Coagulation dependent gene expression and liver injury in rats
given lipopolysaccharide with ranitidine but not with famotidine

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Text pages: 35
Figures: 7
Tables: 3
References: 33
Abstract: 249
Introduction: 637
Discussion: 1716

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PCR, polymerase chain reaction; plasminogen activator inhibitor-1, PAI-1; ranitidine, RAN; thrombin-antithrombin dimer, TAT; vehicle, Veh;

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Abstract

In an animal model of drug idiosyncrasy, rats cotreated with nonhepatotoxic doses of lipopolysaccharide (LPS) and ranitidine (RAN) develop hepatocellular injury, whereas rats treated with LPS and famotidine (FAM) do not. The coagulation system and neutrophils (PMNs) are requisite mediators of LPS/RAN-induced liver injury. We tested the hypothesis that unique gene expression in LPS/RAN-treated rats requires coagulation system activation and that these changes are absent in rats given LPS and FAM. Rats were treated with a nonhepatotoxic dose of LPS (44.4 x 10^6 endotoxin units/kg, iv) or its vehicle, then one hour later with heparin (3000 U/kg) or its vehicle. One hour thereafter they were given RAN (30 mg/kg), FAM (6 mg/kg: a pharmacologically equi-efficacious dose, or 28.8 mg/kg: an equimolar dose), or vehicle (iv). They were killed 2 or 6 h after drug treatment for evaluation of hepatotoxicity, coagulation system activation, and liver gene expression (2 h only). Statistical filtering of gene array results and real-time PCR identified groups of genes expressed in LPS/RAN-treated rats but not LPS/FAM-treated rats that were either changed or unchanged by heparin administration. For example, LPS/RAN-induced mRNA expression of the inflammatory mediators IL-6, COX-2, and MIP-2 was reduced by anticoagulation. Enhancement of serum MIP-2 and PAI-1 concentrations in LPS/RAN-treated rats was prevented by anticoagulation. The results suggest crosstalk between hemostasis-induced gene expression and inflammation (e.g., PMN function) in the genesis of hepatocellular injury in LPS/RAN-treated rats. In contrast, neither the expression of such genes nor hepatocellular necrosis occurred in rats treated with LPS/FAM.
Introduction

Idiosyncratic drug reactions are adverse responses that occur in a small fraction of people taking a drug, and the liver is a frequent target organ. The unpredictable nature and severity of idiosyncratic liver injury have significant impact both on human health and the pharmaceutical industry. Although numerous studies have proposed that idiosyncratic hepatotoxicity occurs as a consequence of metabolic polymorphism and/or drug-specific immunity (for reviews see Ju and Uetrecht, 2002; Pirmohamed et al., 1996), for the vast majority of drugs the mechanisms underlying idiosyncratic responses are unclear. Recent studies have suggested that an inflammatory response might precipitate idiosyncratic liver injury from some drugs, and animal models have been developed to examine drug-inflammation interaction (Buchweitz et al., 2002; Luyendyk et al., 2003b; Roth et al., 2003).

The histamine2 (H2)-receptor antagonist ranitidine (RAN) causes idiosyncratic hepatotoxicity in a small fraction of people. In animal models, RAN is not hepatotoxic in naïve rats, but toxicity develops in rats cotreated with the inflammatory stimulus, bacterial lipopolysaccharide (LPS, Luyendyk et al., 2003b; Roth et al., 2003). In this LPS/RAN animal model, features of hepatotoxicity in rats bore resemblance to clinical observations made in cases of RAN idiosyncrasy in people (Luyendyk et al., 2003b). Hepatic parenchymal cell injury occurred in rats cotreated with normally nonhepatotoxic doses of RAN and LPS beginning approximately 3 h after RAN treatment. By contrast, injury was not observed in rats cotreated with LPS and famotidine (FAM), a H2-receptor antagonist not associated with idiosyncratic liver injury (Luyendyk et al., 2003b). Although mechanisms of hepatotoxicity in this model have not been fully characterized, LPS/RAN-induced hepatocellular injury depends on an activated hemostatic system and inflammatory cells (Luyendyk et al., 2005b; Luyendyk et al., 2004a).
addition, liver hypoxia and altered gene expression appear to play critical roles (Luyendyk et al., 2004a; Luyendyk et al., 2004b).

The hemostatic system comprises two branches, coagulation and fibrinolysis, and is also involved in several models of LPS-potentiated hepatotoxicity (Luyendyk et al., 2003a; Yee et al., 2003). In LPS/RAN-treated rats, the coagulation system is activated and hepatic fibrin deposition occurs before the onset of hepatocellular injury. Treatment with heparin attenuated coagulation system activation, hepatic fibrin deposition, and hepatocellular injury (Luyendyk et al., 2004a). It was suggested that the protective effect of heparin might relate, in part, to its ability to prevent liver hypoxia caused by sinusoidal fibrin deposition (Luyendyk et al., 2005b). However, the mechanism by which coagulation system activation causes LPS/RAN-induced hepatocellular injury has not been completely elucidated.

At a time before hepatocellular injury occurred, evaluation of global hepatic gene expression distinguished LPS/RAN-treated rats from rats given either agent alone, and several gene expression changes were identified as being unique to LPS/RAN-treatment (Luyendyk et al., 2004b). Some of these might occur as a consequence of coagulation system activation. For example, signaling pathways activated downstream cleavage of protease activated receptor-1 (PAR-1) on endothelial cells by thrombin can culminate in altered gene expression (McLaughlin et al., 2005). In addition, liver hypoxia resulting from fibrin deposits and local ischemia can alter gene expression (Sonna et al., 2003). Accordingly, direct and indirect effects of coagulation system activation on hepatic gene expression might contribute to the expression signature in livers of LPS/RAN-treated rats.

