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Thrombin and PAR-1 Agonists Promote Lipopolysaccharide-induced Hepatocellular Injury in Perfused Livers

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Abbreviations: PAR-1, protease activated receptor-1; ICAM-1, intercellular cell adhesion molecule; CINC-1, cytokine-induced neutrophil chemoattractant-1; MIP-2, macrophage inflammatory protein-2; LPS, lipopolysaccharide; TRAP, thrombin receptor-activating peptide; PMN, neutrophil; ALT, alanine aminotransferase; SEC, sinusoidal endothelial cell.

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ABSTRACT

Bacterial lipopolysaccharide (LPS) is a potent inflammatory agent capable of producing liver injury, the pathogenesis of which depends on numerous mediators, including thrombin. Previous studies showed that thrombin promotes LPS-induced liver injury independent of its ability to form fibrin clots. In isolated, buffer-perfused livers from LPS-treated rats, thrombin added to the perfusion buffer caused dose-dependent liver injury with an EC_{50} of 0.4 nM, consistent with activation by thrombin of a protease-activated receptor (PAR). Actions of thrombin at PARs can be mimicked by thrombin receptor-activating peptides (TRAPs). TRAPs for PAR-1 reproduced the injury caused by thrombin in isolated livers, suggesting that one mechanism by which thrombin promotes LPS-induced liver injury is by activating PAR-1. Immunocytochemistry demonstrated the presence of PAR-1 on sinusoidal endothelial cells and Kupffer cells but not on parenchymal cells or neutrophils. Previous studies showed that thrombin interacts with neutrophils in the genesis of liver injury after LPS treatment. To explore this interaction further, the influence of thrombin on mediators that modulate neutrophil function were evaluated. Inhibition of thrombin in LPS-treated rats prevented liver injury but did not prevent upregulation of cytokine-induced neutrophil chemoattractant-1 (CINC-1), macrophage inflammatory protein-2 (MIP-2) or intercellular cell adhesion molecule-1 (ICAM-1). Thrombin inhibition did, however, prevent PMN degranulation *in vivo* as measured by plasma elastase levels. In addition, elastase concentration was increased in the perfusion medium of livers isolated from LPS-treated rats and perfused with TRAPs. These results suggest that activation of PAR-1 after LPS exposure promotes PMN activation and hepatic parenchymal cell injury.

Severe sepsis resulting from gram-negative bacterial infections is a major clinical problem (Siegel et al., 1993). It has been proposed that many of the pathophysiological effects of gram-negative bacterial sepsis, including liver injury, are mediated in part by lipopolysaccharide (LPS), a component of endotoxin contained in the cell walls of gram-negative bacteria. Intravenous injection of LPS in rats produces liver injury that is dependent on several soluble and cellular inflammatory mediators, including platelets, neutrophils (PMNs), Kupffer cells, cytokines and an activated coagulation cascade (Hewett et al., 1993; Hewett and Roth, 1995; Iimuro et al., 1994; Jaeschke et al., 1991; Pearson et al., 1995). Complex interactions among these cellular and soluble mediators contribute to liver injury, although the nature of the interactions is not completely understood.

Activation of the coagulation system commonly occurs in animal models of sepsis (Margaretten et al., 1967; Hewett and Roth, 1995). In rats, formation of thrombin is critical for the genesis of LPS-induced liver injury (Margaretten et al., 1967; Hewett and Roth, 1995; Pearson et al., 1996), and recent results indicate that thrombin promotes liver injury independently of its role in formation of fibrin clots (Hewett and Roth, 1995; Pearson et al., 1996; Moulin et al., 1996; Moulin et al., 2001). The mechanism of thrombin's action and how it relates to other mediators, however, remain to be elucidated.

One mechanism by which thrombin might promote inflammatory tissue injury independent of fibrin deposition is through activation on cells of protease-activated receptor-1 (PAR-1). After binding to PAR-1, thrombin cleaves an extracellular domain of the receptor, which exposes a new N-terminal sequence (Vu et al., 1991). This sequence interacts with and activates the receptor, thereby initiating intracellular signal transduction pathways. Several studies have linked activation of this receptor to inflammatory events (for review see Cocks and Moffatt, 2000).

A previous study in isolated, perfused livers showed that thrombin and PMNs interact in the genesis of LPS-induced liver injury (Moulin et al., 2001). Thrombin did not directly prime or activate PMNs *in vitro*, however, suggesting that it modulates PMN function by indirect mechanisms. In other cell types, activation of PAR-1 by thrombin stimulates production of many factors that regulate PMN function. For example, PAR-1 activation stimulates the release from cells of proinflammatory cytokines (Kranzhofer et al., 1996) and chemokines for PMNs (Ueno et al., 1996). In addition, activation of PAR-1 on endothelial cells upregulates several adhesion molecules for PMNs (Anrather et al., 1997; Kaplanski et al., 1997; Kaplanski et al., 1998; Sugama et al., 1992; Zimmerman et al., 1994). Interestingly, several of these proinflammatory events are required for LPS-induced liver injury (for review see Jaeschke and Smith, 1997).

In the studies presented herein, the hypothesis that thrombin can promote LPS-induced liver injury through PAR activation was tested. We show that peptide agonists (thrombin receptor-activating peptides; TRAPs) for PAR-1 reproduced the effect of thrombin during LPS-induced liver injury, suggesting that thrombin may promote injury through activation of PAR-1. Immunocytochemistry demonstrated that sinusoidal endothelial cells (SECs) and Kupffer cells express PAR-1. Inhibition of thrombin after LPS treatment failed to influence expression of PMN chemokines or ICAM-1 but did prevent PMN activation and hepatocellular injury. Accordingly, thrombin may promote LPS-induced liver injury through activation of PAR-1, which results in release of cytotoxic factors from PMNs.

Methods

Animals. Male, Sprague-Dawley rats (Crl:CD BR(SD) VAF/plus, Charles River, Portage, MI) weighing 250-350g were used in these studies. The animals were maintained on a 12 hr light/dark cycle under controlled temperature (18-21^0 C) and humidity (55±5%). Food (Rat chow, Teklad, Madison, WI) and tap water were allowed *ad libitum*. All procedures on animals were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* promulgated by the NIH.

Cell Isolation and Culture. Under pentobarbital anesthesia (50 mg/kg, i.p.), the abdominal cavity of the rat was opened, and the portal vein was cannulated and perfused with approximately 150 ml of Mg^{2+}-free, Ca^{2+}-free Hanks' balanced salt solution (Sigma, St. Louis, MO). The liver was then perfused with 375 ml of collagenase type H (Boehringer-Mannheim, Indianapolis, IN) containing 2.5% fetal bovine serum (FBS, Intergen, Purchase, NY), and the liver digest was collected and filtered through gauze. The digestion product was subsequently centrifuged at 50Xg for two minutes to pellet the hepatocytes. Kupffer cells and SECs remained in the supernatant. Hepatocytes were resuspended in Williams' medium E (Gibco, Grand Island, NY) containing 10% FBS and 1% gentamicin (Gibco) and plated in Falcon 4-well culture slides (Becton Dickinson, Franklin Lakes, NJ) at a density of 5X10^4/ml, 1 ml/well. After a three-hour attachment period, the medium with unattached cells was removed, and fresh, serum-free medium was added. Normally, 98% of the cells in the final preparation were hepatic parenchymal cells and the viability of the isolated hepatocytes was >90% by the criterion of trypan blue (Sigma) exclusion.

Kupffer cells and SECs were further purified from the supernatant (Braet et al., 1994). The supernatant was centrifuged at 350X*g* for 10 minutes, and the pellet was

resuspended in 20 ml of modified 1X PBS (phosphate-buffered saline, 10X modified PBS = 0.1 M Na_2HPO_4, 0.03 M KH_2PO_4, 1.6 M NaCl, pH 7.4). Twenty-five and 50% percoll solutions (Sigma) were made by diluting a stock percoll solution (10 ml 10X modified PBS and 90 ml percoll) with 1X modified PBS. The cells (10 ml) were layered on a percoll gradient consisting of 15 ml 50% percoll (bottom) and 20 ml of 25% percoll (top), and the gradient was spun for 20 minutes at 900X*g*. The SECs (contained in a band at the interface of the 25% and 50% solutions) and Kupffer cells (contained in the 50% percoll solution) were removed and diluted with an equal volume of 1X modified PBS and centrifuged 10 minutes at 900X*g*. Kupffer cells were resuspended in RPMI-1640 medium (Sigma) containing 10% FBS and gentamicin and were plated in Falcon 4-well culture slides at a density of $1X10^5$ cells/ml, 1ml/well. The cells were allowed to plate for 15 minutes. The cells were then washed to remove unattached cells and fresh medium added. Normally, >95% of the cells in the final preparation were Kupffer cells as determined by immunohistochemical staining with the ED2 antibody (BioSource, Camarillo, CA), which binds to rat Kupffer cells but not other cell types in the liver. SECs were resuspended in MCDB-131 medium (Boehringer-Mannheim) containing 10% FBS and gentamicin and plated in 100 mm dishes for 15 minutes to allow attachment of contaminating Kupffer cells. Unattached cells were removed from the 100 mm dishes and plated in Falcon 4-well culture slides coated with rat tail collagen (Sigma) at a density of $8X10^5$ cells/ml, 1ml/well. Normally, >95% of the cells in the final preparation were sinusoidal endothelial cells as determined by immunohistochemical staining with the RECA-1 (rat endothelial cell antigen-1, Serotec, Raleigh, NC) antibody which binds to

rat endothelium but not other cell types. All cells were maintained in culture for 24 hours before immunostaining.

