Thrombin and Protease-Activated Receptor-1 Agonists Promote Lipopolysaccharide-Induced Hepatocellular Injury in Perfused Livers

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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} value 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 up-regulation of cytokine-induced neutrophil chemoattractant-1, macrophage inflammatory protein-2, or intercellular adhesion molecule-1. Thrombin inhibition did, however, prevent neutrophil (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 (Jaeschke et al., 1991; Hewett et al., 1993; Imuro et al., 1994; Hewett and Roth, 1995; 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, 2001). The mechanism of thrombin’s action and how it relates to other mediators, however, remains 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

ABBREVIATIONS: LPS, lipopolysaccharide; PMN, neutrophil; PAR, protease-activated receptor; TRAP, thrombin receptor-activating peptide; SEC, sinusoidal endothelial cell; ICAM-1, intercellular adhesion molecule-1; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SFFLRN, Ser-Phe-Phe-Leu-Arg-Asn; TFLLR, Thr-Phe-Leu-Leu-Arg; NRLFSS, Asn-Arg-Leu-Phe-Phe-Ser; RLLFT, Arg-Leu-Leu-Phe-Thr; ALT, alanine aminotransferase; DAPI, 4’6-diamidino-2-phenylindole dihydrochloride; CINC-1, cytokine-induced neutrophil chemoattractant-1; MIP-2, macrophage inflammatory protein-2; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; EU, endotoxin unit.
(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 up-regulates several adhesion molecules for PMNs (Sugama et al., 1992; Zimmerman et al., 1994; Anrather et al., 1997; Kaplanski et al., 1997, 1998). 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 promotes 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.

Materials and Methods

Animals. Male, Sprague-Dawley rats (Cr:CD BR(SD) VAF/plus; Charles River, Portage, MI) weighing 250 to 350 g were used in these studies. The animals were maintained on a 12-h light/dark cycle under controlled temperature (18–21°C) and humidity (55 ± 5%). Food (Rat Chow; Teklad, Madison, WI) and tap water were added 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 National Institutes of Health.

Cell Isolation and Culture. Under pentobarbital anesthesia (50 mg/kg i.p.), the abdominal cavity of the rat was opened, and the liver is not exposed to significant concentrations of activated coagulation factors before isolation for perfusion. Livers were revascularized 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 a hepatotoxic dose of LPS (96 × 10⁶ EU/kg; Sigma-Aldrich) as a bolus injection in the tail vein 2 h before removal of the liver for perfusion (Fig. 1A). The specific activity of the LPS was 24 × 10⁶ EU/mg as determined using a kinetic, chromogenic modification of the Limulus amebocyte lysate assay from BioWhittaker (Walkersville, MA). Within 2 h 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 h (Fig. 1B; Pearson et al., 1995). As shown in Fig. 1B, coagulation activation (as marked by a decrease in plasma fibrinogen) occurs between 2 and 3 h 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 (Moulin et al., 1996) 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); Ser-Phe-Phe-Leu-Arg-Asn (SPFLLRN, PAR-1 agonist, 10 μM; Multiple Peptide Systems, San Diego, CA); Thr-Phe-Leu-Leu-Arg (TFLLR, 4.102 EU/mg, as determined with a kinetic chromogenic assay from BioWhittaker) was added to the perfusate 2 h after liver isolation.

Kupffer cells and SECs were further purified from the supernatant (Braet et al., 1994). The supernatant was centrifuged at 350 × g for 2 min, and the pellet was resuspended in 50 ml modified PBS and 90 ml of Percoll with 1 × modified PBS. The cells (10 ml) were layered on a Percoll gradient consisting of 15 ml of 50% Percoll (bottom) and 20 ml of 25% Percoll (top), and the gradient was spun for 20 min at 900g. 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 1 × modified PBS and centrifuged 10 min at 900g. Kupffer cells were resuspended in RPMI 1640 medium (Sigma-Aldrich) containing 10% FBS and gentamicin and were plated in Falcon 4-well culture slides at a density of 1 × 10⁵ cells/ml, 1 ml/well. The cells were allowed to plate for 15 min. The cells were then washed to remove unattached cells and fresh medium added. Normally, ≥95% of the cells in the final preparation were hepatic parenchymal cells, and the viability of the isolated hepatocytes was >80% by the trypan blue (Sigma-Aldrich) exclusion method.

