Chronological expression of PAR isoforms in acute liver injury and its amelioration by PAR2 blockade in a rat model of sepsis

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Summary

The liver can be injured and its functions altered by activation of the coagulation and inflammatory processes in sepsis. The objective of the present study was to investigate the pattern of protease-activated receptors (PARs) over time in a model of acute liver injury induced by lipopolysaccharide (LPS); and whether PARs play a role in this process and exert their effects through inflammation and coagulation. Levels of tumor necrosis factor-α (TNF-α) were significantly expressed 1 h after LPS administration followed by: i) an increase in levels of tissue factor, factor VIIa, thrombin and plasminogen activator inhibitor-1; ii) unchanged or steady levels of tissue factor pathway inhibitor; and iii) subsequent deposition of fibrin in the liver tissue, that led to the elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are associated with liver injury.

Key words

Coagulation, endotoxin, inflammation, liver injury, protease-activated receptor (PAR)

Introduction

The liver can be injured and its functions altered by the activation of coagulation and inflammatory processes in sepsis (1). Both ex-vivo and in-vitro studies have demonstrated that tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are released in response to lipopolysaccharide (LPS), primarily by Kupffer cells (2–4). LPS stimulates Kupffer cells to secrete TNF-α which, subsequently, contributes to the pathogenesis of LPS-induced liver injury by a direct or an indirect polymorphonuclear leukocyte-dependent mechanism (3–6). The disturbance of hepatic microcirculation by sinusoidal fibrin deposition is also another condition that leads to the development of LPS-induced hepatic injury (7). In LPS-treated monkeys, tissue factor mRNA and protein were induced not only in monocytes and endothelial cells, but also in neutrophils that infiltrate the liver (8). Mochida et al. (9) indicated that fibrin deposition and microcirculatory disturbances, caused by tissue-factor activity in Kupffer cells after endotoxin administration, cause severe hepatic and endothelial-cell injury in heptatectomized rats. LPS injection induces plasminogen activator inhibitor-1 (PAI-1) mRNA in hepatocytes and endothelial cells via a TNF-α-dependent mechanism (11–13). Collectively, these studies clearly suggest that TNF-α, neutrophils, tissue factor, PAI-1, and fibrin, are likely to be the main mechanisms that underlie LPS-induced liver injury.

In the past few years, structural and functional studies have increasingly demonstrated the interplay between the inflammatory and coagulation systems (13). It is now clear that factors such as protease-activated receptors (PARs) are important candidates for this kind of interaction (13–15). To date, four distinct PARs isoforms, namely PAR1, 2, 3, and 4, have been described. Thrombin activates PAR1, 3, and 4 (14, 15), whereas, according to recent studies, tissue factor/factor VIIa (FVIIa) complex and tissue factor/FVIIa/FXa ternary complex are selective ligands for PAR1, 2, and 4.
for PAR2 (16, 17). In addition to their classic roles in blood coagulation, thrombin, tissue factor, FVIIa, and FXa all exert a number of proinflammatory and profibrinotic effects that are also mediated by the proteolytic activation of PARs (14, 15).

Copple et al. (18) demonstrated that thrombin receptor-activating peptides (TRAPs) of PAR1 promote neutrophil activation that causes injury of hepatic parenchymal cell. LPS, TNF-α and IL-1 induce expression of PAR2 in endothelial cells. Expression of PAR2, together with PAR4, is also induced by TNF-α and IL-1 in human coronary artery tissue (19, 20). In contrast, the PAR1 gene is not induced by any of these inflammatory mediators (19). These data clearly indicate that PAR2, but not PAR1, likely plays a role in the pathogenesis of acute inflammatory response, and that PAR2 is also involved in LPS-induced liver injury. The three main cell types that contribute to LPS-induced liver injury are Kupffer cells, hepatocytes, and sinusoidal endothelial cells (1, 2). Kupffer cells, the resident macrophages of the liver, appear to play the most critical role in LPS-induced liver injury (3, 6, 9). However, no studies to date have elucidated the expression and cellular localization patterns of all the four PAR isoforms over a course of time in the liver, following LPS administration.

Thus, in the present study, we examined the specific chronological and cellular expression of these PAR isoforms (protein and mRNA) in liver tissues of an LPS-induced endotoxemic rat model. Furthermore, we assessed LPS-induced acute liver injury, with the associated changes in levels of TNF-α and coagulation factors of the tissue factor-dependent pathway and PAI-1, including fibrin deposition within the liver tissues. Finally, the specific role of PAR2 in LPS-induced acute liver injury, associated with inflammation and coagulation, was also assessed.

