Roles of the Hemostatic System and Neutrophils in Liver Injury From Co-exposure to Amiodarone and Lipopolysaccharide

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Received April 1, 2013; accepted July 30, 2013

It has been demonstrated that co-treatment of rats with amiodarone (AMD) and bacterial lipopolysaccharide (LPS) produces idiosyncrasy-like liver injury. In this study, the hypothesis that the hemostatic system and neutrophils contribute to AMD/LPS-induced liver injury was explored. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6 × 10^6 endotoxin units/kg, iv) or saline (Sal). AMD did not affect the hemostatic system by itself but significantly potentiated LPS-induced coagulation activation and fibrinolysis impairment. Increased hepatic fibrin deposition and subsequent hypoxia were observed only in AMD/LPS-treated animals, starting before the onset of liver injury. Administration of anticoagulant heparin abolished AMD/LPS-induced hepatic fibrin deposition and reduced AMD/LPS-induced liver damage. Polymorphonuclear neutrophils (PMNs) accumulated in liver after treatment with LPS or AMD/LPS, but PMN activation was only observed in AMD/LPS-treated rats. Rabbit anti-rat PMN serum, which reduced accumulation of PMNs in liver, prevented PMN activation and attenuated AMD/LPS-induced liver injury in rats. PMN depletion did not affect hepatic fibrin deposition. Anticoagulation prevented PMN activation without affecting PMN accumulation. In summary, both the hemostatic system alteration and PMN activation contributed to AMD/LPS-induced liver injury in rats, in which fibrin deposition was critical for the activation of PMNs.

Key Words: amiodarone; idiosyncratic drug-induced liver injury; hemostatic system; neutrophils; hypochlorous acid.

Amiodarone (AMD; 2-butyl-3-[3′,5′-diiodo-4′α-diethylamino-ethoxybenzoyl]-benzofuran), a class III antiarrhythmic used to treat myocardial infarction and congestive heart failure, is known to cause idiosyncratic, drug-induced liver injury (IDILI) in human patients (Rotmensch et al., 1984). Cases of severe liver reactions, or even fatalities caused by fulminant hepatic failure, are reported after both long-term oral or acute iv administration of AMD (Babatin et al., 2008; Lewis et al., 1989; Rätz Bravo et al., 2005). Like other drugs that can cause idiosyncratic liver injury, in naive laboratory animals AMD is not hepatotoxic by itself. However, AMD interacts with modest inflammation caused by lipopolysaccharide (LPS) to produce pronounced liver injury in rats (Lu et al., 2012).

The importance of tumor necrosis factor-α (TNF) in AMD/LPS-induced liver injury has been demonstrated (Lu et al., 2012), but the role of other LPS-induced proinflammatory mediators, e.g., the hemostatic system and/or innate immune cells, has not been explored. Administration of LPS can lead to activation of tissue factor (TF) and release of plasminogen activator inhibitor-1 (PAI-1) by activated Kupffer cells, sinusoidal endothelial cells, and other cell types (Levi et al., 2003). TF is the major inducer of thrombin-mediated coagulation activation, and PAI-1 is the major inhibitor of plasminogen-mediated fibrinolysis (Dahlbäck, 2000). In LPS/drug co-exposure studies, drugs that cause human IDILI enhanced LPS-induced coagulation activation and fibrinolysis impairment, leading to hepatic fibrin deposition, tissue hypoxia, and liver damage (Luyendyk et al., 2004; Shaw et al., 2009a; Zou et al., 2009). Anticoagulation by heparin significantly reduced hepatic fibrin deposition and attenuated hepatotoxicity, indicating a key role for a dysregulated coagulation system in liver damage in these models.