We tested the hypothesis that unique gene expression in LPS/RAN-treated rats requires coagulation system activation and that these changes are absent in rats given LPS and FAM. To
this end, the effect of heparin on global hepatic gene expression was evaluated using gene arrays in rats cotreated with LPS/RAN or LPS/FAM. Genes were grouped in sets based on their expression pattern, and the expression of 7 genes was confirmed by real-time PCR. For a few genes potentially related to the protective effect of heparin, ELISA was used to determine whether the increased mRNA expression resulted in enhanced protein concentration and whether this was reduced by heparin treatment.
Materials and Methods

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Heparin sodium salt (H-3149, Sigma Chemical Co., St. Louis, MO) with an activity of 180 U/mg and LPS derived from *E. coli* serotype O55:B5 with an activity of $6.6 \times 10^6$ EU/mg (Cat. No. L-2880, Lot # 51K4115) were used for these studies. This activity was determined using a QCL Chromogenic LAL Endpoint Assay from Cambrex (East Rutherford, NJ).

Animals. Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR; Charles River, Portage, MI) weighing 250-350 grams were used for these experiments. Animals were fed standard chow (Rodent chow/Tek 8640, Harlan Teklad, Madison, WI) and allowed access to water *ad libitum*. They were allowed to acclimate for 1 week in a 12-h light/dark cycle prior to use.

Experimental protocols. In a previous study, rats cotreated with normally nonhepatotoxic doses of LPS and RAN developed hepatocellular injury (Luyendyk et al., 2003b). Two doses of FAM were selected for studies evaluating coagulation system activation: 1) a dose equivalent to the pharmacologic efficacy of RAN was selected (FAM) based on relative potencies of RAN and FAM in antagonizing H2-receptors (Scarpignato et al., 1987; Lin, 1991) and 2) a dose equimolar to that of RAN (FAM-EM). Rats fasted for 24 hours were given $44.4 \times 10^6$ endotoxin units (EU)/kg LPS or its saline vehicle (Veh) i.v., and food was then returned. Two hours later they were given 30 mg/kg RAN, 6 mg/kg FAM (FAM-EE), 28.8 mg/kg FAM (FAM-EM), or sterile phosphate-buffered saline (PBS), i.v. Two or 6 h later, rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.) for assessment of coagulation system activation and hepatocellular injury. To simplify treatment nomenclature for the remainder of the report, the following group designations have been applied: Saline/PBS.
(Veh/Veh), Saline/RAN (Veh/RAN), Saline/FAM (Veh/FAM), Saline/FAM-EM (Veh/FAM-EM), LPS/PBS (LPS/Veh), LPS/FAM, LPS/FAM-EM, and LPS/RAN.

In a separate group of rats, animals were treated heparin (3000 U/kg, s.c.) or sterile saline 1 h after LPS administration then given FAM or RAN as described above. Rats were anesthetized 2 or 6 h later as above for evaluation of hepatic gene expression (2 h) and hepatocellular injury (2 and 6 h). Gene expression was evaluated at the time of onset of hepatocellular injury (2 h) so that changes could be associated with the pathogenesis. Another group of animals was killed at 6 h to confirm the occurrence of liver injury.

**Sample collection.** Blood drawn from the dorsal aorta was collected rapidly in BD Vacutainer™ Plus Plastic Citrate Tubes (Becton-Dickinson, Franklin Lakes, NJ) or allowed to clot at room temperature. Citrated-plasma and serum were collected and aliquots stored at -80°C until use. Three, 100 mg midlobe pieces of the right medial liver lobe were flash-frozen in liquid nitrogen for RNA isolation. Slices (3-4 mm thick) of the ventral portion of the left lateral lobe were collected and fixed in 10% neutral buffered formalin.

**Hepatotoxicity assessment.** Alanine aminotransferase (ALT) activity was evaluated using a Hitachi 917 Chemistry Analyzer (Roche Diagnostics, Inc.). Formalin-fixed sections of liver were routinely embedded in paraffin, sectioned at approximately 5 μm, and stained with hematoxylin and eosin. Three sections of each liver were examined. Acute, multifocal, hepatic necrosis was scored as described previously (Luyendyk et al., 2003b) in which a score of 0 represents no significant lesion and 5 represents a severe lesion.

**Measurement of blood proteins.** Serum MIP-2 concentration was evaluated using an ELISA from Biosource International (Camarillo, CA). Total serum PAI-1 concentration was evaluated using an ELISA from American Diagnostica, Inc. (Greenwich, CT). This ELISA
measures total PAI-1 (i.e., active, inactive and tissue plasminogen activator/PAI-1-complexed forms). Plasma fibrinogen was determined from thrombin clotting time of diluted samples using a fibrometer and a commercially available kit (B4233) from Dade-Behring, Inc. (Deerfield, IL). Plasma thrombin-antithrombin (TAT) concentration was determined using ELISA kit #OWMG15 from Dade-Behring.

**RNA isolation and purification.** Total RNA was isolated from a small piece of frozen liver tissue using Trizol® (Invitrogen Corporation, Carlsbad, CA) and purified using RNeasy spin columns (Qiagen, Valencia, CA) according to the manufacturers’ instructions. Complete removal of DNA was achieved by using a Qiagen's RNase-Free DNase Set. The quality of the RNA was evaluated by measuring the 260:280 nm absorbance ratio, and the integrity of 18S and 28S ribosomal RNA bands was assessed by electrophoresis on RNA 6000 Nano labchips (Agilent Technologies, Palo Alto, CA). RNA concentrations were determined from absorbance values at a wavelength of 260 nm using a SpectraMax spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA).