PMNs were isolated from the peritoneal cavity of rats by glycogen elicitation as described in detail previously (Ho et al., 1996). Rat aortic smooth muscle cells were kindly provided by Dr. Stephanie Watts (Michigan State University, East Lansing, MI). Rat platelets were isolated by centrifugation from blood (Pearson et al., 1995).

Isolation and Perfusion of Rat Livers. The recirculating perfusion system used in these experiments has been described in detail previously (Moulin et al., 1996). Experiments were performed using two identical systems, allowing simultaneous perfusion of treated and control livers.

Perfusion of Isolated Livers with Thrombin and TRAPs. Donor rats received an hepatotoxic dose of LPS ($96X10^6$ EU/kg, Sigma) as a bolus injection in the tail vein two hours before removal of the liver for perfusion (Fig. 1A). The specific activity of the LPS was $24X10^6$ EU/mg as determined using a kinetic, chromogenic modification of the *limulus amebocyte* lysate (LAL) assay from BioWhittaker (Walkersville, MA). Within 2 hours after treatment of rats with LPS, many critical inflammatory events (e.g., platelet and neutrophil accumulation, cytokine release) have occurred in the liver *in vivo*; however, activation of the coagulation system does not occur within the first 2 hours (Fig. 1B; Pearson et al., 1995). As shown in Figure 1B, coagulation activation (as marked by a decrease in plasma fibrinogen) occurs between 2 and 3 hours after LPS-treatment *in vivo*, and liver injury (increased ALT in plasma) begins shortly thereafter. Thus, the liver is not exposed to significant concentrations of activated coagulation factors before isolation for perfusion. Livers were removed as described above and perfused in a recirculating manner with Krebs-Henseleit buffer containing 2% bovine serum albumin. Human α-thrombin (0, 0.04, 0.4, 4 or 40 nM, 3048 NIH U/mg, Enzyme Research Laboratories,

Inc., South Bend, IN), SFFLRN (Ser-Phe-Phe-Leu-Arg-Asn, PAR-1 agonist, 10 μM, Multiple Peptide Systems, San Diego, CA), TFLLR (Thr-Phe-Leu-Leu-Arg, PAR-1 agonist, 10 μM, Multiple Peptide Systems) or the inactive, reverse sequence peptides (NRLFFS, Asn-Arg-Leu-Phe-Phe-Ser or RLLFT, Arg-Leu-Leu-Phe-Thr, 10 μM, Multiple Peptide Systems) were added to the perfusion medium. Perfusate samples (350 μL) were taken after 2 hours of perfusion for determination of ALT activity. At the end of perfusion, livers were perfused for ten minutes with 10% buffered formalin in a nonrecirculating manner. Liver slices were embedded in paraffin, and 6 μm sections were stained with hematoxylin and eosin.

Assessment of Hepatocellular Injury. Hepatic injury was evaluated by measuring the activity of ALT in the plasma or perfusion medium using Sigma kit 59-UV.

Immunohistochemistry. For ICAM-1 and PAR-1 immunostaining, livers were frozen in isopentane (Sigma) immersed in liquid nitrogen for 5 minutes. Sections of frozen liver were fixed in acetone (-20°C) for 5 minutes. Immunostaining was performed using Vectastain Elite ABC Kit as per manufacturer's recommendations (Vector Laboratories). Sections were incubated with either mouse anti-rat ICAM-1 (Accurate Chemical Co., Westbury, NY) diluted (1:1000) in PBS containing 10% horse serum (Vector Laboratories) or mouse anti-rat PAR-1 (Kaufmann et al., 1998) diluted (1:1000) in PBS containing 10% horse serum for 1 hour. ICAM-1 and PAR-1 were visualized in liver sections using Sigma Fast. Sections were counterstained with hematoxylin. For PMN, PAR-1 and ICAM-1 immunostaining, no staining was observed in controls in which the primary antibody or the secondary antibody was removed.

For immunostaining of cells for PAR-1, cells were fixed in acetone (-20°C, 5 minutes), blocked with 10% goat serum in PBS (blocking solution, 30 minutes), and then incubated with PAR-1 (1:500, 1 hour) antibody in blocking solution. Cells were then incubated with secondary antibody conjugated to Alexa 594 (red staining, Molecular Probes, Eugene, OR) in blocking solution containing 1 μg/ml DAP-I (4'6-diamidino-2-phenylindole dihydrochloride, blue nuclear staining, Molecular Probes) for 30 minutes. Cells were then counterstained with cell specific antibodies to ensure that the appropriate cell type was being visualized. Platelets were immunostained with rabbit anti-platelet polyclonal antibody (Pearson et al., 1995), Kupffer cells with ED-2 (1:500, 1 hour), sinusoidal endothelial cells with RECA-1 (1:20, 18 hours), and PMNs with rabbit anti-rat PMN polyclonal antibody (1:4000, 16 hours; Hewett et al., 1992). No staining was observed in controls in which the primary or secondary antibody was eliminated from the staining protocol. Parenchymal cells were confirmed by morphological evaluation.

Western analysis. Cells were washed with PBS followed by lysis on ice with lysis buffer (0.01 M dibasic sodium phosphate, pH 7.2, 0.15 M sodium chloride, 10% Triton X-100, 12.7 mM deoxycholate, 1 mM sodium fluoride, 100 ug/ml phenylmethylsulphonyl fluoride, 100 ug/ml aprotinin, 1 mM sodium orthovanadate, 1 ug/ml Pepstatin, and 1 ug/ml Leupeptin). Protein concentrations of the samples were determined using the BCA assay (Pierce, Rockford, IL). Aliquots (30 μg) of cell lysates were analyzed by 10% SDS-polyacrylamide gel electrophoresis, and protein in the gel was transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA). The blot was then probed with mouse monoclonal antibody to PAR-1 followed by incubation with goat, antimouse antibody conjugated to horseradish peroxidase (Santa Cruz

Biotechnology, Santa Cruz, CA). The bands were detected using the ECL Western blotting detection kit (Amersham, Arlington Heights, IL).

Analysis of CINC-1 and MIP-2 mRNA Levels. Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) was used to estimate changes in mRNA levels for CINC-1 (cytokine-induced neutrophil chemoattractant-1) and MIP-2 (macrophage inflammatory protein-2). PrimeScreen rat chemokine primer pairs for CINC-1 and MIP-2 were purchased from BioSource (Camirillo, CA). 18S RNA was amplified and used as an internal standard in this semi-quantitative RT-PCR analysis (QuantumRNA 18S internal standards, Ambion, Inc., Austin, TX). RT-PCR was performed on total liver RNA as described in detail previously (Cho et al., 1999). RT-PCR cycling conditions were 4 minute incubation at 95°C, followed by a 3-step temperature cycle, denaturation at 95°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds, for 35 cycles. A final extension step at 72°C for 7 minutes was included after the final cycle to complete polymerization.

The abundance of chemokine mRNAs was semiquantitatively determined by densitometric analysis of ethidium bromide-stained agarose gels (3%, Nusieve:agarose = 3:1) using a Gel Doc 1000 analysis system (BioRad Laboratories, Inc., Hercules, CA) and Molecular Analyst Software Version 2.1. The volume density of the chemokine cDNA band was divided by the volume of the 18S cDNA band.

Measurement of CINC-1, MIP-2 and Elastase. CINC-1 (Assay Designs, Inc., Ann Arbor, MI), and MIP-2 (BioSource) were measured in the plasma using enzyme-linked immunosorbent assays (ELISA).