Materials and methods

**Animals and treatments**

Adult male Wistar rats (8 weeks old, 246 ± 25 g) were used in all experiments (n=15). In the first series of our study, endotoxiaemia was induced by administering a single (2 ml) intra-peritoneal injection of LPS (dissolved in sterile saline) derived from *Escherichia coli* 055:B5 (15 mg/kg body weight). Animals were killed in groups, under a lethal injection of sodium pentobarbital (80 mg/kg body weight intraperitoneal [i.p.]) at different time points after LPS administration (1, 3, 6, and 10 hours [h]). The control group received an equal volume of sterile saline (2 ml/rat) without LPS. Liver tissues were carefully harvested, immediately frozen in liquid nitrogen, and stored at −80°C. For paraffin sections, after tissue harvest, the liver was postfixed in 4% paraformaldehyde overnight and processed routinely for paraffin embedding. All the experimental procedures of the present study were performed in accordance with the institutional guidelines of the Hokkaido University Graduate School of Medicine Animal Care and Use Committee.

In the second part of the study, in order to investigate the potential role of PAR2 in LPS-induced acute liver injury, rats were treated with PAR2-blocking peptide (PAR2 BP), prior to and also after administration of LPS. Briefly, the left jugular veins of some rats were cannulated for drug administration under urethane anesthesia [35% ethyl carbamate (Wako Pure Chemical Industries, Osaka, Japan) + 4% alpha-chloralose (Wako) saline wt/vol, 0.4 to 0.8 g/kg, i.p.]. All the tested drugs were administered intravenously (i.v.) as a slow bolus injection. LPS (15 mg/kg, i.v.) was administered through the jugular vein, in different groups of rats, at an interval of 1 h, 3 h, and 10 h (n=15 for each time point) and then sacrificed at different time points. Thirty minutes (min) before LPS administration, the PAR2 BP (sc-9278P, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was administered i.v. as a slow bolus injection (100 µg/kg, 500 µl/kg PBS) in the PAR2 BP-treated group. PAR2 BP was then continuously infused (20 µg/h, 100 µl PBS/h) using a pump to different groups for different time duration (1 h, 3 h, and 10 h, n=15 for each time point). Non-treated rats were used as a control group (n=15).

**Measurements of hemodynamic and biochemical parameters**

On the day of the experiment, the rats were anesthetized with sodium pentobarbital (40 mg/kg body weight i.p.) and a microtip pressure transducer catheter (SPC-320, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery. Both the arterial blood pressure and heart rate were monitored with a pressure transducer (model SCK-590, Gould, OH, USA) and recorded using a polygraph system (amplifier, AP-601G; Tachometer, AT-601G; thermal-pen recorder, WT-687G; Nihon Kohden, Tokyo, Japan). We then collected and processed plasma and serum samples. The proportion of blood to sodium citrate dehydrate anticoagulant volume was 9:1.

Plasma bilirubin and lactate dehydrogenase (LDH), serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by using kits from Wako Pure Chemical Industries, LTD (Osaka, Japan) (bilirubin and LDH) and Iatron Laboratories, Inc. (Tokyo, Japan) (AST and ALT).

**Histopathologic examination**

For histopathology, the tissue specimens were fixed in 4% buffered formalin solution, dehydrated through an ethanol series, embedded in paraﬃn, and sliced into 5-µm-thick sections. After deparaffinization, sections were stained with hematoxylin and eosin (HE) using the standard method.

**Immunohistochemistry and immunofluorescent labeling**

Immunohistochemistry and immunofluorescent labeling of liver tissues were performed as described in our previous study (21). Briefly, after tissues were cut (5 µm thick), the sections were deparaffinized and treated for 20 min with citrate buffer (10 mM citric acid, pH 6.0) in a microwave oven (750 W) before immunostaining. In some cases, frozen sections were fixed in acetone and air-dried. After blocking the non-specific staining that arises from secondary antibodies, the sections were incubated with primary antibodies overnight at 4°C, followed by incubation with appropriate secondary antibodies that are coupled to horseradish peroxidase. The sections were then viewed by light microscopy using AEC (3-amino-9-ethylcarbazole) peroxidase substrate solution.