Polymorphonuclear neutrophils (PMNs) are an essential part of the innate immune system (Amulic et al., 2012). The main function of PMNs is to eliminate invading microorganisms and remove dead or dying cells. On the other hand, the proteases and excess of reactive oxygen species, e.g., hypochlorous acid (HOCl, generated by the myeloperoxidase-hydrogen peroxidase-halide system; Arnhold and Flemmig, 2010), released by activated PMNs can lead to tissue damage, rendering PMNs a contributor to the pathogenesis of many acute inflammatory diseases. Examples include liver injury from endotoxemia (Hewett et al., 1992;
Jaeschke et al., 1991), alcoholic hepatitis (Bautista, 1997), concanavalin A exposure (Bonder et al., 2004), and ischemia reperfusion (Jaeschke et al., 1990). PMNs are also involved in animal models in which LPS potentiated hepatotoxicity from agents such as aflatoxin B1 (Barton et al., 2000), monocrotaline (Yee et al., 2003), and allyl alcohol (Kinser et al., 2004). Particularly germane to the studies presented here, in some animal models in which LPS co-exposure with IDILI-associated drugs led to liver injury, PMNs were critical for hepatotoxicity (Luyendyk et al., 2005; Shaw et al., 2009b; Zou et al., 2011). For other drugs, including AMD, this relationship has not been investigated, and determining if there are common mechanisms shared among these animal models could improve our understanding of the etiology of IDILI. The purpose of this study was to test the hypothesis that the hemostatic system and PMNs are critically involved in AMD/LPS-induced liver injury in rats. When the results demonstrated a significant importance of both coagulation activation and neutrophil activation, the causal relationship between the two was explored.

MATERIALS AND METHODS

Materials. Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (St Louis, MO). The activity of LPS (Lot 075K4038, derived from Escherichia coli serotype O55:B5) was 3.3 × 10^10 endotoxin units (EU)/mg, which was determined by a Limulus amebocyte lysate endpoint assay kit from Cambrex Corp. (Kit 50-650U; East Rutherford, NJ).

Animals. Male, Sprague Dawley rats (Crl:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250–370 g were used for studies in vivo. They were fed standard chow (Rodent Chow/Tek 8640; Harlan Teklad, Madison, WI) and allowed access to water ad libitum. Animals were allowed to acclimate for 1 week in a 12-h light/dark cycle prior to experiments. They received humane care according to the criteria in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental protocol. All experimental protocols were kept identical to previous studies (Lu et al., 2012). During development of the model, AMD/LPS-induced liver injury was similar in both fed and fasted rats, but the mortality rate was greater in fed rats. Therefore, all rats were fasted for 12 h before administration of LPS, and food was returned thereafter. A 20-mg/ml solution of AMD was made in its vehicle (0.18% [wt/vol] saline), and plasma (0.32% [wt/vol] sodium citrate, final concentration) were prepared from whole blood. The right medial lobe of the liver was rapidly frozen in liquid nitrogen for immunohistochemistry, and the left lateral lobe of liver was fixed in 10% (vol/vol) neutral-buffered formalin and then stored in 70% ethanol for histopathology.

Evaluation of liver injury. Hepatic parenchymal cell damage was estimated from the activity of alanine aminotransferase (ALT) in serum. ALT activity was measured using Infinity-ALT reagent from Thermo Electron Corp. (Waltham, MA). Formalin-fixed liver samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The stained liver sections were examined using a light microscope.

Evaluation of coagulation system and liver hypoxia. The plasma concentration of thrombin-antithrombin complex (TAT) as a biomarker of thrombin activation was determined with an ELISA kit (Enzygnost TAT Micro; Siemens Healthcare Diagnostics, Newark, DE). The plasma concentration of active PAI-1 was measured with Rat PAl-1 assay kit from Molecular Innovations Inc. (Novi, MI).

Immunohistochemical staining for hepatic fibrin deposition was performed as described previously (Copple et al., 2002). Briefly, frozen liver sections were fixed in 10% (vol/vol) buffered formalin containing 2% (vol/vol) acetic acid for 30 min at 25°C. This fixation procedure solubilized fibrinogen and fibrin monomers, leaving only cross-linked fibrin in the liver sections. Sections were blocked with 10% (vol/vol) horseradish peroxidase-mediated saline (PBS), incubated with goat anti-rat fibrinogen antibody (1:1000; ICN Pharmaceuticals, Aurora, OH) at 4°C overnight, and then incubated with Alexa 594-labeled donkey anti-goat secondary antibody (1:1000; Molecular Probes) for 3 h at 25°C.