PMN elastase concentration was measured in the plasma and in liver perfusate by ELISA. Briefly, diluted aliquots of plasma, perfusion medium and elastase standards (Calbiochem, San Diego, CA) were plated on Immulon-4 ultra-high binding 96 well plates (Thermo Labsystems, Franklin, MA) and incubated at 37°C for 18 hours. The wells were washed with PBS and then incubated with PBS containing 3% goat serum (Vector) for 30 minutes at 37°C. PMN elastase antibody (Calbiochem) diluted 1:1000 in PBS containing 3% goat serum was added to the wells and incubated for 1.5 hours at 37°C. The wells were washed and the remaining steps were performed using the Vectastain Elite ABC Kit as per manufacturer's recommendations (Vector Laboratories). Briefly, anti-rabbit secondary antibody was added to the wells and incubated at room temperature for 30 minutes. The wells were washed 4 times, and ABC reagent was added to the wells and incubated at room temperature for 30 minutes. Tetramethylbenzidine (Sigma) was added to the wells and incubated for 30 minutes followed by addition of 1 N sulfuric acid (stop solution). The absorbance at 450 nm was measured in each well using a PowerWave$_X$340 plate reader (BioTek Instruments, Winooski, VT). The concentration of PMN elastase in each sample was determined from a standard curve.

Statistical analysis. Results are presented as the mean $\pm$ standard error of the mean (SEM). For all studies, *n* represents the number of repetitions of the experiment, each repetition consisting of plasma samples, mRNA samples or a liver from a different rat. In the isolated liver studies, changes in ALT activity and elastase concentrations were analyzed using a one-way analysis of variance (ANOVA). Data from studies investigating the effect of heparin on CINC-1 and MIP-2 mRNA and protein levels and

plasma elastase levels were analyzed using a 2 X 2 multifactorial, completely random ANOVA. ANOVAs were performed on log-transformed data in instances in which variances were not homogeneous. Multiple comparisons were performed using Student-Newman-Keuls test. For all studies, the criterion for significance was $p<0.05$.

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RESULTS

Thrombin produces dose-dependent injury to perfused livers isolated from LPS-treated rats. To determine the concentration-dependence of liver damage in response to thrombin, livers from LPS-treated rats were perfused with buffer containing various concentrations of thrombin. In these studies, rats were treated with LPS 2 hours before removal of the liver for perfusion. At this time, the liver has been exposed to many critical inflammatory factors *in vivo*; however, neither activation of the coagulation system nor liver injury has occurred (Pearson et al., 1995; Fig. 1). Perfusion of these livers *ex vivo* for an additional 2 hours with perfusion medium alone resulted in minimal ALT activity in the recirculating medium (Fig. 2). Addition of thrombin to the perfusion medium caused dose-dependent hepatocellular injury as measured by ALT release (Fig. 2). The EC_{50} was approximately 0.4 nM and the maximum effect of thrombin occurred at 4 nM. Perfusion of naive rat livers with buffer alone or with buffer containing 40 nM thrombin resulted in medium ALT activity of 42±11 and 90±40 U/L, respectively, after 2 hours of perfusion. These values were not significantly different.

TRAPs cause injury to perfused livers isolated from LPS-treated rats. Isolated livers from rats treated with LPS 2 hours earlier were perfused with buffer containing SFFLRN, TFLLR or the inactive, reverse-sequence peptides (NRLFFS or RLLFT; controls). A pronounced increase in ALT activity was detected in the perfusion medium from livers perfused for 2 hours with either SFFLRN or TFLLR compared to those perfused with the control peptides (Fig. 3). The amount of ALT released was comparable to that obtained by perfusing similar livers with 4.0 nM thrombin (Fig. 2).

Typical in the livers isolated from LPS-treated rats and perfused with TRAPs was midzonal hepatocellular necrosis and PMN accumulation both within and outside of the foci of necrosis. Figure 4A shows a representative photomicrograph of an SFFLRN-perfused liver. Similar results were observed in TFLLR-perfused livers (data not shown).

In about half of the livers, the lesions extended from midzonal into centrilobular regions. The lesions comprised either groups of parenchymal cells with eosinophilic cytoplasm and small, darkly staining nuclei (pyknosis) or groups of cells with slightly smaller than normal nuclei and normally staining to slightly eosinophilic cytoplasm with large vacuoles. Some areas had adjacent lesions of each type, suggesting that they represented a continuum of severity. The lesions were much larger and more frequent in TRAP-perfused livers than the occasional lesions observed in control peptide-perfused livers. The midzonal necrosis that occurred in livers perfused with TRAPs resembled lesions produced *in vivo* by an hepatotoxic dose of LPS (Fig. 4B).

PAR-1 is Expressed in Rat Liver. The ability of PAR-1-specific TRAP to produce liver damage after LPS exposure suggests that thrombin may promote LPS-induced liver injury by activating PAR-1. Therefore, studies were conducted to determine which cell types in the liver express this receptor. First, frozen sections of rat liver were subjected to immunohistochemical staining for PAR-1, which appears as dark brown staining in the liver sections (Fig. 5A). Livers from naïve rats or from rats treated 6 hours earlier with saline had light and diffuse staining that was localized to the sinusoids. Other rats were treated with an hepatotoxic dose of LPS ($96X10^6$ EU/mg) before removal of the livers for analysis of PAR-1 to determine if LPS treatment alters PAR-1 protein levels. Livers from these rats had pronounced and specific staining that was localized in the sinusoids (Fig. 5B). The intensity of staining was much greater than that observed in livers from naive or saline-treated rats, which suggested that PAR-1 increased on sinusoidal cells during inflammatory liver injury.

Kupffer Cells and Sinusoidal Endothelial Cells Express PAR-1. To determine which cells in the liver express PAR-1, cells were isolated from livers, grown in culture and analyzed for PAR-1 using immunohistochemistry and Western blot analysis. Primary, rat aortic smooth muscle cells were used as a positive control for

PAR-1 immunohistochemical staining (Fig. 6A; red staining, PAR-1; blue staining, DNA). Inasmuch as previous studies showed that rat platelets do not express PAR-1 (Kinlough-Rathbone et al., 1993), rat platelets were used as a negative control (Fig. 6B). Immunohistochemical staining revealed the presence of PAR-1 on Kupffer cells (Fig. 6C) and sinusoidal endothelial cells (SECs, Fig. 6D), whereas, hepatic parenchymal cells (Fig. 6E) and PMNs (Fig. 6F) did not stain for PAR-1. An occasional signal was observed on parenchymal cells; however, this result did not occur consistently. These results were confirmed by Western blot analysis (Fig. 7), which showed that PAR-1 was expressed as a 66 kD protein (i.e., similar to rat aortic smooth muscle cells used as the positive control, lane 1) in both Kupffer cells and SECs immediately after isolation from the liver and after 24 hours in culture. PAR-1 expression was analyzed just after cell isolation from the liver to ensure that its expression did not change after the cells were placed in culture. A 52 kD band was observed inconsistently in the PMN lane; however, N-terminal protein sequence analysis of this band indicated that it was not PAR-1.

Thrombin is not Required for Induction of ICAM-1, CINC-1 or MIP-2 in the Liver after Exposure of Rats to LPS. Next, it was determined whether inhibition of thrombin *in vivo* would abrogate LPS-mediated induction of the PMN adhesion molecule, ICAM-1, and the PMN chemotactic factors CINC-1 and MIP-2 in the liver. In these studies, rats were treated with an hepatotoxic dose of LPS or an equal volume of saline. Heparin, an inhibitor of thrombin, was injected 1.5 hours after LPS. Livers were removed 6 hours later, and frozen sections were subjected to immunohistochemical staining for ICAM-1, which appears as dark brown staining (Fig. 8). In addition, levels of CINC-1 and MIP-2 mRNA in the liver and protein in the plasma were quantified. LPS administration produced significant hepatocellular injury in these studies as confirmed by measurement of plasma ALT (data not shown). In addition, the dose of heparin used completely prevented LPS-induced hepatocellular injury, as described previously

(Hewett and Roth, 1995). ICAM-1 was not detected in livers from saline-treated rats (Fig. 8A), but the level of ICAM-1 dramatically increased in the liver sinusoids after treatment with LPS (Fig. 8B). This increase was not prevented by heparin administration (Fig. 8C).

Treatment with LPS caused upregulation of CINC-1 (Fig. 9A) and MIP-2 (Fig. 9B) mRNAs in the liver by 2 hours after treatment. Upregulation of both chemokines was unaffected by heparin treatment (Figs. 9A and 9B). Plasma levels of CINC-1 (Fig. 9C) and MIP-2 (Fig. 9D) protein were increased by 2 hours after treatment with LPS. These increases were not prevented by heparin (Figs. 9C and 9D).