Alternatively, for immunofluorescent labeling, following the overnight incubation with an appropriate primary antibody, the sections were exposed to the fluorescence secondary antibody, Cy3-conjugated AffiniPure anti-rabbit IgG or fluorescein-conjugated AffiniPure goat anti-rabbit, anti-goat, anti-sheep or...
anti-mouse IgG (Jackson Immunoresearch Laboratories), for 2 h, according to the manufacturer’s instructions.

**Western blot analysis**

Immunoblotting was performed, as described in our previous report (21). Briefly, samples of tissue homogenate (5–20 µg of protein) were run on a 4–15% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride filter membrane. The membrane was then blotted with the antibody of interest and processed via chemiluminescence.

**Antibodies**

For immunological detection (immunohistochemistry, immunofluorescence and immunoblot analysis) the following antibodies were used: anti-human PAR1 rabbit polyclonal antibody, anti-human PAR2 goat polyclonal antibody, anti-mouse PAR3 goat polyclonal antibody and anti-mouse PAR4 goat polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-human fibrin mouse monoclonal antibody (Chemicon International, Temecula, CA, USA), anti-human thrombin goat polyclonal antibody (R&D Systems Inc., Minneapolis, MN, USA), anti-human coagulation FVII goat polyclonal antibody (R&D Systems Inc.), rabbit polyclonal factor X (FX) (H-120) antibody (Santa Cruz Biotechnology), rabbit polyclonal tissue factor pathway inhibitor (TFPI) antibody (Santa Cruz Biotechnology), anti-rabbit tissue factor sheep polyclonal antibody (American Diagnostica, Stamford, CT, USA), rabbit anti-rat PAI-1 antibody (American Diagnostica), and anti-Xenopus laevis β-actin mouse monoclonal antibody (Abcam, Cambridge, UK). In most cases, preliminary experiments were performed to determine the specificity of each antibody by blocking its (primary antibody) immunoreactivity using excess amounts of a competing peptide. Additionally, these antibodies were tested for their ability to recognize the target peptide of interest within the rat. It should be noted that the immunoreactivity was expected to disappear when non-immune IgG was used instead of primary antibodies.

**RNA preparation and real-time quantitative PCR**

Total RNA were prepared from liver tissue using the guanidinium thiocyanate-phenol-chloroform single-step extraction method with Isogen (Nippon Gene, Toyama, Japan), used routinely in our laboratory (22). After the RNA was isolated, treated with DNase I and quantified, it was reverse transcribed to cDNA by omniscript reverse transcribe using a first-strand cDNA synthesis kit (Qiagen GmbH, Hilden, Germany). The reaction was performed at 37°C for 60 min.

The levels of PARs mRNA were analyzed by real-time quantitative PCR with a TaqMan probe using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA), as previously described (23). The gene-specific primers and TaqMan probes were synthesized from Primer Express v. 1.61 software (Perkin-Elmer, Boston, MA, USA), according to the published cDNA sequences for each gene. The sequences of the oligonucleotides were as follows:

- PAR1 forward: 5'-CCCCCCCTTAAGATCAGCTACTACTTC-3'
- PAR1 reverse: 5'-GGCGAAGCGACACATTCC-3'
- PAR1 probe: 5'-CCCGAACTGCCAATCG-3'
- PAR2 forward: 5'-CCTTGACATCACCCACTGTC-3'
- PAR2 reverse: 5'-GGGAGAGGAAGTAACTGAACATGTC-3'
- PAR2 probe: 5'-GGGAGAGGAAGTAACTGAACATGTC-3'
- PAR3 forward: 5'-CCACAGAGGACGAACTAGTACG-3'
- PAR3 reverse: 5'-CCAAAGGACGAACTAGTACTACG-3'
- PAR3 probe: 5'-CCACAGAGGACGAACTAGTACTACG-3'
- PAR4 forward: 5'-CTCACTGATCGTACGTGG-3'
- PAR4 reverse: 5'-GCACATTGCTAGGTGTGAAAGC-3'
- PAR4 probe: 5'-CTCACTGATCGTACGTGG-3'
- β-actin forward: 5'-GGCCGGGACCTGACA-3'
- β-actin reverse: 5'-GCTGTGGTGGTGAAGCTGTAG-3'
- β-actin probe: 5'-ACTACCTCATGAAGATCC-3'