Pimonidazole (PIM), a 2-nitroimidazole hypoxia marker, was used to identify hypoxic regions in liver (Arteel et al., 1995). PIM hydrochloride (Hyoxyprobe-1 from HPI Inc., Burlington, MA) was given to rats at 120 mg/kg, iv, 2 h before they were euthanized. Immunohistochemical staining of PIM adduct was performed as described previously (Copple et al., 2004).

Quantification of immunohistochemical staining. Quantification of images was performed using Image J software: The fraction of the area that was positively stained was measured in at least 10 randomly chosen microscope fields. The fraction of positively stained area is defined as the size of positively stained area divided by the total size of image evaluated.

Anticoagulation. Inhibition of activation of coagulation was performed by administration of heparin. Rats were treated with AMD/LPS as described in Experimental protocol, and heparin (3000 units/kg, s.c.) or Sal was administered 0.5 h after LPS. The animals were euthanized 10 h after LPS for sample collection.

Hepatic PMN accumulation and activation. Immunohistochemical staining for hepatic PMNs was performed as described previously (Yee et al., 2003). PMNs within liver sections were stained with anti-PMN immunoglobulin isolated from serum of rabbits immunized with rat PMNs (Hewett et al., 1992). After incubation with the primary antibody, tissue sections were incubated with biotinylated goat anti-rabbit immunoglobulin G, avidin-conjugated alkaline phosphatase, and Vector Red substrate. PMNs were identified by positive staining and nuclear morphology. The numbers of PMNs in 10–20 randomly selected, high-power fields (HPF; ×400) in liver sections were counted and an average calculation for each rat.

Staining for HOCI-modified epitopes is a sensitive and specific method for evaluating the activation of the myeloperoxidase-hydrogen peroxidase-chloride system in PMNs; the HOCI-modified epitopes are persistent (Hazell et al., 1996; Malle et al., 1997). The monoclonal antibody (2D10C9, subtype IgG2b) is specific for HOCI-modified epitopes and does not cross-react with epitopes generated by oxidative reactions involving nitrating species, transition metals, or lipid peroxidation reactions (Malle et al., 1995), and its efficacy and specificity have been demonstrated by us and others (Deng et al., 2008; Hanumegowda et al., 2003; Hasegawa et al., 2005; Hazell et al., 1996; Malle et al., 1995, 1997; Zou et al., 2011). Immunohistochemical staining for HOCI-modified epitopes in frozen liver sections was performed as described previously (Deng et al., 2007). Frozen liver sections were fixed in 4% (vol/vol) formalin for 10 min at 25°C. After washing three times with PBS, the slides were blocked with 3% (vol/vol) goat serum for 1 h. Then, 2D10C9 antibody (1:10 dilution in 3% [vol/vol] goat serum) was applied for 2 h. After washing three times with PBS, Alexa Fluor 594-labeled goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA; diluted 1:500 in 3% [vol/vol] goat serum) was applied for 3 h. After washing three times with PBS, fluorescent pictures were recorded and quantified as described previously (Deng et al., 2007).

PMN depletion. A rabbit anti-rat PMN serum (antineutrophil serum [NAS]; Intercell Technologies, Jupiter, FL) was used to deplete circulating PMNs. A previous study in rats demonstrated the efficacy of this NAS to deplete circulating PMNs (Snipes et al., 1995). Rats were given control serum deposited.
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EU/kg, iv) or Sal. Plasma samples were collected at 2, 4, 6, or 10 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. Plasma samples were collected at 2, 4, 6, or 10 h after LPS administration. Concentrations of TAT (A) and active PAI-1 (B) in plasma were measured. #, significantly different from respective groups not given LPS; *, significantly different from respective group not given AMD. p < 0.05, n = 4–9.