Thrombin and TRAPs Promote PMN Activation after LPS Exposure. PMN elastase is a serine protease contained within the azurophilic granules of PMNs that is released upon activation of these cells (Ho et al., 1996). Numerous investigators have used plasma elastase levels as a biomarker of PMN activation *in vivo*. In the present studies, rats were treated with an hepatotoxic dose of LPS with or without heparin as described above, and plasma PMN elastase was measured by ELISA 6 hours later. Plasma levels of elastase were increased 6 hours after treatment with LPS (Fig. 10A). This increase was completely prevented by heparin cotreatment (Fig. 10A).

To determine if activation of PAR-1 promotes PMN activation in livers from LPS-treated rats, rats were treated with an hepatotoxic dose of LPS. Livers were removed 2 hours later (i.e., before the onset of injury) and perfused as described above. Perfusion with buffer containing either SFFLRN or TFLLR (PAR-1 TRAPS) resulted in a significant increase in elastase levels in the medium compared to livers perfused with buffer containing the control peptides NRLFFS and RLLFT (Fig. 10B).

Discussion

Activation of the coagulation system occurs frequently in patients with gram-negative bacterial sepsis (Penner, 1998) and in animal models of endotoxemia (Margaretten et al., 1967; Hewett and Roth, 1995). In rats, coagulation system activation is required for LPS-induced liver injury. Studies have shown that thrombin is the critical component of the coagulation system necessary for LPS-induced liver injury and that it promotes liver injury independently of its role in fibrin clot formation (Hewett and Roth, 1995; Pearson et al., 1995; Pearson et al., 1996). However, its exact role has not been elucidated.

In cell cultures, thrombin activates cellular responses through receptor-mediated mechanisms at concentrations between 0.5-50 nM (De Caterina and Sicari, 1993). In the isolated, perfused liver thrombin addition caused LPS-induced hepatocellular injury, with an EC_{50} of approximately 0.4 nM (Fig. 2). This concentration is consistent with the hypothesis that thrombin promotes LPS-induced liver injury through activation of a cellular receptor.

One receptor that is activated by thrombin is PAR-1. To determine if activation of this receptor promotes LPS-induced liver injury, livers isolated from LPS-treated rats were perfused with buffer containing TRAPs. TRAPs are short peptides identical or similar to the N-terminal sequence of thrombin-cleaved PARs. These peptides are highly selective agonists for PAR receptors and bind to and activate the receptor but have no proteolytic activity. PAR activation by TRAPs reproduces the actions of thrombin at PARs but bypasses the need for receptor proteolysis (Chao et al., 1992). SFFLRN and TFLLR are TRAPs which activate PAR-1, and these were used to investigate whether or not the effects of thrombin in the isolated liver were linked to activation of this receptor.

SFFLRN is an agonist for the rat PAR-1 receptor and is identical to the N-terminal sequence of thrombin-cleaved rat PAR-1. This peptide can activate both PAR-1 and PAR-2 receptors (Blackhart et al., 1996). TFLLR is a specific agonist for the rat PAR-1 receptor and does not activate any of the other known PARs (Hollenberg et al., 1997). A pronounced increase in ALT activity occurred in media from livers perfused for two hours with either SFFLRN or TFLLR compared to those perfused with the inactive control peptides (Fig. 3). The magnitude of ALT release was comparable to that obtained by perfusing livers with 4 nM thrombin (see Fig. 2). Importantly, the histopathological changes that occurred in the liver after perfusion with TRAPs were very similar to those that occur *in vivo* after administration of an hepatotoxic dose of LPS to rats (Fig. 4). Although the SFFLRN TRAP can also activate PAR-2, the TFLLR TRAP can only activate PAR-1, which suggests that the effects of these peptides were mediated through activation of the PAR-1 receptor. These results support the hypothesis that one mechanism by which thrombin promotes LPS-induced liver injury is by activating PAR-1. Examining the effects of PAR-1 antagonists on the development of liver injury after LPS exposure *in vivo* would be of interest; however, these tools unfortunately are not yet commercially available. PAR-1 knockout mice are available (Darrow et 1996), although it is difficult to produce liver injury in mice with high doses of LPS unless they are sensitized with an additional agent such as galactosamine. Studies suggest that the mechanisms involved in the development of liver injury by LPS alone and LPS given with galactosamine are different (Mignon et al., 1999).

PAR-1 expression has not been analyzed in rat liver during inflammation. Therefore, we examined the localization of PAR-1 protein in the liver using immunohistochemistry. Livers from naive animals showed modest and diffuse sinusoidal

staining (Fig. 5). Livers from LPS-treated rats, however, had pronounced and specific staining that was localized to the sinusoids, and the intensity of the staining was greater than that observed in livers from naive rats (Fig. 5). This result suggests that PAR-1 is upregulated on cells within the sinusoids of the liver during LPS-induced liver injury. Similar upregulation of PAR-1 has been observed in skeletal muscle during inflammation in which TNF-α and IL-1β induced PAR-1 on myotubules (Mbebi et al., 2001). Interestingly, both of these proinflammatory cytokines are released after injection of LPS in rats and may contribute to induction of PAR-1 in the liver after LPS-treatment.

To determine which cells in the liver express PAR-1, cells were isolated from livers, grown in culture and analyzed for PAR-1 using immunocytochemistry and Western blot analysis. The results revealed that sinusoidal endothelial cells and Kupffer cells express PAR-1, whereas hepatic parenchymal cells, PMNs and platelets do not (Fig. 6 and 7). Although hepatic stellate cells were not analyzed in this study, others have shown that human stellate cells express the PAR-1 receptor (Marra et al., 1998). Activation of PAR-1 on any one or all of these sinusoidal cell types could contribute to LPS-induced liver injury.

PMNs accumulate rapidly in the liver after LPS treatment (Pearson et al., 1995, see Fig. 1), and these cells are required for hepatocellular injury (Jaeschke et al., 1991; Hewett et al., 1992). Our previous results suggested that thrombin interacts with PMNs in the genesis of LPS-induced liver injury (Moulin et al., 2001). In those studies, however, thrombin was unable to activate PMNs directly or prime them for activation by other agents, suggesting that thrombin promotes PMN-dependent liver injury by indirect mechanisms. Through activation of PAR-1, thrombin regulates many mediators that modulate PMN function. For example, thrombin can mediate firm adhesion of PMNs to vascular endothelial cells through induction of ICAM-1 (Sugama et al., 1992), and this

adhesion molecule appears to be responsible for firm adhesion and transmigration of PMNs in the liver vasculature (Jaeschke and Smith, 1997). Therefore, whether inhibition of thrombin *in vivo* would abrogate LPS-mediated induction of ICAM-1 in the liver was determined. ICAM-1 was increased in the liver vasculature after LPS treatment (Fig. 8); however, this increase was not prevented by inhibition of thrombin with heparin (Fig. 8).

Chemokines are chemotactic factors for PMNs that may be necessary for transendothelial migration of PMNs from the liver sinusoid into the hepatic parenchyma, which is a requirement for LPS-induced liver injury (Jaeschke and Smith, 1997). Thrombin can stimulate production of the human PMN chemotactic factor IL-8 from endothelium (Ueno et al., 1996). In addition, during hepatic ischemia-reperfusion, inhibition of coagulation system activation attenuated production of the rat PMN chemokine, CINC-1 (Yamaguchi et al., 1997). Therefore, studies were conducted to determine if inhibition of thrombin *in vivo* would prevent upregulation of the PMN chemokines, CINC-1 and MIP-2, in liver after LPS treatment. CINC-1 and MIP-2 mRNAs were upregulated in liver, and these cytokines increased in the plasma after LPS treatment (Fig. 9). Upregulation of neither chemokine, however, was prevented by heparin treatment. These studies suggest that thrombin is not required for upregulation of CINC-1 and MIP-2 in liver after LPS treatment.

Although inhibition of thrombin did not prevent upregulation of ICAM-1 or PMN chemokines, it did prevent PMN activation after LPS treatment. As discussed, PMN elastase is a serine protease contained within the azurophilic granules of PMNs, and it is released upon activation of these cells. This protease damages hepatic parenchymal cells *in vitro* (Ho et al., 1996), and PMN elastase inhibitors prevent LPS-induced

hepatocellular injury *in vivo* (Ishii et al., 2002). Therefore, release of this protease from activated PMNs *in vivo* is important for the pathogenesis of LPS-induced liver injury. Many investigators use plasma elastase levels as an *in vivo* biomarker of PMN activation. In the present studies, treatment of rats with LPS caused an increase in plasma PMN elastase, indicating PMN activation (Fig. 10A). This increase was completely prevented by heparin treatment, suggesting that it prevented PMN activation. Heparin can have many effects on factors that regulate PMN function, independent of thrombin inhibition, and it is possible that heparin prevented PMN activation through one of these nonspecific effects. However, the observation that perfusion of livers from LPS-treated rats with medium containing PAR-1 TRAPs caused the release of PMN elastase (Fig. 10B) supports the conclusion that activation of PAR-1 in the liver promotes PMN activation. The mechanism by which thrombin and activation of PAR-1 promote PMN activation after LPS exposure remains unknown, but it is possible that activation of PAR-1 stimulates the release of mediators or upregulates other adhesion molecules that are important for activation of PMNs. Additional studies will be required to explore these possibilities.