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<th>LPS 3 h</th>
<th>LPS 6 h</th>
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<td>Systolic BP (mmHg)</td>
<td>142±4</td>
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<td>Diastolic BP (mmHg)</td>
<td>94±8</td>
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<td>Bilirubin (mg/dl)</td>
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<td>1.5±0.3</td>
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<td>AST (KU)</td>
<td>9.8±1.8</td>
<td>11.0±1.3</td>
<td>18.2±2.1**</td>
<td>19.3±3.8*</td>
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<td>ALT (KU)</td>
<td>56.0±6.6</td>
<td>68.3±9.5</td>
<td>98.1±19.5*</td>
<td>120.5±37.7**</td>
<td>176.2±79.6**</td>
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<td>TNF-α (Plasma, pg/ml)</td>
<td>28±6</td>
<td>225±1067**</td>
<td>497±143*</td>
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<td>TNF-α (Liver, pg/mg)</td>
<td>27±6</td>
<td>120±63**</td>
<td>55±14*</td>
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<td>PAI-1 (Serum, pg/ml)</td>
<td>307±43</td>
<td>1959±779**</td>
<td>8285±812**</td>
<td>8123±165**</td>
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<td>PAI-1 (Liver, pg/mg)</td>
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<td>863±19**</td>
<td>843±199**</td>
<td>577±215**</td>
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<td>TF (Liver, AU)</td>
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<td>5.1±2.0**</td>
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<td>TFPI (Liver, AU)</td>
<td>1.0±0.0</td>
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<td>0.97±0.1</td>
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Table 1: Effects of LPS on blood pressures and biochemical markers.

**LPS:** Lipopolysaccharide; **BP:** blood pressure; **PAI-1:** plasminogen activator inhibitor-1; **TF:** tissue factor; **TFPI:** tissue factor pathway inhibitor; **KU:** Karmen unit; **AU:** arbitrary unit. *p < 0.05, **p < 0.01.
Immunoassays
The levels of TNF-α in the plasma and liver tissues were detected using a rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc.). A rat PAI-1 Activity ELISA kit (ZYMUTEST Rat-PAI-1-Activity, Hyphen Biomed, 95000 Neuville-sur-Oise, France) was employed for detecting PAI-1 in rat serum and liver tissues. For measurement of liver tissue FVIIa, an IMUBIND® Factor VIIa ELISA Kit was used (American Diagnostica). Plasma thrombin-antithrombin complex (TAT) was also measured by ELISA (SRL, Tokyo, Japan).

Statistical analysis
The results are expressed as the mean ± SD, and the sample numbers are equivalent to the number of animals in each group. Means were compared by one factor analysis of variance, followed by the Scheffé’s test for multiple comparisons. Differences were considered significant at p < 0.05.

Results

Part 1
The results showing the chronological expression of the different factors (proteins/genes) in LPS-administered rats are summarized below. It should be noted that these time-course studies were also performed in the control rats, and there was no significant change observed in the levels of the molecules of interest (data not shown).

The effects of LPS on blood pressure, biochemical markers, and TNF-α expression
Table 1 summarizes the values for blood pressure, biochemical markers, and TNF-α expression for control and LPS-treated rats. Levels of bilirubin, AST, and ALT, increased significantly at 3 h after LPS administration, when compared to the control rats. Plasma LDH levels, however, were unchanged after LPS administration in comparison to the control group (data not shown). The plasma and hepatic levels of TNF-α were elevated after administration of LPS and peaked after one hour (80-fold and 4.6-fold of control, respectively).

Effect of LPS on the hepatic expression of PARs
PARs protein. Immunoblot analysis was performed using specific antibodies of the four PAR isoforms (Fig. 1A, C). The specific antibody of PAR1 reacted with a band of ~62 kDa (18), gradually increasing and peaking at 10 h (4.4-fold) post-LPS administration. The levels of PAR2 were detected as a band of ~55 kDa, whose intensity increased over the course of time, peaking at 6 h (3.6-fold) post-LPS administration. The upregulatory trends of PAR3 and PAR4 slightly tapered-off at 3 h post-LPS administration, but, thereafter, increased in a time-dependent manner. No band was observed without the addition of primary anti-PAR antibodies to the tissue sections.

PARs mRNA. Quantitative real-time PCR was performed to determine the effects of LPS on gene transcription (Fig. 1D). We found that mRNA expressions for PAR1 and PAR2 increased in the hepatic tissues in a time-dependent manner. PAR3 and PAR4 showed similar expression patterns for mRNA in liver tissues.