Figure 1. Plasma markers of hemostatic system alteration after treatment with AMD and/or LPS. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. Plasma samples were collected at 2, 4, 6, or 10 h after LPS administration. Concentrations of TAT (A) and active PAI-1 (B) in plasma were measured. #, significantly different from respective groups not given LPS; *, significantly different from respective group not given AMD. p < 0.05, n = 4–9.

RESULTS

AMD Enhanced the Alterations in the Hemostatic System Caused by LPS

Hepatocellular injury from AMD/LPS co-treatment begins between 4 and 6 h after administration of LPS and progresses through 10 h (Lu et al., 2012). Coagulation activation was measured at times before (2 and 4 h) and during injury progression using the plasma concentration of TAT as a marker for generation of thrombin (Fig. 1A). LPS alone caused an increase in TAT concentration in the plasma, which peaked at 2 h or earlier and returned to baseline by 10 h after LPS. AMD alone did not affect TAT concentration. The concentration of TAT in plasma of AMD/LPS-treated rats was not different from that in LPS-treated rats until 10 h, at which time it was greater in the AMD/LPS-co-treated animals.

An increase in plasma concentration of PAI-1 suggests reduced fibrinolysis. LPS increased the concentration of active PAI-1 in plasma, an effect that started at or before 2 h, peaked between 2 and 4 h, and returned to baseline at 10 h after LPS treatment (Fig. 1B). AMD did not alter the concentration of active PAI-1 by itself but significantly enhanced the LPS-induced active PAI-1 peak from 4 to 10 h.

AMD/LPS Co-treatment Induced Fibrin Deposition and Hypoxia in the Liver

Fibrin deposition is a consequence of coagulation system activation and impaired fibrinolysis and can lead to tissue hypoxia. Little fibrin was detected in the livers of rats treated with vehicle, AMD, or LPS alone at either 4 or 10 h after LPS (Fig. 2A). After 4 and 10 h, respectively, fibrin deposition was observed in rats co-treated with AMD/LPS, with the fraction of positively stained area about twofold greater than any of the control groups. Fibrin deposition was panlobular and appeared to be sinusoidal in AMD/LPS-treated rats (Fig. 2B).

Next, liver hypoxia was evaluated by quantification of immuno-histochemical staining of PIM adducts 4 h after LPS (Fig. 3A), which is before the onset of hepatic parenchymal damage. Increased PIM adduct staining was only observed in liver sections from AMD/LPS-treated rats, whereas treatment with Veh/LPS or AMD/Sal did not cause an increase compared with the Veh/Sal-treated group. Figure 3B shows representative photomicrographs of PIM adduct staining at 4 h after LPS treatment. Minimal staining was observed in Veh/Sal-, Veh/LPS-, or AMD/Sal-treated groups. Positive staining in the AMD/LPS-treated group was localized mainly to the midzonal regions of the liver lobules.

Anticoagulant Heparin Prevented Hepatic Fibrin Deposition and Attenuated AMD/LPS-induced Liver Injury

Treatment with heparin abolished the AMD/LPS-induced hepatic fibrin deposition at 10 h after LPS treatment (Fig. 4A). Heparin also diminished liver damage: ALT activity in serum was reduced by 60% (Fig. 4B), and the areas of hepatocellular necrosis were smaller after heparin treatment (Fig. 4C).

AMD Affected LPS-induced Hepatic PMN Accumulation

Treatment of rats with LPS alone caused an increase in the number of PMNs in the liver at 4 h, and this effect remained stable up to 10 h (Fig. 5A). AMD alone did not cause PMN accumulation in the liver. At 4 h, before the onset of liver injury (Lu et al., 2012), co-treatment with AMD/LPS led to impaired PMN accumulation in the liver compared with Veh/LPS treatment. At 10 h, which is near the peak of liver injury, livers from AMD/LPS-treated rats had slightly increased hepatic PMN accumulation. This accumulation displayed a panlobular distribution in livers of rats treated with Veh/LPS or AMD/LPS before the onset of liver injury (4 h; data not shown). At 10 h, PMNs in AMD/LPS-treated rats formed clusters around the necrotic regions (Fig. 5B). Hepatic PMNs in Veh/LPS-treated animals retained panlobular distribution.