**Probe preparation and microarray hybridization.** Sample labeling, hybridization, and staining were carried out according to the Eukaryotic Target Preparation protocol in the Affymetrix® Technical Manual for GeneChip® Expression Analysis. In summary, 5 µg of purified total RNA was used to generate double-stranded cDNA using Superscript reverse transcriptase (Invitrogen Life Technologies) and a T7-oligo (dT) primer. The resulting cDNA was purified using the GeneChip Sample Cleanup Module according to the manufacturer's protocol. The purified cDNA was amplified using BioArray high yield RNA transcription labeling kit (Enzo) according to the manufacturer's instructions to produce biotin-labeled cRNA (complementary RNA) which was then purified using GeneChip Sample Cleanup Module and
quantified. Twenty (20) µg of labeled cRNA (per chip) were fragmented at 94°C for 35 min. Fifteen (15) µg of the fragmented cRNA were then hybridized to the Affymetrix Rat 230.2 arrays for 16 h at 45°C. The hybridized arrays were washed and stained using Streptavidin–Phycoerythrin (Molecular Probes, Carlsbad, CA) and amplified with affinity-purified, biotinylated anti-streptavidin (Vector Laboratories, Inc, Burlingame, CA) using a GeneChip® Fluidics Station 450. The arrays were scanned in Affymetrix high-resolution GeneChip scanner 3000 at 570 nm using Genechip Operating software (GCOS, Ver.1.2).

**Data Analysis.** Raw Affymetrix scan data (CEL files) that met manufacturer's recommended quality criteria were imported into Rosetta Resolver. Downstream analysis was done with the Rosetta Resolver gene expression analysis software (version 5.0, Rosetta Biosoftware, Seattle, WA). Intrachip normalization and background corrections were applied to the hybridizations or profiles, and the replicate profiles were combined in an error-weighted fashion to create ratio experiments with each treatment group as the baseline. Interchip scaling was done to normalize intensity brightness, both across multiple microarrays of the same pattern and of different patterns. Error-model-based transformation was then applied to intensity profiles, and the transformed data were corrected for nonlinearity of expression levels. Error-weighted ANOVA was performed on the input data that were partitioned into groups (ratio experiments) to determine whether any statistically significant differences existed among the group means. Genes were considered active if p<0.01 and the fold change for a comparison was at least +/- 1.5-fold compared to Veh/Veh-treated rats. Clustering analysis was performed using an agglomerative hierarchical clustering algorithm where error-weighted Euclidean distance-based measure (emphasizes the magnitude of the fold changes based on the sum of squares of differences in each direction) was used as similarity measurement.
Real-time polymerase chain reaction (PCR) analysis. Changes in selected transcript levels determined from microarray analyses were also confirmed by real-time PCR. Five (5) µg of RNA were reverse transcribed to cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using 2X SYBR Green master mix (Eurogentec, San Diego, CA). Amplification was carried out as follows: 50°C for 2 min (for uracil N-glycosylase incubation); 95°C for 10 min (denaturation); 40 cycles of 95°C for 15 s and 60°C for 30 s (denaturation/amplification). Dissociation curves were created by adding the following steps to the end of the amplification reaction: 95°C for 15 s (denaturation) and 60°C for 15 s, then gradually increasing to 95°C over 20 min, with a final hold at 95°C for 15 s. Primers were designed for selected genes using Primer Express (v2.0, Applied Biosystems), and checked for specificity by BLAST searches. In addition, primers were only used when they gave rise to a single amplicon as revealed by melting curve analysis. Sequences of forward and reverse primers for target genes purchased from Sigma Genosys (The Woodlands, TX) are listed in Table 1. Twenty (20) ng of cDNA samples were amplified in duplicate using 100nM primers. Eighteen (18)s rRNA was used as an endogenous control to normalize the mRNA target for the differences in the amount of total RNA added to each reaction. Standard curves were constructed for the target mRNA and the endogenous control (18s rRNA) by serial dilution (60, 20, 6.67, 2.22, 0.74ng cDNA) of the mixture of cDNA samples obtained from the LPS/Veh group. The amount of target gene and endogenous control in samples was determined by linear regression analysis, and the target mRNA abundance was expressed as the ng target gene/ng 18s rRNA ratio.

Statistics. Two-way analysis of variance (ANOVA) with Student-Newman-Keuls’s test for multiple comparisons was used for comparison of all data with the exception of gene
expression filtering, which was performed as described above. The criterion for significance was $p < 0.05$. 
Results

Effect of RAN or FAM cotreatment on LPS-induced coagulation system activation.

Rats were given either LPS or its vehicle and cotreated with RAN, FAM-EE, FAM-EM, or Veh. Coagulation system activation was estimated 2 h after drug administration from the decrease in plasma fibrinogen and from an increase in plasma TAT concentration. Plasma fibrinogen concentration decreased (Fig. 1A) and plasma TAT concentration increased (Fig. 1B) after LPS treatment, irrespective of drug cotreatment. Confirming previous results (Luyendyk et al., 2004a), RAN cotreatment caused an additional increase in plasma TAT concentration (Fig 1A and 1B). Treatment with RAN alone caused a decrease in plasma fibrinogen (Fig. 1A). Consumption of fibrinogen after treatment with RAN was not associated with significantly increased TAT concentration (Fig. 1B). FAM did not cause any of the changes in the coagulation system imposed by RAN.

Effect of anticoagulation on hepatotoxicity after LPS/RAN-cotreatment. Since neither FAM-EE nor FAM-EM treatments augmented LPS-induced coagulation system activation, the more pharmacologically comparable dose (i.e., FAM-EE) was selected for studies to evaluate the effect of anticoagulation on gene expression. As depicted in Fig 1A, LPS administration caused a significant decrease in plasma fibrinogen concentration. LPS/RAN-cotreatment caused a more pronounced decrease in plasma fibrinogen compared to LPS/Veh-treated rats, whereas FAM cotreatment had no further effect. Coadministration of heparin significantly attenuated this decrease in both LPS/FAM and LPS/RAN-cotreated rats at 2 and 6 h (Fig. 2A). Confirming previous results (Luyendyk et al., 2003b), LPS/RAN cotreatment, but not LPS/FAM cotreatment, caused a significant increase in serum ALT activity (normal ~50 U/L) at 2 h that became more pronounced by 6 h (Fig. 2B). Midzonal hepatocellular necrosis, which has
been described previously in this model (Luyendyk et al., 2003b), developed in 3 of 8 LPS/Veh/RAN-treated rats by 2 h and was more prevalent (6 of 7) at 6h. Consistent with the reduction in ALT activity, heparin prevented these changes completely at 2 h and markedly reduced the prevalence (1 of 5 rats) at 6 h (Table 2).