Kupffer cells and SECs were further purified from the supernatant (Braet et al., 1994). The supernatant was centrifuged at 350 × g for 2 min, and the pellet was resuspended in 20 ml of modified 1× phosphate-buffered saline (PBS) (10 × modified PBS = 0.1 M Na₂HPO₄, 0.03 M KH₂PO₄, 1.6 M NaCl, pH 7.4). Twenty-five and 50% Percoll solutions (Sigma-Aldrich) were made by diluting a stock Percoll solution (10 ml of 10× modified PBS and 90 ml of Percoll) with 1× modified PBS. The cells (10 ml) were layered on a Percoll gradient consisting of 15 ml of 50% Percoll (bottom) and 20 ml of 25% Percoll (top), and the gradient was spun for 20 min at 900g. 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 1× modified PBS and centrifuged 10 min at 900g. Kupffer cells were resuspended in RPMI 1640 medium (Sigma-Aldrich) containing 10% FBS and gentamicin and were plated in Falcon 4-well culture slides at a density of 1 × 10⁵ cells/ml, 1 ml/well. The cells were allowed to plate for 15 min. The cells were then washed to remove unattached cells and fresh medium added. Normally, ≥95% of the cells in the final preparation were sinusoidal endothelial cells as determined by immunohistochemical staining with the ED2 antibody (BioSource International, Camarillo, CA), which binds to rat Kupffer cells but not other cell types in the liver. SECs were resuspended in MCDB-131 medium (Roche Diagnostics) containing 10% FBS and gentamicin and plated in 100-mm dishes for 15 min 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-Aldrich) at a density of 5 × 10⁶ cells/ml, 1 ml/well. Normally, ≥95% of the cells in the final preparation were sinusoidal endothelial cells as determined by immunohistochemical staining with 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 h 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 a hepatotoxic dose of LPS (96 × 10⁶ EU/kg; Sigma-Aldrich) as a bolus injection in the tail vein 2 h before removal of the liver for perfusion (Fig. 1A). The specific activity of the LPS was 24 × 10⁶ EU/mg as determined using a kinetic, chromogenic modification of the Limulus amebocyte lysate assay from BioWhittaker (Walkersville, MA). Within 2 h 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 h (Fig. 1B; Pearson et al., 1995). As shown in Fig. 1B, coagulation activation (as marked by a decrease in plasma fibrinogen) occurs between 2 and 3 h 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 (Moulin et al., 1996) 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); Ser-Phe-Phe-Leu-Arg-Asn (SPFLLRN, PAR-1 agonist, 10 μM; Multiple Peptide Systems, San Diego, CA); Thr-Phe-Leu-Leu-Arg (TFLLR,
Fig. 1. Protocol and rationale for isolated, perfused liver experiments. A, isolated liver protocol. LPS (96 × 10^6 EU/kg i.v.) was injected into rats. Two hours later, livers were isolated and perfused for 2 h 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., tumor necrosis factor-α) into plasma. All of these events occur within the first 2 h 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.

