± SEM; n = 5–6 rats/group. a, Significantly different from the veh/veh/veh group. b, Significantly different from the veh/LPS/RAN group.
Coagulation-dependent liver damage in LPS/RAN-cotreated rats could activate inflammatory cells such as PMNs. Depletion of circulating PMNs attenuated LPS/RAN-induced liver injury, suggesting their importance in the pathogenesis (Luyendyk et al., 2005). In models of PMN-dependent liver injury, PMNs not only accumulate in liver sinusoids but must also transmigrate through sinusoidal endothelium and become activated in close proximity to HPCs to cause injury to these cells (Jaeschke, 2006). Interestingly, anticoagulation did not inhibit PMN accumulation in LPS/RAN-treated rats, but it did reduce plasma concentration of the PMN chemokine, MIP-2 (Luyendyk et al., 2006), which can activate PMNs and elicit the release of toxic products (Biedermann et al., 2000; Wang and Thorlacius, 2005). TNF can prompt the accumulation of PMNs in tissues by activating endothelial cells (Bradham et al., 1998; Vassalli, 1992) and can also prime PMNs for activation.

FIG. 10. Release of ALT and TNF-α from HPC/KC cocultures exposed to LPS and RAN or FAM. HPCs and KCs were isolated and cocultured as described in “Materials and Method” section. LPS (10 ng/ml) and drug at the indicated concentrations were added at the same time, and the medium was collected after 6 h. ALT activity and TNF-α concentration were measured in the cell-free supernatant. ALT activity (A and B) in the medium is expressed as a percent of the total ALT activity. TNF-α concentration (C and D) was determined by ELISA. n = 4–5 isolations from different rats. a Significantly different from vehicle-treated group at the same concentration of drug; b significantly different from corresponding vehicle-treated group in the absence of drug.

FIG. 11. Release of TNF-α from KCs exposed to LPS and RAN or FAM. KCs were isolated and cultured as described in “Materials and Method” section. LPS (10 ng/ml) and drug at the indicated concentrations were added at the same time, and medium was collected after 6 h. TNF-α concentration was measured in the cell-free supernatant. n = 3–4 isolations from different rats. a, Significantly different from corresponding vehicle-treated group at the same concentration of drug; b significantly different from vehicle-treated group in the absence of drug.
Analogous to the protection afforded by anticoagulants in this model, inhibition of TNF did not impact hepatic PMN accumulation but did reduce serum MIP-2 concentration (Fig. 9). The observations that TNF inhibition reduced the concentration of MIP-2 in plasma and attenuated coagulation system activation and that inhibition of coagulation reduced MIP-2 concentration (Luyendyk et al., 2006) are consistent with the hypothesis that TNF activation of coagulation induces the expression of MIP-2, which could activate PMNs accumulated in the liver.

Recently, we reported that inflammatory mediator expression elicited by xenobiotic/LPS coexposure in rats could be recapitulated using a KC/HPC coculture system (Tukov et al., 2006). In the present study, RAN enhanced TNF release in the presence of LPS in KC/HPC cocultures but only at a large RAN concentration that is unlikely to be achieved in the LPS/RAN animal model (Fig. 10). FAM had a similar effect. Thus, this in vitro cytokine system failed to distinguish a drug that causes IADRs in humans from one that does not. The MIP-2 response in vitro also differed from the response in rats (Luyendyk et al., 2006, and Fig. 12). The disparity between these in vitro and in vivo responses suggests that the RAN-induced enhancement of TNF and MIP-2 production seen in vivo likely occurs by indirect mechanisms, perhaps involving other cell types.

LPS/RAN treatment of rats selectively increased COX-2 mRNA expression, whereas LPS/FAM treatment did not (Luyendyk et al., 2006). Interestingly, treatment with RAN produced a similar increase in PGE₂ compared with treatment

**FIG. 12.** Release of MIP-2 and PGE₂ from HPC/KC cocultures exposed to LPS and RAN or FAM. HPCs and KCs were isolated and cocultured as described in “Materials and Methods” section. LPS (10 ng/ml) and drug at the indicated concentrations were added at the same time, medium was collected after 6 h, and MIP-2 (A and B) and PGE₂ (C and D) were measured in the cell-free supernatant. n = 3–5 isolations from different rats. a, Significantly different from vehicle-treated group at the same concentration of drug; b, significantly different from corresponding vehicle-treated group in the absence of drug.

**FIG. 13.** Working hypothesis for the pathogenesis of LPS/RAN-induced hepatotoxicity. Previous studies (see “Discussion” and Luyendyk et al. 2004a and b; 2005) have implicated the hemostatic system and neutrophils (PMNs) as critical players in the hepatotoxic interaction between LPS and RAN. The present results suggest that TNF-α is important as a proximal mediator in the chain of events leading to hepatocellular injury.
with FAM, irrespective of LPS cotreatment (Fig. 2H). In KC/HPC cocultures, RAN prompted the release of PGE₂, whereas FAM had little effect (Figs. 12C and 12D). These drug effects were not influenced by LPS treatment of the cells. Thus, increased PGE₂ release did distinguish the two drugs in vitro, whereas it failed to do so in vivo.

In summary, RAN cotreatment potentiated the LPS-mediated production of TNF at a time before the onset of liver injury in LPS/RAN-cotreated rats. Inhibition of TNF biosynthesis or signaling significantly attenuated LPS/RAN-induced hepato-cellular injury. Interference with TNF also reduced the serum concentrations of other proinflammatory cytokines and chemokines in LPS/RAN-treated rats. In addition, TNF inhibition decreased coagulation system activation, PAI-1 production, and fibrin deposition. Together, these results suggest that TNF decreased coagulation system activation, PAI-1 production, kines in LPS/RAN-treated rats. In addition, TNF inhibition attenuated LPS/RAN-induced hepato-cellular injury during hepatic ischemia-reperfusion and other acute inflammatory conditions.

**REFERENCES**


**ACKNOWLEDGMENTS**

The authors are grateful to Theresa Eagle and Sandra Newport for technical assistance.

**FUNDING**

Grant DK061315 from the National Institutes of Health; Training Grant 2 T32 ES07255 from the National Institute of Environmental Health Sciences to F.F.T. and J.P.L.; the Society of Toxicology’s Colgate Palmolive Postdoctoral Fellowship in *In Vitro* Toxicology to F.F.T.; and Society of Toxicology’s Novartis Predoctoral Fellowship to J.P.L.