Hierarchical clustering of hepatic gene expression. Hepatic gene expression was evaluated 2 h after drug treatment. Affymetrix 230 2.0 probesets defined as active (see Materials and Methods) were subjected to hierarchical clustering. The resulting dendrogram is displayed in Fig 3. Two clusters were apparent, segregating animals by RAN or FAM cotreatment. Additional clustering by heparin treatment within each drug cluster was not observed, suggesting that heparin altered the expression of few genes.

Effect of heparin on LPS/RAN-induced gene expression. Two criteria were established to identify individual genes that had the potential to be mechanistically relevant to LPS/RAN-induced liver injury and that were regulated by coagulation system activation. Inasmuch as hepatocellular injury occurred in LPS/Veh/RAN- but not LPS/Veh/FAM-treated rats, we first identified genes expressed to a different degree in these groups as those that might be mechanistically relevant to the injury. Accordingly, probesets for which gene expression was different in LPS/Veh/RAN-treated rats relative to LPS/Veh/FAM-treated rats were first selected (i.e., Criterion A). This group of probesets, denoted as set A, is listed in Supplemental Table 1. Next, probesets were identified for which heparin coadministration altered expression in LPS/RAN-treated rats (Criterion B). Heparin altered the expression of 145 probesets in LPS/RAN-treated rats (Supplemental Table 2). Probesets that met both criteria were denoted subset AB and are listed in Supplemental Table 3, and those with annotation currently available are shown in Table 3. The AB set contains only twenty nine probesets (Supplemental Table 3)
and annotation was available for 15 (Table 3). These genes are perhaps the most interesting because they distinguish the drug treatment that produces hepatocellular injury from one that does not, and an agent that reduces injury in LPS/RAN-treated rats (i.e., heparin) prevents or reduces their expression. These included several genes encoding products involved in inflammation (i.e., Csf3, Cxcl2, IL6, Ptgs2, Gzmb), in transporting agents across membranes (i.e., Atp1b1, Slc5a3), and signal transduction (i.e., Stk2, Rac1, Klf4). Many of them were also associated with hypoxia (Atp1b1, Egln3, Cxcl2, Ptgs2, Rac1, Tfrc; Table 3).

Some gene products were changed in LPS-treated rats by RAN coexposure but not by FAM cotreatment and were, in addition, not affected by heparin (ie, met criterion A but not B). These might be important for LPS/RAN-induced liver injury, but the cause of their altered expression is unrelated to activation of the coagulation system. This subset was denoted A.1 and is shown in Supplemental Table 3. This group included PAI-1, egr-1, and btg2, three genes expressed to a greater degree in rats treated with LPS/RAN compared to treatment with RAN or LPS alone (Luyendyk et al., 2004b).

**Real-time PCR.** Real-time PCR was used to verify selected gene expression changes observed from microarray analysis. Four genes (PAI-1, BNIP3, Atf3, MAPKAPK-2) from the A.1 subset (Fig. 4) and 3 genes from the AB subset (COX-2, Cxcl2 [MIP-2], IL-6) were selected for confirmation (Fig. 5). The expression of PAI-1, BNIP3, and MAPKAPK-2, was greater (1.6-fold, 2-fold, 1.6-fold, respectively) in LPS/Veh/RAN-treated rats compared to LPS/Veh/FAM-treated rats, and the expression of each gene was not significantly affected by heparin coadministration in any treatment group (Fig. 4A, B, C). The expression of Atf3 was greater (3-fold) in LPS/Veh/RAN-treated rats compared to LPS/Veh/FAM-treated rats and was
significantly reduced by heparin (Fig. 4D). However, heparin did not reduce Atf3 expression to that observed after LPS/heparin/FAM treatment.

The expression of Ptgs2 (i.e., COX-2, cyclooxygenase-2) was greater (2.3-fold) in LPS/Veh/RAN-treated rats compared to LPS/Veh/FAM-treated rats (Fig. 5A). Heparin was without effect in LPS/FAM-treated rats but prevented the enhanced expression of COX-2 in LPS/RAN-treated rats (Fig. 5A). Cxcl2 (MIP-2, macrophage inflammatory protein-2) expression was greater (2-fold) in LPS/Veh/RAN-treated rats compared to LPS/Veh/FAM-treated rats. Heparin was without effect on MIP-2 in LPS/FAM-treated rats but prevented its enhanced expression in LPS/RAN-treated rats (Fig. 5B). Expression of the inflammatory cytokine IL-6 (i.e., interleukin-6) mRNA was greater (1.8-fold) in LPS/Veh/RAN-treated rats compared to LPS/Veh/FAM-treated rats (Fig. 5C), and this increase was prevented by heparin administration.

Effect of heparin on serum MIP-2 concentration. MIP-2 is a PMN chemokine, and its upregulation is of interest since LPS/RAN hepatotoxicity depends on PMNs (Luyendyk et al., 2005b). The concentration of MIP-2 in serum of naïve rats was ~20 pg/ml (data not shown). 2h after RAN administration, serum MIP-2 concentration increased markedly in LPS-treated rats, but this increase was much greater after RAN cotreatment (39 ng/ml; Fig. 6) than after FAM cotreatment (18 ng/ml). Consistent with its reduction in MIP-2 gene expression at 2 h, heparin reduced serum MIP-2 concentration in LPS/RAN-treated rats at both 2 and 6 h, but it was without a statistically significant effect in LPS/FAM-treated rats (Fig. 6). Despite this overall decrease, serum MIP-2 concentration remained elevated in LPS/Veh/RAN-treated rats compared to LPS/Veh/FAM-treated rats.