Immunohistochemical analysis. At 10 h after LPS administration, PAR1 was strongly expressed in hepatocytes (++++), with minimal expression in Kupffer cells (+). A pronounced immunostaining of PAR1 was also observed in the structures of the portal areas (portal vein, hepatic artery, bile duct) (++++). The endothelium of the portal vein, all the layers of the hepatic artery, including endothelial and vascular smooth muscle cells and the bile duct epithelium, showed positive immunoreactivity. The endothelia of the central vein also showed strong immunoreactivity for PAR1 (++++).

PAR2 immunoreactivity was located predominantly in Kupffer cells (++++) and partly in hepatocytes (+) (Fig. 1B). It was also observed in the endothelium of central veins (+) at 10 h post-LPS administration in the septic liver. A few and discrete immuno-reactive positive cells for PAR2 were observed in the
portal areas [portal vein (endothelium), hepatic artery (all layers) and bile duct epithelium] (+).

Extensive PAR3 immunostaining was observed in hepatocytes (++++) while Kupffer cells (+) showed only minimum staining for PAR3. Moderate immunoreactivity was also seen in the endothelium of central veins (+). Structures of the portal area [portal vein (endothelium), hepatic artery (all layers), bile duct epithelium] (+) also exhibited discrete staining patterns for PAR3.

PAR4 expression was predominantly observed in Kupffer cells (+++) and partly in hepatocytes (++). Moderate to strong immunoreactivity of PAR4 was found in the endothelium of the central veins (+~++). The endothelia of some central veins showed diffuse staining pattern, while others had discrete immunoreactivity. Limited discrete immunoreactivity for PAR4 was also seen in the structures of the portal area (portal vein, hepatic artery, bile duct) (+).

In the control group (without LPS administration), immunostainings for the four PARs were negligible.

Levels of coagulation and fibrinolytic markers in the blood and in the liver tissues

The quantitative analysis of immunoblotting for tissue factor (a single band migrating at 45 kDa) in rat hepatic tissues showed elevated levels in the LPS-treated group in comparison to the control group (Table 1). TFPI (a single band migrating at
35kDa), however, was almost unchanged between the control and LPS-treated rats (Table 1). Levels of plasma TAT were significantly increased at 3 h after LPS administration compared to control rats (Fig. 2A). In addition, the relative levels of alpha-active thrombin (target-molecular weight around 37 kDa) and FVIIa were significantly upregulated at 3 h after LPS administration, whereas those of total FVII (target-molecular weight 50 kDa) decreased (Fig. 2B-D). Consistent to the findings of FVII, the expression of FX in liver tissue (target-molecular weight 56 kDa) decreased after LPS administration (Fig. 2E), suggesting that production of FXa increased in liver tissue after LPS administration. Table 1 shows statistically high levels of PAI-1 in the serum and in the liver tissues after LPS administration compared to control rats. The relative amounts of immunodetectable fibrin/fibrinogen progressively increased following LPS administration (Fig. 2F). By 10 h after LPS administration, fibrin deposition was evidently identified intra- and extra-vascularly, as well as in hepatic sinusoids (data not shown). Fibrin was barely detectable in the control liver.

Histopathology after LPS administration
An hour after induction of sepsis, mild hepatocyte atrophy and scattered neutrophil infiltration (+) were observed in the liver tissue. Three hours later, necrosis in a few hepatocytes (+) was seen. By 6 h post-LPS treatment, hepatocyte necrosis became more evident (+) than at the 3 h time-point. Furthermore, modest (+++) neutrophil infiltration was also observed at this stage in the liver. By 10 h post-LPS treatment, necrosis became more extensive (+++) and neutrophil infiltration aggravated (+++). Some of the representative images from hematoxylin and eosin (HE) staining are shown in Figure 2G.

Part 2
In order to gain more insight into the significance of the differential cellular localization and chronological expression pattern of PARs in the pathology of LPS-induced liver injury, the action of PAR2 was blocked by PAR2 BP at different time points (1 h, 3 h and 10 h), prior to and after LPS injection. From our investigation, we found that the neutralization or blockage of PAR2 was optimal at 3 h, in terms of reversing liver injury, levels of inflammatory cytokines, and coagulation and fibrinolytic factors. It should be noted that the dosage of PAR2 BP used in the present study was selected based on findings obtained from dose-dependent preliminary experiments. Moreover, the patterns or trends in levels of the different molecules associated with LPS-induced liver injury, was almost identical irrespective of the route of LPS administration, i.e. i.p. versus i.v. Finally, administration of PAR2 BP to control rats had no effect on the levels of the different molecules of interest that are associated with liver injury.