In summary, previous results pointed to thrombin as a critical mediator of LPS-induced liver injury and suggested that it may act in a manner independent of its ability to form occlusive fibrin clots. Studies herein showed that perfusion of livers from LPS-treated rats with buffer containing thrombin or PAR-1 TRAPs produced hepatocellular injury. Inhibition of thrombin prevented LPS-induced liver injury *in vivo* but did not prevent upregulation of ICAM-1, CINC-1 or MIP-2. Inhibition of thrombin did, however, prevent PMN activation, and perfusion of livers from LPS-treated rats with PAR-1 TRAPs promoted PMN activation. These studies suggest that thrombin, through

activation of PAR-1, promotes PMN activation that results in hepatic parenchymal cell injury.

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FIGURE LEGENDS

Fig. 1. Protocol and rationale for isolated, perfused liver experiments. (A) Isolated liver protocol. LPS ($96X10^6$ EU/kg, iv) was injected into rats. Two hours later, livers were isolated and perfused for 2 hours with buffer containing thrombin or TRAPs. (B) Events occurring *in vivo* after exposure of rats to LPS (Pearson et al., 1995). After treatment of rats with LPS there is a rapid accumulation of platelets and neutrophils in the liver and release of inflammatory mediators (i.e., TNF-α) into plasma. All of these events occur within the first 2 hours after LPS treatment. Activation of the coagulation system (as marked by a decrease in plasma fibrinogen) and hepatic parenchymal cell injury (increase in plasma ALT activity) occur later *in vivo*.

Fig. 2. Injury to livers isolated from LPS-treated rats and then perfused with thrombin. Donor rats received LPS ($96X10^6$ EU/kg, iv) two hours before liver isolation. Isolated livers were perfused in a recirculating manner with Krebs-Henseleit bicarbonate buffer containing 2% BSA as described in the Materials and Methods. Various concentrations of human α-thrombin were added to the perfusion buffer (n=9 for each thrombin concentration). At the end of two hours of perfusion, a sample of medium was taken for determination of ALT, a marker of hepatocellular injury. *Significantly different from livers perfused with thrombin-free medium (P<0.05).

Fig. 3. Injury to livers isolated from LPS-treated rats and perfused with TRAPs. Donor rats received LPS ($96X10^6$ EU/kg, iv) two hours before liver isolation. Isolated livers were perfused in a recirculating manner with Krebs-Henseleit bicarbonate buffer containing 2% BSA as described in Materials and Methods. SFFLRN (rat PAR-1 agonist, n=6), NRLFFS (control, n=6), TFLLR (rat PAR-1 agonist, n=4) or RLLFT (control, n=4) were added to the perfusion buffer (final concentration, 10 μM). After two

hours, aliquots of perfusate were removed for determination of ALT activity. *Significantly different from corresponding control peptide (P<0.05).

Fig. 4. Histopathological comparison of livers isolated (A) from LPS-treated rats and perfused with SFFLRN or (B) from rats treated with an hepatotoxic dose of LPS ($96X10^6$ EU/kg, iv) *in vivo* 6 hours earlier. Livers were fixed in buffered formalin, and 6-μm paraffin-embedded sections were stained with hematoxylin and eosin. Areas of hepatocellular necrosis are indicated by arrows. PP, periportal region; CL, centrilobular region; MZ, midzonal region. Scale bar, 100 μm. Representative photomicrographs from an n=4.

Fig. 5. Expression of PAR-1 in rat liver. Livers were isolated from (A) untreated rats or (B) rats treated with an hepatotoxic dose of LPS ($96X10^6$ EU/kg, iv) 6 hours earlier. Frozen sections were fixed in acetone and stained for PAR-1 (dark brown staining) using immunohistochemistry. Representative photomicrographs from groups of 3 rats.

Fig. 6. Expression of PAR-1 by isolated cells. Cultured rat aortic smooth muscle cells (A) were used as a positive control for PAR-1 staining, and rat platelets (B) were used as a negative control. Light microscopy and immunostaining using an anti-platelet antibody confirmed the presence of platelets in the field shown in (B). Kupffer cells (C), sinusoidal endothelial cells (D), and hepatic parenchymal cells (E) were isolated by collagenase perfusion of rat livers. PMNs (F) were isolated by glycogen-elicitation from the peritoneal cavity of rats. The identity of Kupffer cells, sinusoidal endothelial cells and PMNs were confirmed by immunostaining with cell specific antibodies and morphological evaluation as outlined in the Methods section. Cells were stained for PAR-1 with mouse anti-rat PAR-1 antibody followed by Alexa-594-labeled goat anti-

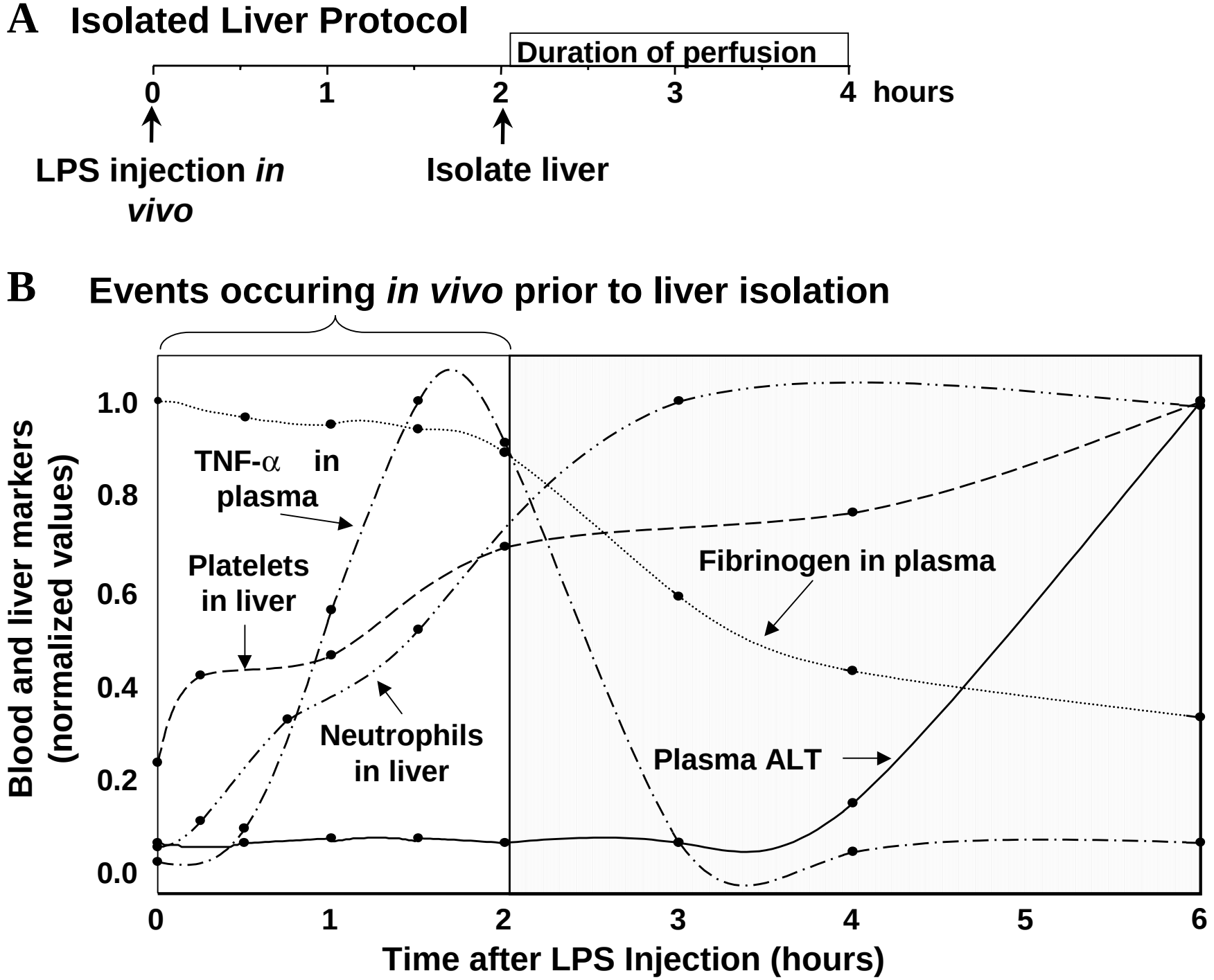
mouse secondary antibody containing DAP-I. Positive PAR-1 staining is red. Blue staining is DAP-I, which stains nuclear DNA. Representative photomicrographs from cells from 3 rats.