Effect of heparin on serum PAI-1 concentration. An earlier study showed that serum PAI-1 was increased by LPS treatment and that this increase was markedly enhanced by
cotreatment with RAN (Luyendyk et al., 2004b). Heparin significantly reduced serum PAI-1 concentration in LPS/Veh/RAN-treated rats (Fig. 7) to a value similar to that seen in rats treated only with LPS (data not shown). In striking contrast, heparin significantly increased PAI-1 concentration in LPS/FAM-treated rats (Fig. 7). In a separate timecourse study, the LPS/RAN-induced increase in serum PAI-1 concentration was reduced by heparin at both 3 and 6 h by more than 50% (data not shown).
Discussion

Previous studies demonstrated that LPS/RAN-cotreatment, but not LPS/FAM-cotreatment, results in hepatocellular injury, and that anticoagulation attenuates injury in this model (Luyendyk et al., 2003b; Luyendyk et al., 2004a). Treatment with RAN alone decreased fibrinogen concentration without a corresponding activation of thrombin, suggesting a thrombin-independent consumption of fibrinogen (Fig. 1). Interestingly, a similar effect was not observed in rats given FAM alone, suggesting that this effect is unique to RAN. The enzyme responsible for this decrease has not yet been identified, but it is known that other enzymes, including metalloproteinases, can degrade fibrinogen (Bini et al., 1996).

Treatment with LPS alone caused an increase in TAT and a decrease in plasma fibrinogen concentration that were augmented by RAN cotreatment but not FAM-cotreatment (Fig. 1 and 2A). Since heparin attenuated coagulation system activation and hepatocellular injury in LPS/RAN-treated rats (Fig. 2, Table 2), these results suggest that enhanced thrombin generation as well as hepatocellular injury are specific to LPS/RAN-cotreatment and independent of H2-receptor antagonism.

The effect of anticoagulation on hepatic gene expression in LPS/RAN- and LPS/FAM-treated rats was examined at a time near the onset of hepatocellular injury, thereby minimizing the likelihood that a reduction in gene expression by heparin is a consequence of lessened injury. Hierarchical clustering distinguished LPS/RAN and LPS/FAM groups but did not distinguish heparin-cotreated rats from those cotreated with Veh (Fig. 3). This indicates that, despite its near complete reduction of hepatocellular injury, heparin altered the expression of a small number of genes in LPS/RAN rats (i.e., only ~0.5% of probesets). However, heparin changed even fewer probesets in LPS/FAM-treated rats (~0.06%), a result consistent with heparin altering gene
expression only in association with the treatment that caused pronounced hepatic coagulation system activation (Fig. 1). Coagulation system activation might cause injury by altering expression of one or more of the relatively few gene products that were affected in LPS/RAN-treated rats. Alternatively, the protective effect of heparin in this model might be independent of changes in gene expression.

Several probesets were differentially expressed in LPS/RAN-cotreated rats compared to LPS/FAM-treated rats. These gene products are of obvious potential mechanistic interest, since liver injury occurred only in LPS/RAN-treated rats. To determine whether for any of these genes the difference was mediated by the coagulation system, their expression was compared after heparin coadministration, and two subsets were generated: 1) subset A.1: LPS/Veh/RAN different from LPS/Veh/FAM, but not different from LPS/heparin/RAN (i.e., genes met criteria A, but not B, Supplemental Table 3) and 2) subset AB: LPS/Veh/RAN different from LPS/Veh/FAM and different from LPS/heparin/RAN (i.e., genes met both criterion A and B, Supplemental Table 3). Gene products comprising the A.1 subset might be important for LPS/RAN-induced liver injury but were unaffected by heparin, suggesting that their differential expression is independent of coagulation system activation. Real-time PCR confirmed that heparin did not prevent LPS/RAN-mediated expression of genes encoding PAI-1, BNIP3, and MAPKAPK-2 (Fig. 5). In the case of Atf3, real-time PCR indicated an attenuation of Atf3 expression by heparin, suggesting that Atf3 should actually be segregated to the AB subset.

PAI-1 is one of the genes in the A.1 subset that has received attention in the LPS/RAN-model due to the importance of fibrin clots and tissue hypoxia (Luyendyk et al., 2004a). In LPS/RAN-treated rats, heparin treatment reduced both sinusoidal fibrin deposition and liver hypoxia (Luyendyk et al., 2004a; Luyendyk et al., 2005b). Inasmuch as PAI-1 is a hypoxia-
inducible gene (Kietzmann et al., 1999), one possibility is that fibrin clot-mediated liver hypoxia causes induction of PAI-1 (Luyendyk et al., 2005b). However, although it prevented fibrin deposition, heparin did not significantly attenuate the augmentation of PAI-1 gene expression in LPS/RAN-treated rats, suggesting that enhanced expression of PAI-1 mRNA in LPS/RAN-treated rats occurs through a hemostasis-independent mechanism. Since heparin also markedly reduces hypoxia in livers of LPS/RAN-treated rats, PAI-1 mRNA expression appears to be independent of hypoxia as well. Interestingly, although heparin did not affect the enhanced PAI-1 mRNA expression, it reduced the increase in serum PAI-1 protein concentration (Fig. 7). One potential source of PAI-1 is platelets, which release preformed PAI-1 upon stimulation with thrombin (Brogren et al., 2004). Taken together, the data indicate that enhanced PAI-1 appearance in LPS/RAN-treated rats is mediated through both transcription-dependent and -independent mechanisms, the latter occurring during coagulation.