AMD/LPS Co-treatment Induced PMN Activation

No staining for HOCI-modified epitopes was detected in any treatment group at 4 h after LPS treatment (Fig. 6A). At 10 h after LPS, positive staining for HOCI-modified epitopes was
observed only in the AMD/LPS-treated group. The staining localized mainly to the midzonal regions of the liver lobules (Fig. 6B).

PMN Depletion Attenuated AMD/LPS-induced Liver Injury

Next, AMD/LPS-treated rats were pretreated with NAS or CS, and the numbers of circulating total leukocytes, lymphocytes, and PMNs were quantified at 10 h after LPS (Table 1). CS/AMD/LPS-treated rats had significant lymphocytopenia and neutrophilia compared with CS/Veh/Sal-treated rats. NAS treatment dramatically decreased the number of circulating PMNs after AMD/LPS treatment without affecting the number of circulating lymphocytes. The number of circulating PMNs in NAS/AMD/LPS-treated rats (275 ± 40/µl) was even smaller compared with CS/Veh/Sal-treated rats (579 ± 150/µl).

As expected, there were few PMNs in livers of rats treated only with CS. Treatment with CS did not affect the PMN accumulation in the livers of AMD/LPS-treated rats (compare Figs. 5A and 7A). NAS attenuated the AMD/LPS-induced hepatic PMN accumulation by ~30%, but the number of hepatic PMNs in NAS/AMD/LPS-treated rats was still substantially greater than that of the CS/Veh/Sal-treated rats. PMNs in NAS/AMD/LPS-treated rats were distributed across the lobules, and clustering of PMNs in the necrotic regions that was observed in the livers of CS/AMD/LPS-treated rats was not seen in rats treated with NAS/AMD/LPS (Fig. 7B). Despite the considerable PMN accumulation, NAS completely abolished the AMD/LPS-induced positive staining of HOCl-modified epitopes (Fig. 7C).

Effects of PMN depletion on AMD/LPS-induced liver injury were assessed at 10 h after LPS. NAS reduced the AMD/LPS-induced increase in ALT activity by 40% (Fig. 7D) and

FIG. 2. Hepatic fibrin deposition after treatment with AMD and/or LPS. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. Liver tissue samples were collected at 4 or 10 h after LPS administration. (A) Fibrin polymers deposited in the liver were immunohistochemically stained and quantified as described in Materials and Methods. #, significantly different from respective group not given LPS; *, significantly different from respective group not given AMD. p < 0.05, n = 4–9. (B) Representative photomicrographs (×100) of hepatic fibrin deposition at 4 h after LPS.

FIG. 3. Hepatic hypoxia after treatment with AMD and/or LPS. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. PIM hydrochloride (120 mg/kg, iv) was given to rats 2 h after LPS, and liver tissue samples were collected at 4 h after LPS. (A) PIM-adducted proteins were immunohistochemically stained and quantified as described in Materials and Methods. #, significantly different from respective group not given LPS; *, significantly different from respective group not given AMD. p < 0.05, n = 3–6. (B) Representative pictures (×100) of PIM adduct staining at 4 h after LPS.
Figure 4. Effect of heparin on hepatic fibrin deposition and liver injury induced by AMD/LPS. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. Heparin (3000 units/kg, sc) or Sal was administered 0.5 h after LPS. Animals were euthanized 10 h after LPS for sample collection. Hepatic fibrin deposition (A) and ALT activity in serum (B) were quantified as described in Materials and Methods. #, significantly different from Veh/Sal/Sal; *, significantly different from AMD/LPS/Sal. p < 0.05, n = 3–7. (C) Representative photomicrographs (×200) of H&E-stained liver from rats treated with AMD/LPS/Sal (upper panel) or AMD/LPS/Heparin (lower panel). Black arrows indicate necrotic foci.