Fig. 7. Western blot analysis of PAR-1 expression in isolated cells. PMNs and platelets were analyzed for PAR-1 after isolation from the rat. Hepatocytes, Kupffer cells and SECs were analyzed for PAR-1 either after isolation (0 hour) from the rat or after 24 hours in culture (24 hours). Representative Western blot from cells from 3 rats.

Fig. 8. Effect of heparin on LPS-mediated induction of ICAM-1 in rat liver. Male rats were treated with (A) saline, (B) LPS ($96X10^6$ EU/kg, iv), or (C) LPS ($96X10^6$ EU/kg, iv) followed by 2000 U/kg heparin 1.5 hours after LPS. Six hours after saline or LPS, livers were removed and frozen sections were stained for ICAM-1 (dark brown-staining) using immunohistochemistry. Representative photomicrograph from groups of 3 rats.

Fig. 9. Effect of heparin on LPS-induced upregulation of CINC-1 and MIP-2 mRNA and protein levels. Rats were treated with either LPS ($96X10^6$ EU/kg, iv) or saline vehicle. They then received either 2000 U/kg heparin or saline 1.5 hours after LPS. Total RNA was isolated from livers 2, 3, 4 and 6 hours after LPS treatment and (A) CINC-1 and (B) MIP-2 mRNAs were analyzed by RT-PCR. Plasma (C) CINC-1 and (D) MIP-2 proteins were analyzed by ELISA 2, 4, and 6 hours after LPS treatment. Data from saline-treated rats evaluated at different times were combined into 1 group because no differences were observed among them. Data are expressed as means $\pm$ SEM; n = 4 rats/group.
*Significantly different ($p<0.05$) from corresponding saline-treated control.

Fig. 10. Effect of heparin and TRAPs on activation of PMNs after LPS treatment. (A) Rats were treated with either LPS ($96X10^6$ EU/kg, iv) or saline vehicle. They then received either 2000 U/kg heparin or saline 1.5 hours after LPS. PMN elastase concentrations were measured in the plasma by ELISA 6 hours after LPS treatment. *Significantly different ($p<0.05$) from corresponding saline-treated control. **Significantly different ($p<0.05$) from rats treated with LPS and saline. (B) Donor rats received LPS ($96X10^6$ EU/kg, iv) two hours before liver isolation. Isolated livers were perfused in a recirculating manner and SFFLRN (rat PAR-1 agonist, $n=6$), NRLFFS (control, $n=6$), TFLLR (rat PAR-1 agonist, $n=4$) or RLLFT (control, $n=4$) were added to the perfusion buffer (final concentration, 10 μM). After two hours, PMN elastase concentrations were measured in the perfusate by ELISA. *Significantly different from corresponding control peptide ($P<0.05$).



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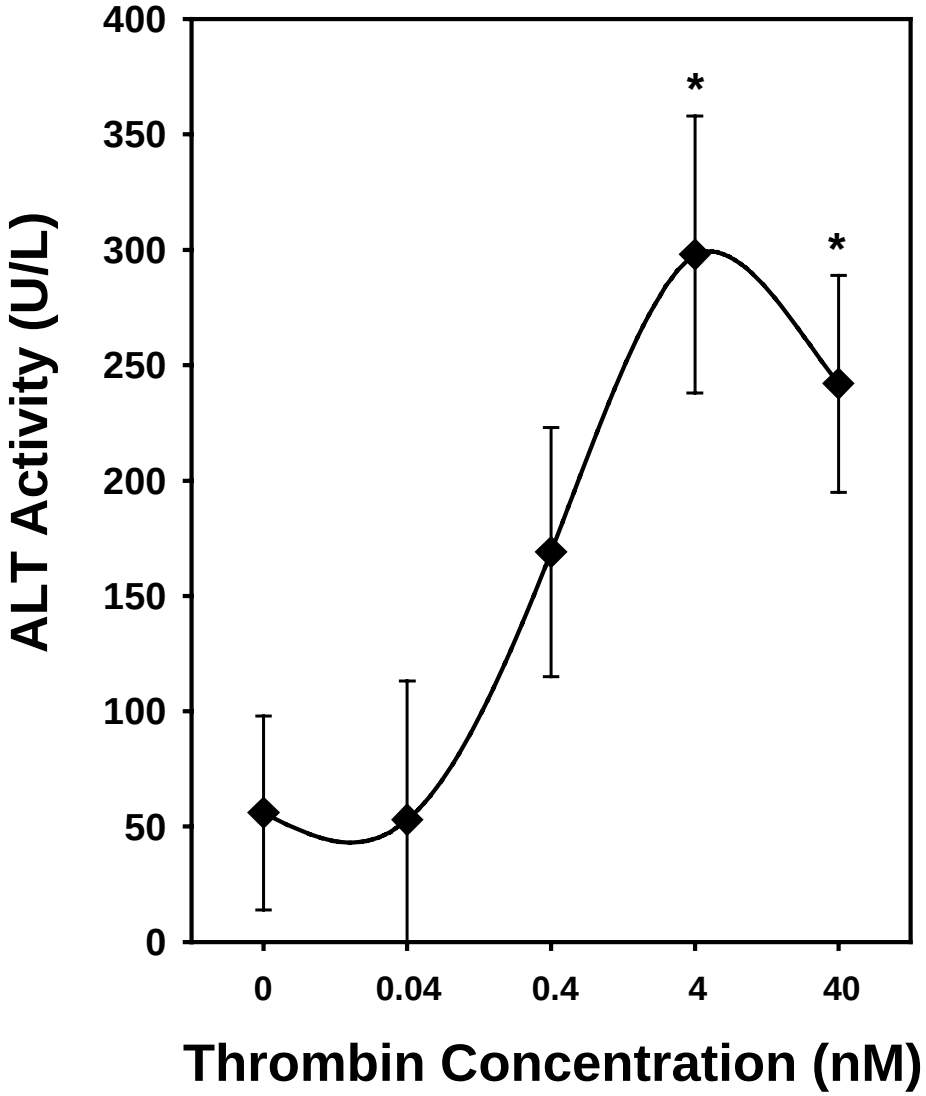

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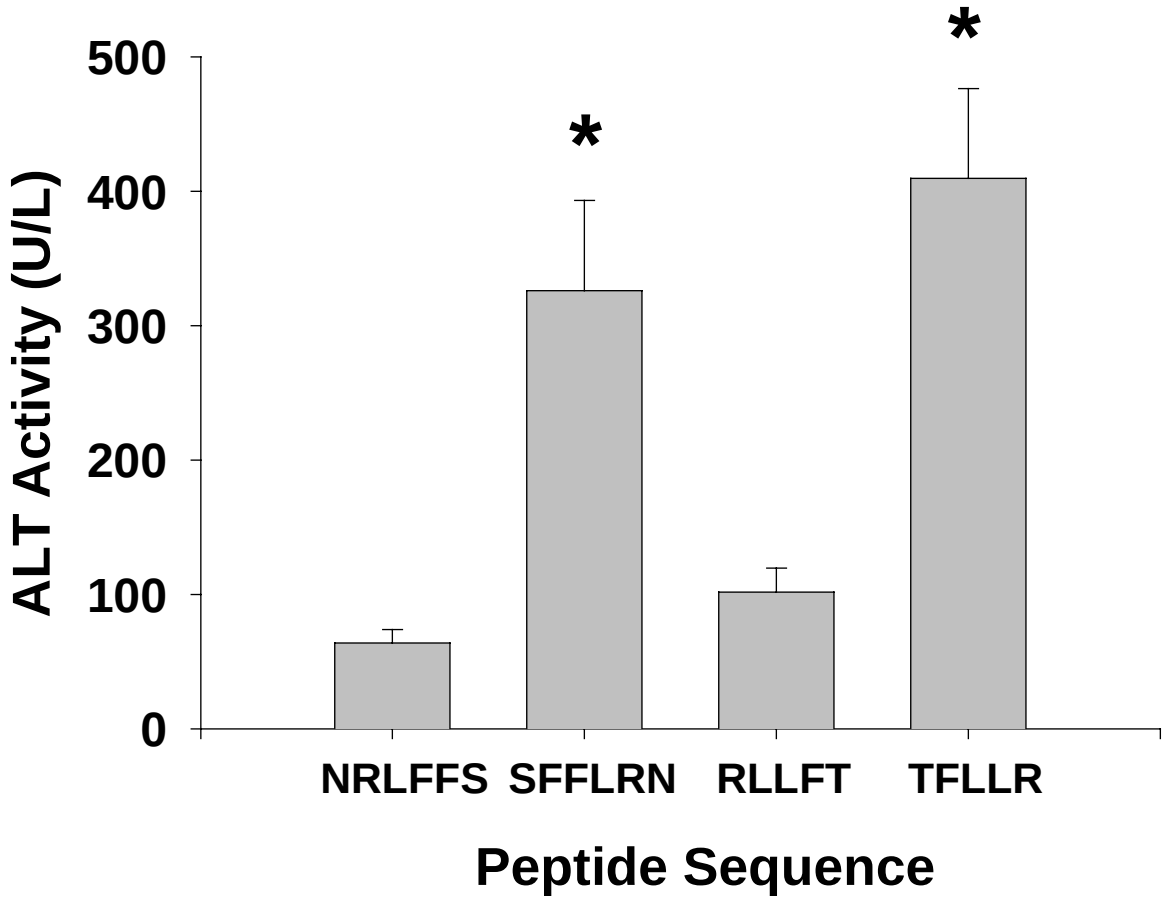