Unexpectedly, in LPS/FAM-treated rats heparin increased serum PAI-1 concentration, but not hepatic expression of PAI-1 mRNA (Fig. 7). The mechanism causing this increase is not clear, but it might be a response to excessive anticoagulation. Whereas LPS-induced coagulation and plasma PAI-1 concentration were enhanced by RAN cotreatment, FAM lacked these effects. Anticoagulation in LPS/RAN-treated rats reduced serum PAI-1 and liver injury, whereas anticoagulation in uninjured LPS/FAM-treated rats might have increased PAI-1 as a compensatory response to “rebalance” the hemostastic system. Whether or not this effect of FAM on PAI-1 concentration resulted from H2-receptor antagonism is currently unknown. Regardless, the effect of heparin on PAI-1 in LPS/FAM-treated rats is likely not relevant to the mechanism of injury, since injury did not occur in these rats.
Genes comprising the AB subset are potentially related to the mechanism by which the coagulation system contributes to LPS/RAN-induced liver injury, since heparin prevented the difference in expression between LPS/RAN and LPS/FAM groups. This gene subset contained several hypoxia-inducible genes, consistent with the reduction in liver hypoxia by heparin in this model (Luyendyk et al., 2005b). Indeed, the expression of several of these, (i.e., Atp1b1, Cxcl2, Egln3, ptgs2, and tfrc) as well as genes involved in hypoxic signaling (i.e., Rac1) was prevented by heparin. However, several hypoxia-inducible genes were also identified in the A.1 subset (e.g., BNIP3, egr-1, PAI-1), suggesting that their expression occurred by hypoxia-independent factors. All of these genes are likely controlled by several transcription factors activated by numerous initiators of intracellular signaling, one of which is hypoxia. For example, induction of egr-1 expression is regulated by hypoxia and inflammatory cytokines (Yan et al., 1999; Cao et al., 1992), both of which are enhanced in LPS/RAN-treated rats. Additional studies are required to determine the relative contributions of inflammatory mediators and hypoxia to their regulation.

One gene product in subset AB for which regulation by hypoxia might be important is MIP-2, a neutrophil chemokine. In LPS/RAN-treated rats, PMNs accumulated in liver at a time near the onset of hepatotoxicity, and a PMN-depleting antibody attenuated hepatocellular necrosis (Luyendyk et al., 2005b). The role of chemokines in LPS/RAN-induced liver injury is unknown. In some, but not all studies of PMN-dependent liver injury, evidence supports involvement of chemokines such as MIP-2 and CINC-1 (cytokine-induced neutrophil chemoattractant-1; KC) in the pathogenesis of hepatocellular injury (Li et al., 2004; Dorman et al, 2005; Jaeschke and Bajt, 2004). Similarly, effects of heparin on chemokine expression are model- and perhaps tissue-dependent (Copple et al., 2003; Yamaguchi et al., 2000; Frank et al., 2005). In the present study, CINC-1 expression was not differentially regulated in LPS/RAN-
treated rats compared to LPS/FAM-treated rats or compared to rats given LPS alone (i.e., transcript 1387316_at did not meet criterion A). Moreover, heparin administration affected neither CINC-1 plasma concentration nor hepatic PMN accumulation in LPS/RAN-treated rats (Luyendyk et al., 2005b). In contrast to CINC-1, MIP-2 expression was increased in livers of LPS/RAN-treated rats but not by LPS/FAM-treatment, and heparin prevented this increase (Figs. 5B and 6). This suggests that MIP-2 expression is regulated by hemostasis and/or hypoxia and could have a critical role in LPS/RAN-hepatotoxicity. The reduction in serum MIP-2 by heparin was not accompanied by a reduction in hepatic PMN accumulation (Luyendyk et al., 2005b).

Nevertheless, MIP-2 expression might play a critical role in PMN activation or in migration of PMNs into parenchyma. PMN transmigration through endothelium is required for hepatocellular injury (Jaeschke et al., 1996), and hepatocellular expression of CXC chemokines can drive this process (Maher et al., 1997). It is noteworthy that MIP-2 expression is induced in hepatocytes exposed to hypoxia (Laurens et al., 2005), which occurs in livers of LPS/RAN-treated rats as a consequence of hemostasis. Accordingly, hypoxia-induced transcription, translation, and release of MIP-2 by hepatocytes in LPS/RAN-treated rats could provide the trigger for PMN activation.

Coagulation system activation also appears to be critical for enhanced expression of the COX-2 gene in LPS/RAN-treated rats (Fig. 5). Coagulation-mediated COX-2 (ptgs2) expression might result in the production of cytotoxic lipid mediators, some of which alter hepatocellular cell death signaling pathways (Ganey et al., 2001; Maddox et al., 2004). In a previous study, LPS-inducible COX-2 expression was enhanced by RAN, but not FAM cotreatment (Luyendyk et al., 2005a). Similarly, coagulation system activation was enhanced in LPS-treated rats by RAN, but not FAM cotreatment (Fig. 1). Events that occur during coagulation system activation,
such as protease activated receptor-1 activation (Houliston et al., 2002) and hypoxia (Pichiule et al., 2004) are known to induce COX-2 expression in endothelial cells.

In summary, in our attempt to develop animal models that mimic human idiosyncratic adverse drug reactions (ADRs), we have compared a drug (i.e., RAN) that causes human ADRs with one in the same pharmacologic class that does not share this liability (i.e., FAM). We have confirmed in this study that RAN interacts with LPS to cause liver injury whereas FAM does not. A new finding was that FAM does not share RAN’s ability to enhance coagulation system activation in LPS-treated rats. Another novel and somewhat surprising finding was that, despite nearly eliminating hepatocellular injury, heparin affected very few genes that were selectively altered in expression in LPS/RAN-treated rats. This finding will help to focus on certain genes for further evaluation. For example, both mRNA and protein for MIP-2 were selectively elevated in livers and serum, respectively, of LPS/RAN-treated rats, and this elevation was prevented by heparin. Like MIP-2, COX-2 mRNA was differentially expressed in LPS/RAN-cotreated rats as was the inflammatory cytokine IL-6, and these changes were reduced by heparin. PAI-1 mRNA expression was also elevated in livers of LPS/RAN-, but not LPS/FAM-treated rats; however, in this case heparin did not prevent the increase. Interestingly, heparin did reduce the increase in PAI-1 protein in plasma, suggesting that PAI-1 is released in part from a preformed pool, perhaps in platelets. The results suggest crosstalk between hemostasis-induced gene expression and inflammation (e.g., PMN function) in the genesis of hepatocellular injury in LPS-exposed rats cotreated with RAN. In contrast, neither the expression of such genes nor hepatocellular necrosis occurred in rats treated with LPS/FAM.
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References