The mechanisms by which drugs cause IDILI are not known. IDILI-associated drugs, such as ranitidine (RAN, a H₂-receptor antagonist), trovafloxacin (TVX, a broad spectrum antibiotic) and sulindac (SLD, a nonsteroidal antiinflammatory drug), are safe in the majority of patients but induce idiosyncratic liver injury in a very small fraction of patients. Administration of these drugs to rats or mice, even at large doses, failed to produce liver injury (Luyendyk et al., 2003; Shaw et al., 2007; Zou et al., 2009). On the other hand, coadministration of some IDILI-associated drugs with LPS to induce modest inflammation caused liver injury and has provided animal models with which to study mechanisms of this injury (Roth and Ganey, 2011).

Previous studies demonstrated that AMD/LPS-treated rats developed idiosyncrasy-like liver injury, characterized by elevated activities of ALT and AST in serum and midzonal hepatocellular necrosis, whereas neither AMD nor LPS alone caused hepatotoxicity at the doses used in this model. The onset of liver injury was between 4 and 6 h after LPS administration, and injury progressed through 10 h (Lu et al., 2012). This AMD/LPS model is the only animal model developed for AMD in which pronounced liver injury occurs, and this study is the first to report the contributions of neutrophils and the hemostatic system to AMD-induced hepatotoxicity.
Hepatic fibrin deposition is controlled by both the coagulation system (deposition) and the fibrinolytic system (removal). LPS given alone caused elevations of both TAT (Fig. 1A) and PAI-1 (Fig. 1B), but they were not enough to result in fibrin deposition (Fig. 2A). AMD/LPS-induced hepatic fibrin deposition started at 4h after LPS (Fig. 2A), a time at which the LPS-induced increase in PAI-1 (Fig. 1B) but not TAT (Fig. 1A) was enhanced by AMD. These results suggest that the effect of AMD on LPS-induced PAI-1 at 4h was critical for the formation of hepatic fibrin after AMD/LPS co-treatment. Fibrin...
Fig. 6. Hepatic PMN activation after treatment with AMD and/or LPS. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. Liver tissue samples were collected at 4 or 10 h after LPS injection. (A) HOCl-modified epitopes in liver sections were stained immunochemically and quantified as described in Materials and Methods. #, significantly different from respective group not given AMD; *, significantly different from respective group not given LPS; **, significantly different from respective group not given AMD. ALT: p < 0.05, n = 4–9. (B) Representative photomicrographs (taken at a magnification of ×100) of HOCl-modified epitopes staining at 10 h after LPS.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leukocytes (number/μl)</th>
<th>Lymphocytes (number/μl)</th>
<th>PMNs (number/μl)</th>
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<tr>
<td>CS/Veh/Sal</td>
<td>5188 ± 174</td>
<td>4609 ± 198</td>
<td>579 ± 150</td>
</tr>
<tr>
<td>CS/AMD/LPS</td>
<td>3745 ± 257</td>
<td>1523 ± 173</td>
<td>2222 ± 199</td>
</tr>
<tr>
<td>NAS/AMD/LPS</td>
<td>1407 ± 123</td>
<td>1132 ± 95</td>
<td>275 ± 40</td>
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Note. Rats were treated with AMD (400 mg/kg, ip) or vehicle and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. NAS or CS (0.5 ml per rat, iv) was injected 2 h before AMD, and blood samples were collected 10 h after LPS. Total circulating blood leukocytes, lymphocytes, and PMNs were quantified as described in Materials and Methods. *p < 0.05, n = 5–12.

### Generation and deposition directly. One mechanism by which AMD could potentiate LPS-induced PAI-1 activation is through an effect on PAI-1-inducing cytokines, e.g., TNF, because AMD enhances LPS-induced TNF production in this model (Lu et al., 2012), and TNF can induce PAI-1 production (Nawroth and Stern, 1986).