Changes in the levels of ALT, AST, TAT, and PAI-1, after blockade of PAR2
The increase in serum activities of both ALT and AST at 3 h post-LPS-treatment was significantly suppressed by treating rats with PAR2 BP before and after LPS administration (Fig. 3A, B). In addition, the competitive blockade of PAR2 at 3 h post-LPS treatment appeared to completely normalize the increased plasma levels of TAT in LPS-treated rats (Fig. 3C). In contrast, the upregulated levels of serum PAI-1 remained unchanged, even after treatment with PAR2 BP (Fig. 3D).

The effects of PAR2 blockade on plasma and hepatic levels of TNF-α
Both the plasma and hepatic levels of TNF-α were increased at 3 h in LPS-treated rats compared to the control group. These increases were significantly prevented by the administration of PAR2 BP for 3 h in LPS-treated rats (Fig. 3E, F).

The effects of PAR2 blockade on expression of thrombin, FVII, FVIIa, FX, PAI-1 and fibrin in liver tissues
The upregulated levels of alpha-active thrombin in liver tissues were significantly reversed 3 h after blockade of PAR2 in LPS-treated rats (Fig. 4A). The treatment of PAR2 BP for 3 h in LPS treated rats normalized the elevated FVIIa expression in liver tissues (Fig. 4B) and restored the hepatic expression of FVII (Fig. 4C). Similar to its effect on levels of FVII, the PAR2 BP also normalized those of FX in the liver tissues (Fig. 4D). Although the blockade of PAR2 could not normalize the high serum levels of PAI-1 by 3 h in LPS-treated rats, PAR2 BP was able to significantly lower those of the liver tissues (Fig. 4E). Finally, PAR2 BP
significantly decreased the levels of fibrin in liver tissues in LPS-administered rats (Fig. 4F).

The effects of PAR2 blockade on the morphological features of acute liver injury
Neutrophil-infiltration and swelling of Kupffer cells were seen at 3 h in the liver tissues of LPS-treated rat, while control livers showed no morphological abnormalities. After 3 h treatment with PAR2 BP, neutrophil-infiltration almost completely disappeared, and the swelling of Kupffer cells was normalized (Fig. 4G).

Discussion
Many studies have implicated TNF-α in the pathogenesis of LPS-induced hepatotoxicity (3–6). It has been shown that TNF-α activates neutrophils and increases the gene expression of adhesion molecules, which ultimately leads to neutrophil-mediated liver injuries (1, 2, 5). In addition, TNF-α enhances procoagulant activity through the activation of tissue factor-dependent pathway and via PAI-1-mediated inhibition of fibrinolysis in sepsis (24). Many other studies have also demonstrated the induction of tissue factor and PAI-1 gene and protein expression.
in the liver tissues by LPS-administration, which is followed by sinusoidal fibrin deposition and circulatory disturbance (7–12). LPS-induced liver injuries were significantly attenuated by administration of inhibitors of thrombin, such as by heparin or hirudin. Furthermore, coagulation was controlled by TFPI or recombinant human thrombomodulin (1, 7, 9, 25). Collectively, these results suggest that fibrin thrombosis is another important pathogenic event in LPS-induced liver injury. Here, following the LPS-induced elevation of TNF-α levels, we found increased synthesis of tissue factor-mediated thrombin; a dysfunctional anticoagulant mechanism due to unchanged levels of TFPI; impaired fibrinolysis induced by PAI-1, and, subsequently, a time-dependent elevation of fibrin/fibrinogen levels in liver tissues after LPS administration.