Footnotes:

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**Legends for Figures**

Figure 1: Coagulation system activation after LPS/RAN cotreatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its vehicle, then two hours later with either 30 mg/kg RAN, 6 mg/kg FAM (FAM), 28.8 mg/kg FAM (FAM-EM) or vehicle. Coagulation system activation was estimated 2 h after drug treatment by decreased plasma fibrinogen concentration (A) and increased thrombin-antithrombin (TAT) dimer concentration (B). Data are expressed as mean ± SEM. n=5-8. *Significantly different from respective group not given LPS. #Significantly different from respective group not given drug. (p<0.05).

Figure 2: Effect of anticoagulation on LPS/RAN-induced hepatocellular injury. Rats were treated with 44.4 X 10^6 EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour later rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Coagulation system activation was estimated 2 and 6 h after drug treatment by decreased plasma fibrinogen (A). Hepatocellular injury was estimated 2 and 6 h after drug treatment by increased serum alanine aminotransferase (ALT) activity (B). Data are expressed as mean ± SEM. n=5-8. *Significantly different from respective LPS/FAM group at that time. #Significantly different from respective group not given heparin at that time. (p<0.05).

Figure 3: Hierarchical clustering of hepatic gene expression after LPS/heparin/RAN-treatment. Rats were treated with 44.4 X 10^6 EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour later rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Two hours after drug administration, RNA was isolated from liver, and gene expression was evaluated by Affymetrix 230 2.0 Gene Array. RNA from each rat was analyzed using a separate array.
Affymetrix probesets determined to be active by a statistical filter were subjected to two-way agglomerative hierarchical clustering performed using an unweighted average and Euclidean distance as the similarity measure.

Figure 4: Confirmation of expression of selected A.1 subset genes by real-time PCR. Rats were treated with 44.4 × 10^6 EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour later rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Two h after drug administration, RNA was isolated from whole liver, and SYBR Green real-time PCR was performed for (A) PAI-1, (B) BNIP3, (C) MAPKAPK-2, and (D) Atf3. Data are presented as the ratio of ng PCR product/ng 18s rRNA (bars) and are expressed as mean ± SEM. n=5-8. *Significantly different from respective LPS/FAM group. #Significantly different from respective group not given heparin. (p<0.05). (♦) Normalized signal intensity expressed as mean ± SEM for selected Affymetrix probesets: 1368519_at, PAI-1; 1387805_at, BNIP3; 1371446_at, MAPKAPK-2; 1369268_at, Atf3.

Figure 5: Confirmation of COX-2, MIP-2, and IL-6 expression by real-time PCR. Rats were treated with 44.4 × 10^6 EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour later rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Two h after drug administration, RNA was isolated from whole liver, and SYBR Green real-time PCR was performed for (A) COX-2, (B) MIP-2 and (C) IL-6. Data are presented as the ratio of ng PCR product/ng 18s rRNA (bars) and are expressed as mean ± SEM. n=5-8. *Significantly different from respective LPS/FAM group. #Significantly different from respective group not given
heparin. (p<0.05). (♦) Normalized signal intensity expressed as mean ± SEM for selected Affymetrix probesets: 1368527_at, COX-2; 1368760_at, MIP-2; 1369191_at, IL-6.

Figure 6: Effect of heparin on serum MIP-2 concentration. Rats were treated with 44.4 X 10⁶ EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour thereafter rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Serum MIP-2 concentration was measured 2 and 6 h after drug treatment. Data are expressed as mean ± SEM. n=5-8. Serum MIP-2 concentration in naïve rats is < 0.1 ng/ml. *Significantly different from respective LPS/FAM group. #Significantly different from respective group not given heparin. (p<0.05).

Figure 7: Effect of heparin on serum PAI-1 concentration. Rats were treated with 44.4 X 10⁶ EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour thereafter rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Serum PAI-1 concentration was measured 2 h after drug treatment. Data are expressed as mean ± SEM. n=5-8. Serum PAI-1 concentration in naïve rats is < 10 ng/ml. *Significantly different from respective LPS/FAM group. #Significantly different from respective group not given heparin. (p<0.05).
### Table 1

**SYBR Green real-time PCR primers**

<table>
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<th>Gene</th>
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<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>PAI-1</td>
<td>NM_012620</td>
<td>AACCCAGGCCGACTTCA</td>
<td>CATGCGGGCTGAGACTGAAT</td>
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<td>ATF-3</td>
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<tr>
<td>MAPKAPK-2</td>
<td>NM_178102</td>
<td>ACGCCATCACCGACGACTA</td>
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<tr>
<td>BNIP-3</td>
<td>NM_053420</td>
<td>CTCATCTGTTAGCCATTGAGTT</td>
<td>CAGCGTGAATCCCTACTGTGT</td>
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<td>CXCL2 (MIP-2)</td>
<td>NM_053647</td>
<td>TGCCCTGACGACCTACCAA</td>
<td>TCACCAGCAAGCTCTGGATGT</td>
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<tr>
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<td>AAGGGAGTCTGGAACATTGTGAAC</td>
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<tr>
<td>IL-6</td>
<td>NM_012589</td>
<td>TATGAAACAGCGATGACGTGACTG</td>
<td>TTGCTCTGAATGACTCTGGGTT</td>
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Table 2