A direct consequence of fibrin deposition in the liver is disruption of sinusoidal blood flow, which can lead to hepatic hypoxia. Hypoxia can directly injure isolated hepatocytes (Khan and O’Brien, 1997) or perfused livers (Lemasters et al., 1981). Hypoxia can also sensitize the liver or isolated hepatocytes to secondary stress, such as exposure to hepatotoxins or drugs (Bacon et al., 1996; Sparkenbaugh et al., 2012). Both AMD and hypoxia can cause mitochondrial dysfunction in hepatocytes (Chandel et al., 2000; Spaniol et al., 2001), so it is possible that the combination of the two led to more severe mitochondrial damage and eventually hepatocellular necrosis in AMD/LPS-co-treated animals.

Heparin is a widely used anticoagulant that inhibits thrombin activation by increasing the inhibitory effect of endogenous antithrombin III (Björk and Lindahl, 1982). Heparin administration prevented hepatic fibrin deposition (Fig. 4A) but only provided a 60% reduction of the AMD/LPS-induced ALT increase (Fig. 4B). A similar dose of heparin provided 90% protection against liver damage from a larger, hepatotoxic dose of LPS in rats (Moulin et al., 1996). These results suggest that coagulation activation was not solely responsible for AMD/LPS-induced liver injury; other LPS-induced inflammatory factors also contribute.

The influx of PMNs into the liver was mainly driven by LPS administration (Fig. 5A). Accumulated PMNs distributed evenly across the liver lobules of LPS-treated rats throughout the time examined (Fig. 5B) and in AMD/
FIG. 7. Effect of PMN antiserum on hepatic PMN accumulation and activation and liver injury induced by AMD/LPS. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6×10^6 EU/kg, iv) or Sal. NAS or CS (0.5 ml per rat, iv) was injected 2 h before AMD. Blood and liver tissue samples were collected at 10 h after LPS. (A) Hepatic PMN accumulation (HPF); (B) Representative pictures (taken at a magnification of ×100; insets showing stained PMNs were taken at ×1000) of hepatic PMN accumulation; (C) Staining for HOCl-modified epitopes; (D) ALT activity in serum; (E) representative photomicrographs (taken at ×200) of H&E-stained liver sections were obtained as described in Materials and Methods. #, significantly different from CS/Veh/Sal. *, significantly different from CS/AMD/LPS. p < 0.05, n = 5–12. Black arrows indicate necrotic foci.
LPS-treated rats before the onset of liver injury (data not shown). One mechanism that might contribute to PMN accumulation is the regulation of PMN migration by binding to coagulation proteins including thrombin (Gillis et al., 1997). Accumulation of PMNs in the liver does not necessarily result in damage to hepatocytes. β-integrin/intercellular adhesion molecule (ICAM)-mediated transmigration of PMNs through the endothelial cells and subsequent adhesion to the target cells are required for PMN activation and cytotoxic effects on parenchymal cells (Shappell et al., 1990). PMNs in livers of rats treated with the dose of LPS we used were not activated at any time examined, and LPS given alone did not cause liver damage. Because staining for HOCl-modified epitopes revealed cumulative activation and none was seen by 10 h in rats treated only with LPS, it is not likely that we missed LPS-induced activation of PMNs during the experiment. In AMD/LPS-treated rats, PMNs were not activated at 4 h (Fig. 6A), and injury was not observed at this time (Lu et al., 2012). Similar findings were observed in acetaminophen-induced hepatocellular injury (Lawson et al., 2000) and lithocholic acid-induced cholestatic injury (Fickert et al., 2006). PMNs were activated in livers of AMD/LPS-treated rats by 10 h, a time when hepatic injury was apparent.