PAR-1 agonist, 10 μM; Multiple Peptide Systems); or the inactive, reverse sequence peptides [Asn-Arg-Leu-Phe-Ser (NRLFFS) or Arg-Leu-Leu-Phe-Thr (RLLFT), 10 μM; Multiple Peptide Systems] were added to the perfusion medium. Perfusion samples (350 μl) were taken after 2 h of perfusion for determination of ALT activity. At the end of perfusion, livers were perfused for 10 min 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 a Widmer ALT assay (Widmer, Westbury, NY) diluted (1:1000) in PBS containing 10% horse serum (Vector Laboratories, Burlingame, CA). Sections were incubated with either mouse anti-rat ICAM-1 (Accurate Chemical & Scientific, 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 h. 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 min), blocked with 10% goat serum in PBS (blocking solution, 30 min), and then incubated with PAR-1 (1:500, 1 h) 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 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, blue nuclear staining; Molecular Probes) for 30 min. 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 h), sinusoidal endothelial cells with rat anti-endothelial cell antibodies (1:20, 18 h), and PMNs with rabbit anti-rat PMN polyclonal antibody (1:4000, 16 h; 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 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 mM sodium orthovanadate, 1 μg/ml pepstatin, and 1 μg/ml leupeptin). Protein concentrations of the samples were determined using the bicinchoninic acid assay ( assay Chemical, Rockford, IL). Aliquots (30 μg) of cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis, and protein in the gel was transferred to Immobilon polyvinylidene difluoride transfer membrane (Millipore Corporation, Bedford, MA). The blot was then probed with mouse monoclonal antibody to PAR-1 followed by incubation with goat anti-mouse antibody conjugated to horseradish peroxidase (Santa Cruz Biototechnology, Inc., Santa Cruz, CA). The bands were detected using the enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences, Inc., Piscataway, NJ).

Analysis of Cytokine-Induced Neutrophil Chemotrac tant-1 (CINC-1) and Macrophage Inflammatory Protein-2 (MIP-2) mRNA Levels. Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used to estimate changes in mRNA levels for CINC-1 and MIP-2. PrimeScreen rat chemokine primer sets for CINC-1 and MIP-2 were purchased from BIOSOURCE International. 18S RNA was amplified and used as an internal standard in this semiquantitative RT-PCR analysis (QuantumRNA 18S internal standards; Ambion, 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-min incubation at 95°C, followed by a three-step temperature cycle, denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s, for 35 cycles. A final extension step at 72°C for 7 min 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 kit (Bio-Rad, 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 International) 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 h. The wells were washed with PBS and then incubated with PBS containing 5% goat serum (Vector Laboratories) for 30 min 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 h 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 min. The wells were washed four times, and ABC reagent was added to the wells and incubated at room temperature for 30 min. Tetramethylbenzidine (Sigma-Aldrich) was added to the wells and incubated for 30 min followed by addition of 1 N sulfuric acid (stop solution). The absorbance at 450 nm was measured in each well.
using a PowerWave340 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 ± S.E.M. 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 × 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.

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 h 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 h 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} value 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 h 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 h 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 h with either SFFLRN or TFLLR compared with those perfused with the control peptides (Fig. 3). The amount of ALT released was comparable with that obtained by perfusing similarly treated 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 one-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 a 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

Fig. 2. Injury to livers isolated from LPS-treated rats and then perfused with thrombin. Donor rats received LPS (96 × 10^6 EU/kg i.v.) 2 h before liver isolation. Isolated livers were perfused in a recirculating manner with Krebs-Henseleit bicarbonate buffer containing 2% bovine serum albumin, as described under Materials and Methods. Various concentrations of human α-thrombin were added to the perfusion buffer (n = 8) for each thrombin concentration). At the end of 2 h 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 (96 × 10^6 EU/kg i.v.) 2 h before liver isolation. Isolated livers were perfused in a recirculating manner with Krebs-Henseleit bicarbonate buffer containing 2% bovine serum albumin, as described under 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) was added to the perfusion buffer (final concentration, 10 μM). After 2 h, aliquots of perfusate were removed for determination of ALT activity. *, significantly different from corresponding control peptide (P < 0.05).
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 shows as dark brown staining in the liver sections (Fig. 5A). Livers from naïve rats or from rats treated 6 h earlier with saline had light and diffuse staining that was localized to the sinusoids. Other rats were treated with a hepatotoxic dose of LPS (96 × 10^6 EU/kg i.v.) before removal of the livers for analysis of PAR-1 to determine whether 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 naïve 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). Immunohistochemical staining revealed the presence of PAR-1 on Kupffer cells (Fig. 6C) and 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-kDa 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 h in culture. PAR-1 expression was analyzed just after cell isolation from the liver to evaluate whether its expression did not change after the cells were placed in culture. A 52-kDa 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