Histopathologic evaluation of liver necrosis

<table>
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<tr>
<th>Time after Drug</th>
<th>Score</th>
<th>2 h</th>
<th>6h</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS/FAM-EE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5 (100%)</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Heparin</td>
<td>1 (20%)</td>
<td>5 (100%)</td>
<td></td>
</tr>
<tr>
<td>LPS/RAN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
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<td>2 (25%)</td>
<td>1 (12.5%)</td>
</tr>
<tr>
<td>Heparin</td>
<td>5 (100%)</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
</tr>
</tbody>
</table>

Rats were treated with $44.4 \times 10^6$ EU/kg LPS, then one hour later with heparin (3000 U/kg) or vehicle. One hour later rats were given either 30 mg/kg RAN or 6 mg/kg FAM (FAM). Livers were removed 2 or 6 h after drug treatment, fixed in 10% neutral buffered formalin, and evaluated by light microscopy. Midzonal hepatic necrosis was assigned a histopathology score as described in *Materials and Methods* based on the following scale: 0, no significant lesion; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. No livers were assigned a score >2. n=5-8 rats per group. Data are expressed as the number and % of animals in each group given each score.
**Table 3**

**Differentially expressed genes regulated by coagulation system activation in LPS/RAN-treated rats**

<table>
<thead>
<tr>
<th>Probeset</th>
<th>Gene Name</th>
<th>Expression Ratio</th>
<th>Gene function associated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LPS/Veh/RAN LPS/Veh/FAM-EE</td>
<td>LPS/heparin/RAN LPS/Veh/RAN</td>
</tr>
<tr>
<td>1368223_at</td>
<td>Adamts1</td>
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<td>0.5</td>
</tr>
<tr>
<td>1386937_at</td>
<td>Atplb1</td>
<td>2.6</td>
<td>0.6</td>
</tr>
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<td>1369529_at</td>
<td>Csf3</td>
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</tr>
<tr>
<td>1368760_at</td>
<td>Cxcl2</td>
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<td>0.5</td>
</tr>
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<td>1368174_at</td>
<td>Egln3</td>
<td>3.1</td>
<td>0.5</td>
</tr>
<tr>
<td>1379784_at</td>
<td>Gzmb</td>
<td>1.8</td>
<td>0.6</td>
</tr>
<tr>
<td>1369191_at</td>
<td>Ifih1</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>1387260_at</td>
<td>Klf4</td>
<td>2.7</td>
<td>0.4</td>
</tr>
<tr>
<td>1378396_at</td>
<td>LOC310448</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>1368527_at</td>
<td>Pigs2</td>
<td>2.3</td>
<td>0.6</td>
</tr>
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<td>1391048_at</td>
<td>Rac1</td>
<td>1.6</td>
<td>0.5</td>
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<td>1367940_at</td>
<td>Rdk1</td>
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<td>0.6</td>
</tr>
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<td>1392349_at</td>
<td>Slc5a3</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>1375616_at</td>
<td>Sfk2</td>
<td>1.6</td>
<td>0.6</td>
</tr>
<tr>
<td>1388750_at</td>
<td>Tfre</td>
<td>2.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Genes that met both criterion A and criterion B were identified as described in Materials and Methods. For each comparison, the ratio of the expression signals is shown. Genes are listed alphabetically. Genes were classified based on the relationship of their gene product to one or more functions potentially relevant to LPS/RAN-induced liver injury. Genes in this group that could not be classified or were ESTs are listed in Supplemental Table 3.
Figure 1

A

Fibrinogen (mg/dL)

Veh | RAN | FAM-EE | FAM-EM

Veh | LPS

B

TAT (µg/L)

Veh | RAN | FAM-EE | FAM-EM

Veh | LPS
Figure 2

A

Fibrinogen (mg/dL)

- LPS/Veh/FAM-EE
- LPS/Hep/FAM-EE
- LPS/Veh/RAN
- LPS/Hep/RAN

2 h 6 h

B

ALT Activity (U/L)

- LPS/Veh/FAM-EE
- LPS/Hep/FAM-EE
- LPS/Veh/RAN
- LPS/Hep/RAN

2 h 6 h
Figure 3
Figure 4

(A) mRNA ratio (ng PAI-1/ng 18S)

(B) mRNA ratio (ng BNIP3/ng 18S)

(C) mRNA ratio (ng MAPKAPK-2/ng 18S)

(D) mRNA ratio (ng Atf3/ng 18S)
Figure 5

A

mRNA ratio
(ng COX-2/ng 18S)

Normalized Signal

Veh  Hep  Veh  Hep
LPS/FAM-EE  LPS/RAN

B

mRNA ratio
(ng MIP-2/ng 18S)

Normalized Signal

Veh  Hep  Veh  Hep
LPS/FAM-EE  LPS/RAN

C

mRNA ratio
(ng IL-6/ng 18S)

Normalized Signal

Veh  Hep  Veh  Hep
LPS/FAM-EE  LPS/RAN
Figure 6

![Bar chart showing MIP-2 levels (ng/ml) at different time points after RAN treatment.](chart)

- LPS/Veh/FAM-EE
- LPS/heparin/FAM-EE
- LPS/Veh/RAN
- LPS/heparin/RAN

**Time after RAN (hours)**
- 2 h
- 6 h

- *: Statistically significant difference from control
- #: Statistically significant difference from LPS/Veh/FAM-EE
- a: Statistically significant difference from LPS/Veh/RAN
Figure 7

The figure shows a bar graph with the following conditions:

- **PAI-1 (ng/ml)**
- **Veh** and **Hep**
- **LPS/FAM-EE** and **LPS/RAN**

The graph compares the levels of PAI-1 in different groups labeled as **Veh** and **Hep** for **LPS/FAM-EE** and **LPS/RAN** conditions. The bars are denoted with symbols: 
  - **#**
  - *****

The final version may differ from this version.