The present study is the first to demonstrate that LPS administration induces increase in levels of PAR 1–4 isoforms at both the mRNA and protein levels in the rat liver, suggesting that these increases occur at the level of transcription. Moreover, using immunohistochemical analysis, we also reveal here a differential distribution of PAR1, 2, 3, and 4 in the injured liver. The apparent cooperative interaction previously observed between PAR3 and PAR4 suggests that PAR3 may be a cofactor for PAR4 in murine platelets (26). Consistent with these data, here, we found similar patterns in the expression of PAR3 and PAR4 proteins and mRNAs, as demonstrated by both immunoblotting and real-time PCR, in liver tissue after LPS administration. However, we also noticed differences in the cellular distributions of PAR3 and PAR4, which may suggest existence of distinct roles for these two isoforms (PAR3 and PAR4) in LPS-induced liver injury at the cellular level. Of particular interest is the predominant localization of PAR2 and PAR4 in Kupffer cells. Since several studies suggest that Kupffer cells play a critical role through tissue factor expression, TNF-α and IL-1 release, and, ultimately, lead to coagulation and inflammation activation in LPS-induced liver injuries (1, 3, 6, 9), the distinct localization of PAR2 and PAR4, as seen here, suggests an important role for these PAR isoforms in the pathogenesis of LPS-induced liver injury.

The observed elevated levels of TAT and thrombin, enhancement of tissue factor and FVIIa expression, decrease in total FVII and FX, and fibrin formation following LPS administration in the present study, suggests activation of the extrinsic coagulation pathway. In addition, levels of TFPI were unchanged. Collectively, these data suggest increase in tissue factor/FVIIa and tissue factor/FVIIa/Fxa complexes, which are selective ligands for PAR2 in the liver.

The close relationship between TNF-α and PAR2 is well established (19, 20). PAR2 is known to be involved in leukocyte rolling, adhesion, and transmigration in vivo (26). Furthermore, inhibition of thrombin, the primary ligand of PAR, and PAR2 deficiency reduce inflammation and mortality in mouse models of endotoxemia (27). Taken together, the data of these studies and the present investigation, suggest that the prominent expression of PAR2 in Kupffer cells is potentially related to or underlies the acute inflammatory response activated by the selective ligands (mentioned above) that are generated in the plasma and liver tissues of LPS-treated rats.

A key finding of the present study is the demonstration that blockade of PAR2 action by PAR2 BP significantly prevents elevation of TNF-α levels, a key mediator of LPS-induced liver injury, in both plasma and hepatic tissues. The marked decrease in TNF-α level may help explain the observed effects, such as inactivation of complement and improvement of fibrinolysis, which are associated with decreased fibrin formation in liver tissues. These ameliorations in inflammation, coagulation, and fibrinolysis lead to a decrease in levels of AST and ALT and are associated with the normalization of impaired morphological changes in LPS-induced liver injuries. However, in analyzing these data, it is important to take into account other possibilities that may induce liver injuries in LPS-treated rat models, since PAR2 BP only showed partial blockade of the parameters of interest in the present study.

Copple et al. have shown that thrombin promotes liver injury independent of its role in the formation of fibrin clots, and also demonstrated that PAR1 agonists promote LPS-induced hepato-cellular injury (18). In their study they used two TRAPs, namely TFFLR and SFRLN, that generated the same results. The former is a specific agonist for rat PAR1, but the latter can activate both PAR1 and PAR2 (27). Although they did not provide a comprehensive cellular distribution of this PAR isoform, PAR1 was shown to be upregulated in sinusoid cells. Collectively, their results suggest that PAR2, as well as PAR1, may be involved in LPS-induced liver injuries. PAR4 is activated by the selective ligand thrombin, whose expression was significantly increased by LPS in the present study. Recent studies suggest the redundancy of PAR1 and PAR4, and PAR1, 2, and 4, in microvascular endothelial cells (28, 29). The prominent expression of PAR4 in Kupffer cells, as revealed here and these previous studies, suggest that this PAR isoform may also have a pivotal role in LPS-induced liver injury.

In summary, we demonstrate that the increase in the expression of TNF-α after LPS administration in rats, which is followed by activation of tissue factor-dependent coagulation pathway and fibrinolytic shut-down, gives rise to fibrin deposition that ultimately leads to liver injury. The expression of inflammatory cytokine and procoagulant molecules were accompanied by induction of four PAR isoforms at gene and protein levels, and were differentially localized in the subpopulation of liver cells and had distinct LPS-induced time-dependent expression patterns. The blockade of PAR2 was associated with the suppression of TNF-α elevation, as well as normalization of coagulation and fibrinolysis, ultimately leading to a decrease in fibrin formation and improved healing of LPS-induced liver injury. Collectively, these results suggest that the predominant expression of PAR2 in Kupffer cells may account for the LPS-induced liver injury, which is mediated by inflammation, coagulation and fibrinolytic pathways.

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