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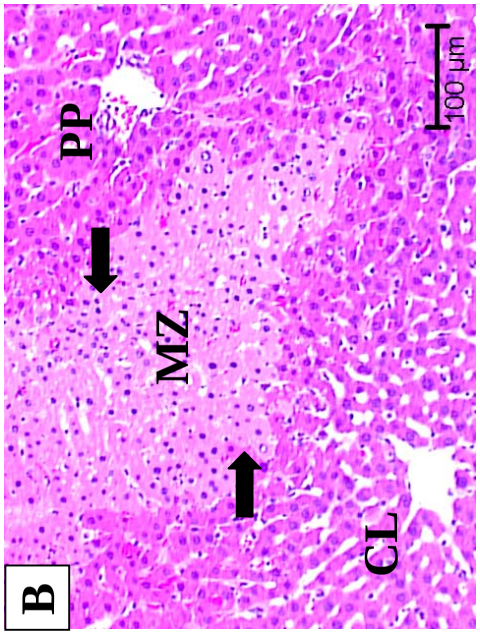

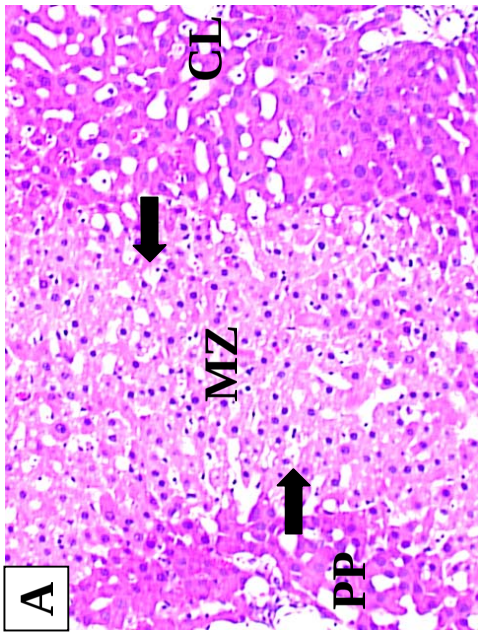

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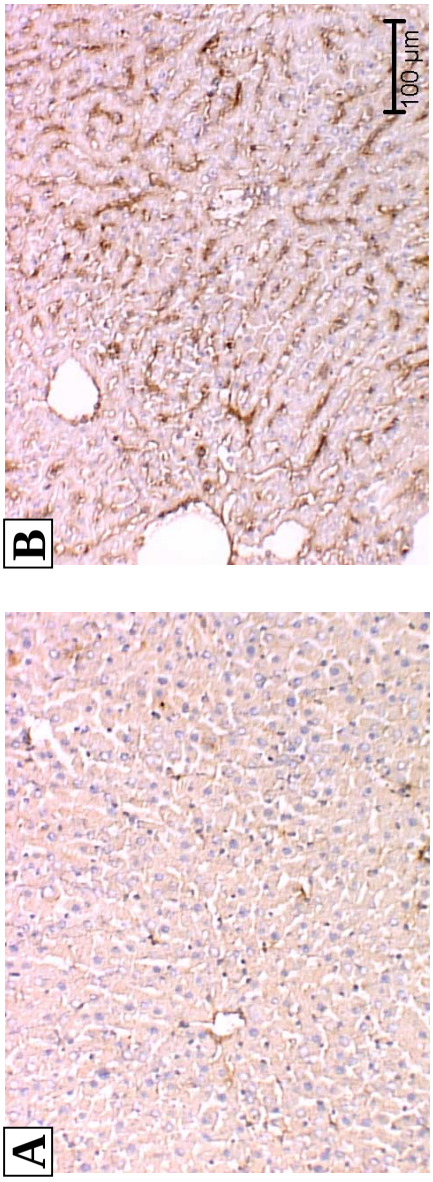

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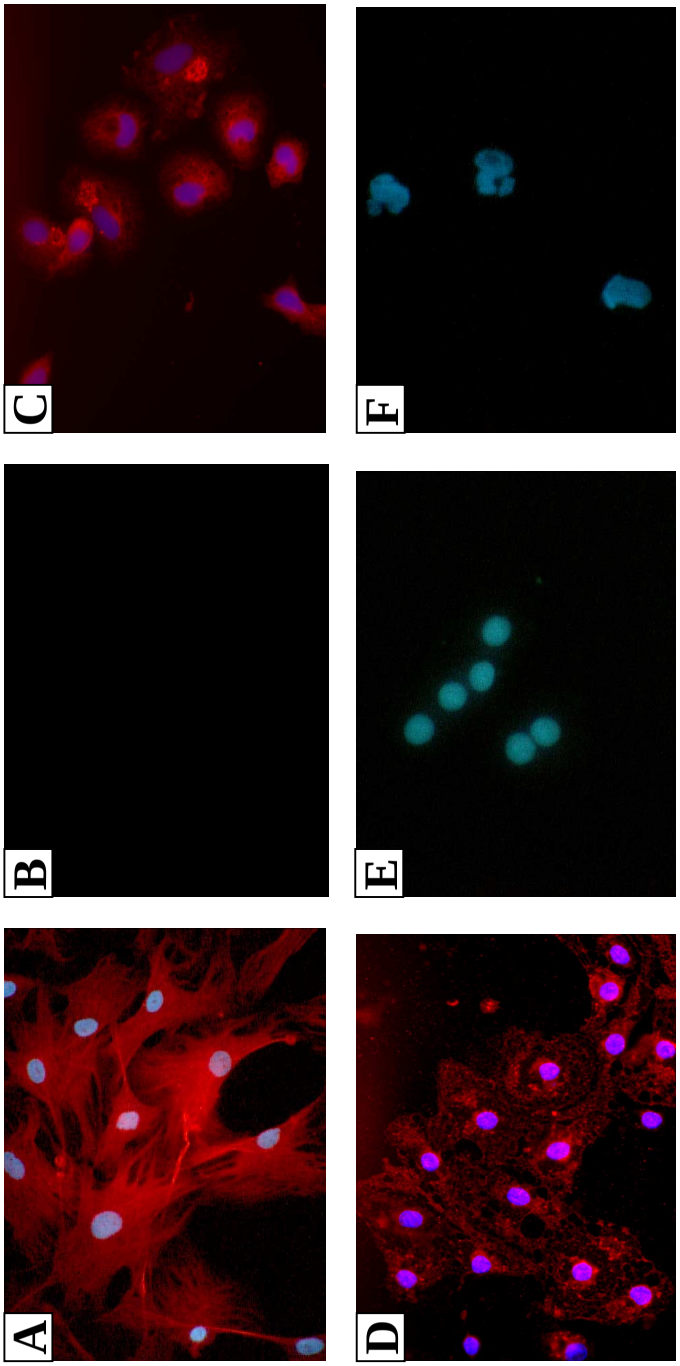