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**Fig. 4.** Histopathological comparison of livers isolated from LPS-treated rats and perfused with SFFLRN (A) or from rats treated with a hepatotoxic dose of LPS (96 × 10^6 EU/kg i.v.) 6 h earlier (B). 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 untreated rats (A) or rats treated with a hepatotoxic dose of LPS (96 × 10^6 EU/kg i.v.) 6 h earlier (B). Frozen sections were fixed in acetone and stained for PAR-1 (dark brown staining) using immunohistochemistry. Representative photomicrographs from groups of three 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 from the peritoneal cavity of rats. The identity of Kupffer cells, sinusoidal endothelial cells, and PMNs was confirmed by immunostaining with cell-specific antibodies and morphological evaluation, as outlined under Materials and Methods. Cells were stained for PAR-1 with mouse anti-rat PAR-1 antibody followed by Alexa 594-labeled goat anti-mouse secondary antibody containing DAPI. Positive PAR-1 staining is red. Blue staining is DAPI, which stains nuclear DNA. Representative photomicrographs from cells from three rats.
were treated with a hepatotoxic dose of LPS or an equal volume of saline. Heparin, an inhibitor of thrombin, was injected 1.5 h after LPS. Livers were removed 6 h later, and frozen sections were subjected to immunohistochemical staining for ICAM-1, which shows 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 up-regulation of CINC-1 (Fig. 9A) and MIP-2 (Fig. 9B) mRNAs in the liver by 2 h after treatment. Up-regulation of both chemokines was unaffected by heparin treatment (Fig. 9, A and B). Plasma levels of CINC-1 (Fig. 9C) and MIP-2 (Fig. 9D) protein were increased by 2 h after treatment with LPS. These increases were not prevented by heparin (Fig. 9, C and D).

**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 a hepatotoxic dose of LPS with or without heparin as described above, and plasma PMN elastase was measured by ELISA 6 h later. Plasma levels of elastase were increased 6 h after treatment with LPS (Fig. 10A). This increase was completely prevented by heparin cotreatment (Fig. 10A).

To determine whether activation of PAR-1 promotes PMN activation in livers from LPS-treated rats, rats were treated with a hepatotoxic dose of LPS. Livers were removed 2 h 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 with 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, 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 and 50 nM (De Caterina and Sicari, 1993). In the isolated, perfused liver thrombin addition caused LPS-induced hepatocellular injury, with an EC$_{50}$ value 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 whether 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). SFLLRN and TFLLR are TRAPs that activate PAR-1, and these were used to investigate whether the effects of thrombin in the isolated liver were linked to activation of this receptor. SFLLRN 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 2 h with either SFLLRN or TFLLR compared with those perfused with the inactive control peptides (Fig. 3). The magnitude of ALT release was comparable with that obtained by perfusing livers with 4 nM thrombin (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 a hepatotoxic dose of LPS to rats (Fig. 4). Although the SFLLRN 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. PAR-1 knockout mice are available (Darrow et al., 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 up-regulated on cells within the sinusoids of the liver during LPS-induced liver injury. Similar up-regulation of PAR-1 has been observed in skeletal muscle during inflammation in which tumor necrosis factor-α and interleukin-1β induced PAR-1 on myotubes (Mbeki 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 (Figs. 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; 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 seems 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 interleukin-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 whether inhibition of thrombin in vivo would prevent up-regulation of the PMN chemokines CINC-1 and MIP-2 in liver after LPS treatment. CINC-1 and MIP-2 mRNAs were up-regulated in liver, and these cytokines increased in the plasma after LPS treatment (Fig. 9). Up-regulation of neither chemokine, however, was prevented by heparin treatment. These studies suggest that thrombin is not required for up-regulation of CINC-1 and MIP-2 in liver after LPS treatment.

Although inhibition of thrombin did not prevent up-regulation 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 non-specific 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 up-regulates 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 up-regulation 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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References


Kaplan D, Braet F, De Zanger R, Sasaoki T, Baekeland M, Janssens P, Smedsrod B, and Wisse E (1993) Thrombin-induced endothelial cell 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 medium containing 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.