PMNs not only help to clear injured or dying cells but also tend to attack hepatocytes stressed by inflammatory cytokines because these hepatocytes express more ICAM-1, which facilitates the recruitment and activation of PMNs (Farhood et al., 1995). Dead or necrotic hepatocytes also release damage-associated molecular pattern molecules (DAMPs), such as high mobility group box-1, heat shock proteins, uric acid, and endogenous DNA fragments (Brenner et al., 2013). DAMPs can also act as chemokines to attract PMNs (Pittman and Kubes, 2013). We explored whether PMNs were recruited to clear damaged hepatocytes after AMD/LPS treatment or exaggerated parenchymal cell injury. PMN depletion was associated with a reduction in liver injury in AMD/LPS-treated rats (Fig. 7D), suggesting that PMNs play a contributory role in the AMD/LPS-induced liver injury.

Interestingly, the contribution of PMNs to AMD/LPS-induced liver damage differs, at least in magnitude, from the contribution of these cells in other models of liver damage arising from LPS interaction with IDILI-associated drugs. For example, PMN depletion offered major protection (around 90%) from liver injury induced by co-treatment with LPS and RAN, TVX, or SLD (Luyendyk et al., 2005; Shaw et al., 2009b; Zou et al., 2011), but it had only moderate effects (around 40% reduction in injury) in the AMD/LPS model.

A causal relationship between hepatic fibrin deposition and PMN activation was explored. Serine proteases released by activated PMNs affect hepatic fibrin deposition (Deng et al., 2007). However, in this study, PMN activation was detected later than the appearance of hepatic fibrin deposition and hypoxia (Figs. 2A, 3A, and 6A), and PMN depletion did not affect hepatic fibrin deposition (Fig. 8). These results suggest that PMN activation did not initiate or contribute to hepatic fibrin deposition. On the other hand, anticoagulation by heparin, which prevented hepatic fibrin deposition, also prevented PMN activation (Fig. 9C) without affecting hepatic PMN accumulation (Fig. 9A). These results suggested that fibrin deposition was associated with activation of PMNs rather than their accumulation. One mechanism by which fibrin deposition could contribute to PMN activation is through hypoxia. Hypoxia is a direct consequence of impaired blood flow after fibrin deposition, and it induces adhesion molecules important for PMN activation (Arnould et al., 1993). Hypoxia can also cause necrosis of hepatocytes (Fassoulaki et al., 1984), which could release PMN-activating DAMPs (Scaffidi et al., 2002). It is possible that hypoxia initiated hepatocellular necrosis starting at 4 h after LPS and that activated coagulation factors together with DAMPs released from necrotic hepatocytes caused activation of PMNs at later times.

In summary, AMD enhanced LPS-induced impairment of the hemostatic system, resulting in elevated fibrin deposition and subsequent hypoxia. LPS drove PMN accumulation in the liver but not PMN activation. Only the PMNs in AMD/LPS-treated rats were activated during the progression phase of liver injury. Anticoagulation and PMN depletion each reduced AMD/LPS-induced liver injury. PMN depletion did not affect hepatic fibrin deposition, whereas an anticoagulant completely prevented PMN activation in the liver. These results suggest that a critical interaction between the hemostatic system and PMNs occurs in the pathogenesis of AMD/LPS-induced liver damage.
FIG. 9. Effect of heparin on AMD/LPS-induced hepatic PMN accumulation and activation. Rats were treated with AMD (400 mg/kg, ip) or vehicle (Veh) and 16 h later with LPS (1.6 × 10^6 EU/kg, iv) or Sal. Heparin (3000 units/kg, sc) or Sal was administered 0.5 h after LPS. Animals were euthanized 10 h after LPS for sample collection. (A) Hepatic PMN accumulation (HPF); (B) Representative photomicrographs (taken at a magnification of ×100; insets showing stained PMNs were taken at ×1000) of hepatic PMN accumulation; (C) Staining for HOCI-modified epitopes was performed as described in Materials and Methods. #, significantly different from Veh/Sal/Sal. *, significantly different from AMD/LPS/Sal. p < 0.05, n = 3–7.

FUNDING
National Institutes of Health (R01DK061315); Austrian Science Fund (FWF 3007, 19074-B05, and W1226-B18).

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