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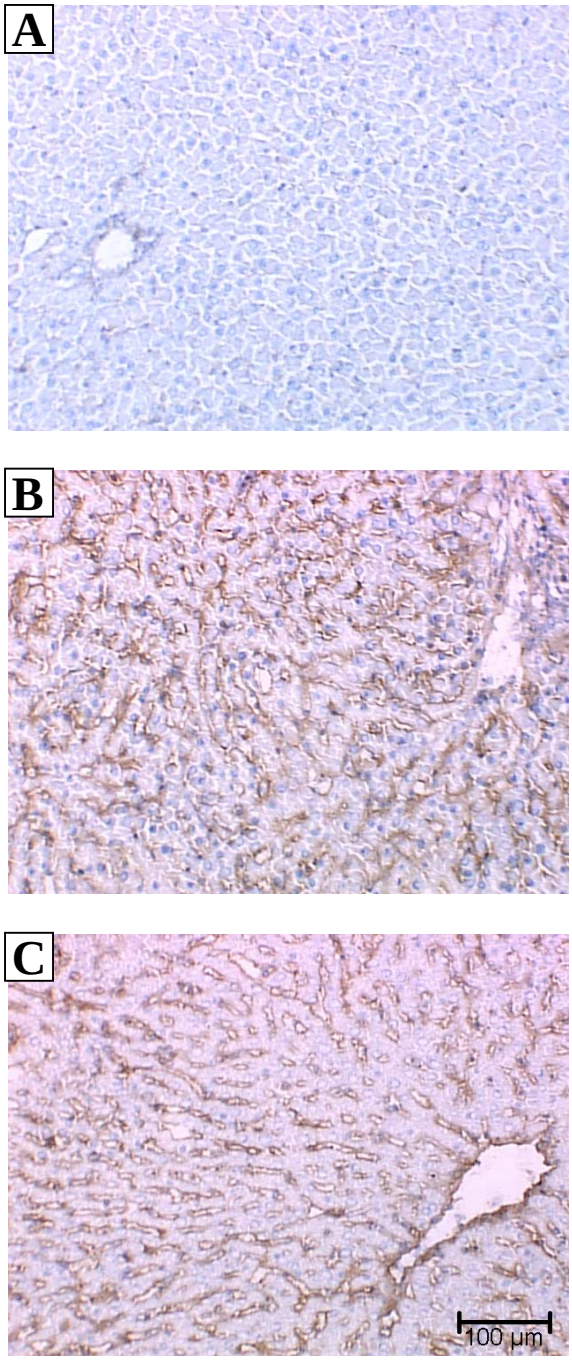

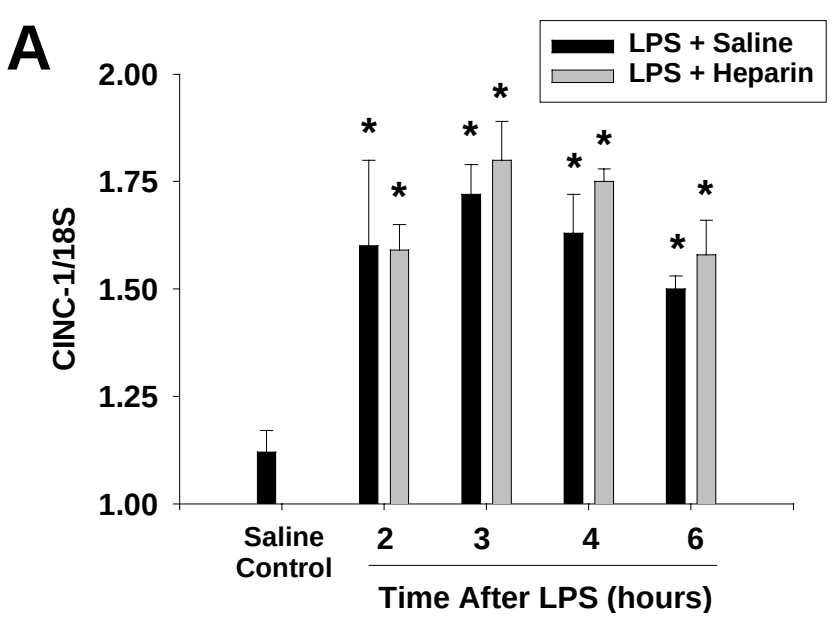

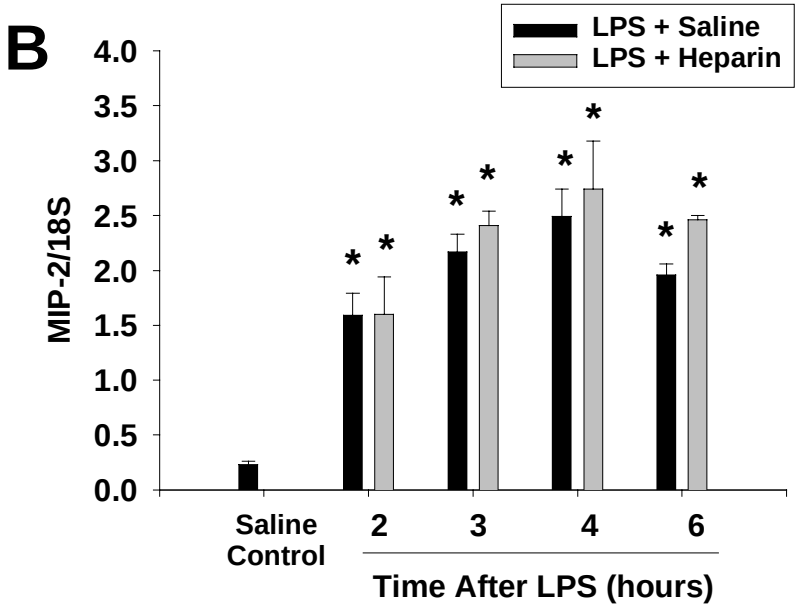

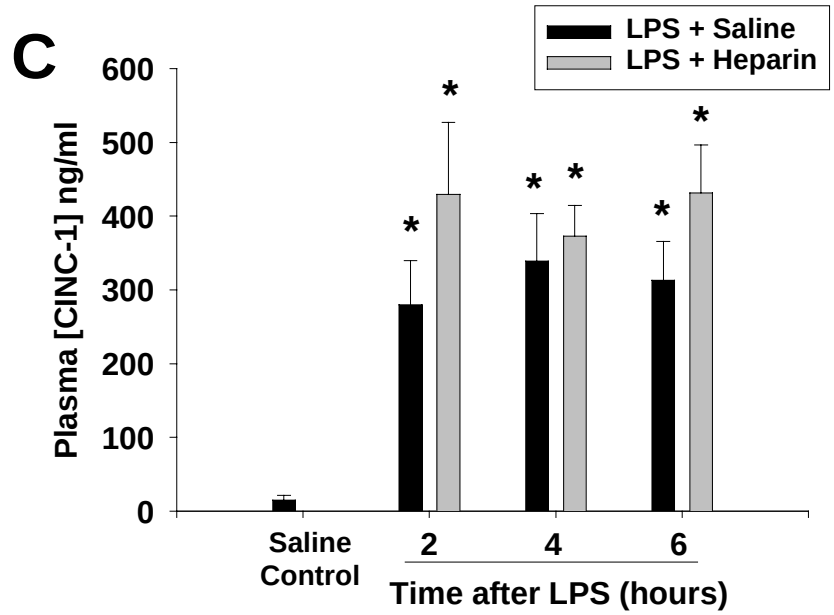

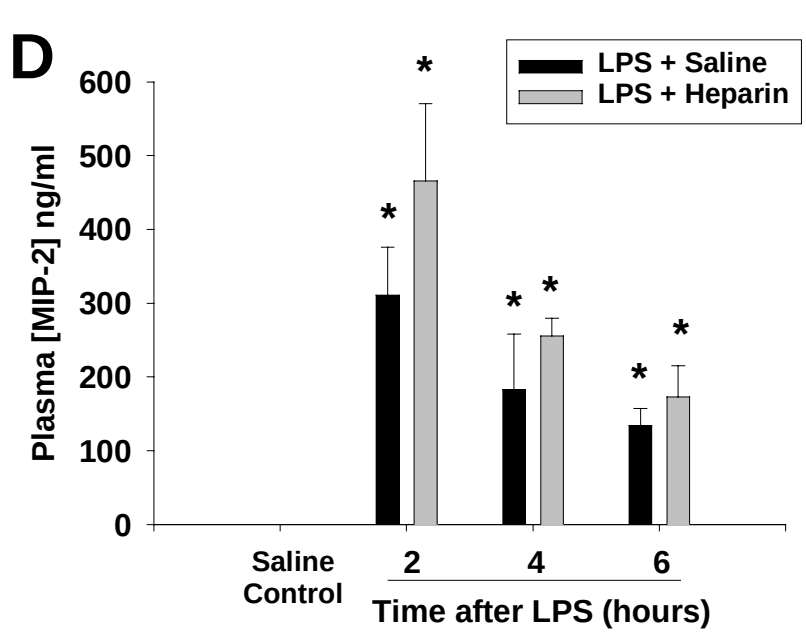

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