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Coagulation-mediated hypoxia and neutrophil-dependent hepatic injury in rats given lipopolysaccharide and ranitidine

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Abbreviations: alanine aminotransferase ALT; hepatocyte, HPC; lipopolysaccharide, LPS; neutrophil, PMN; pimonidazole, PIM; ranitidine, RAN; vehicle, Veh; cytokine-induced neutrophil chemoattractant-1, CINC-1; thrombin-antithrombin dimer, TAT

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Abstract

Idiosyncrasy-like liver injury occurs in rats cotreated with nonhepatotoxic doses of ranitidine (RAN) and bacterial lipopolysaccharide (LPS). Hepatocellular oncotic necrosis is accompanied by neutrophil (PMN) accumulation and fibrin deposition in LPS/RAN-treated rats, but the contribution of PMNs to injury has not been shown. We tested the hypothesis that PMNs are critical mediators of LPS/RAN-induced liver injury and explored the potential for interaction between PMNs and hemostasis-induced hypoxia. Rats were given either LPS (44.4 X 10^6 EU/kg) or its vehicle, then RAN (30 mg/kg) or its vehicle 2 h later. They were killed 3 or 6 h after RAN treatment, and hepatocellular injury was estimated from serum alanine aminotransferase activity and liver histopathology. Plasma PMN chemokine concentration and the number of PMNs in liver increased after LPS treatment at 3 h and were not markedly altered by RAN cotreatment. Depletion of circulating PMNs attenuated hepatic PMN accumulation and liver injury and had no effect on coagulation system activation. Anticoagulation with heparin attenuated liver fibrin deposition and injury in LPS/RAN-treated rats; however, heparin had little effect on liver PMN accumulation or plasma chemokine concentration. Liver hypoxia occurred in LPS/RAN-cotreated rats and was significantly reduced by heparin. *In vitro*, hypoxia enhanced the killing of rat hepatocytes by PMN elastase and shortened its onset, indicating a synergistic interaction between PMNs and hypoxia. The results suggest that PMNs are involved in the hepatocellular injury caused by LPS/RAN-cotreatment, and that hemostasis increases sensitivity to PMN-induced hepatocellular injury by causing liver hypoxia.

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Introduction

Ranitidine (RAN) is a histamine 2 (H2)-receptor antagonist available over the counter and by prescription for treatment of several gastric hypersecretory conditions. RAN is associated with idiosyncratic hepatotoxicity in a small fraction (estimated at <0.1%) of people taking the drug (Vial et al., 1991). Although the mechanism of RAN-induced hepatotoxicity is not understood, inflammation might be a predisposing factor for idiosyncratic hepatotoxicity from this drug and others (Ganey et al., 2004; Roth et al., 2003). Indeed, RAN is rendered hepatotoxic in rats cotreated with a small, noninjurious dose of the potent inflammagen, bacterial lipopolysaccharide (LPS; Luyendyk et al., 2003b).

Hepatocellular injury in rats given a large, hepatotoxic dose of LPS and in several models of LPS-augmented xenobiotic hepatotoxicity occurs by a neutrophil (PMN)-dependent mechanism (Barton et al., 2000; Hewett et al., 1992; Yee et al., 2003a). Activated PMNs release proteases (ie, elastase and cathepsin G) that kill HPCs *in vitro* (Ho et al., 1996), and inhibition of PMN elastase reduces LPS-induced liver injury *in vivo* (Ishii et al., 2002). Histopathological evaluation of livers from LPS/RAN-treated rats revealed midzonal hepatocellular oncotic necrosis accompanied by a marked infiltration of PMNs (Luyendyk et al., 2003b). However, it is not clear whether PMNS are involved causally in LPS/RAN-induced liver injury.

In addition to PMNs, an activated coagulation system is also important for hepatocellular injury in several animal models of LPS-potentiated hepatotoxicity (Kinser et al., 2002; Luyendyk et al., 2003a; Yee et al., 2003b). In some of these models, tissue PMN accumulation and/or activation depends on the coagulation system. For example, in the case of large, hepatotoxic doses of LPS, coagulation system activation is required for activation of PMNs and hepatotoxicity, but it is not important for upregulation of the PMN chemokine, cytokine-induced

neutrophil chemoattractant-1 (CINC-1; Copple et al., 2003). In contrast, coagulation system activation is required for CINC-1 production and liver PMN accumulation in ischemia-reperfusion-induced liver damage (Yamaguchi et al., 1997). Accordingly, crosstalk between coagulation and PMNs can occur during liver injury. In LPS/RAN-treated rats, coagulation system activation and fibrin deposition occurred before the onset of hepatotoxicity, and the ensuing liver injury was prevented by administration of the anticoagulant, heparin (Luyendyk et al., 2004a). Accordingly, interaction between accumulated PMNs and the coagulation system might occur in LPS/RAN-treated rats.

Coagulation system activation in LPS/RAN-treated rats results in sinusoidal fibrin deposition, one consequence of which is liver hypoxia (Luyendyk et al., 2004a). Indeed, liver hypoxia is observed at a time near the onset of hepatotoxicity in this model (Luyendyk et al., 2004a). Although these results are consistent with liver hypoxia as an important, downstream, deleterious consequence of hemostasis in LPS/RAN-treated rats, a direct link between coagulation system activation and hypoxia has not yet been established in this model. Similarly, interaction between hypoxia and PMNs has not been explored. Interestingly, liver injury caused by a large, hepatotoxic dose of LPS is enhanced by exposure of rats to a level of hypoxia that is normally noninjurious (Shibayama, 1987). Inasmuch as LPS-induced liver injury depends on PMNs (Hewett et al., 1992), this result suggests the possibility that hypoxia increases susceptibility of HPCs to toxic inflammatory mediators released by PMNs. Accordingly, hypoxia might be a necessary component of an interaction between PMNs and the coagulation system in LPS/RAN-treated rats.

The purpose of these experiments was to test the hypotheses that hypoxia and PMNs are both critical to LPS/RAN-mediated liver injury, and that the coagulation system increases

sensitivity to PMN-dependent liver injury by causing liver hypoxia. Accordingly, the effects of PMN depletion on LPS/RAN-induced liver injury and on coagulation system activation were evaluated. Furthermore, the effects of anticoagulation on plasma CINC-1, liver PMN accumulation, and liver hypoxia were investigated. In addition, to examine the possibility that hypoxia increases hepatocellular sensitivity to PMN-mediated hepatocellular injury, the influence of hypoxia on sensitivity of hepatocytes (HPCs) to the cytotoxic action of one PMN-derived product, PMN elastase was evaluated.

Materials and Methods

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

Animals. Male, Sprague-Dawley rats (Crl:CD (SD)IGS BR; Charles River, Portage, MI) weighing 250-350 grams (*in* vivo studies) or 90-150 grams (*in vitro* studies) were used for these experiments. Animals were fed standard chow (Rodent chow/Tek 8640, Harlan Teklad, Madison, WI) and allowed access to water *ad libitum*. They were allowed to acclimate for 1 week in a 12-h light/dark cycle prior to use.

Experimental protocol. Rats fasted for 24 hours were given 44.4 X 10^6 EU/kg LPS or its saline vehicle (Veh) i.v., and food was then returned. Two hours later, 30 mg/kg RAN or sterile phosphate-buffered saline (PBS) Veh was administered (i.v.). RAN solution was administered at 2 ml/kg at a rate of approximately 0.15 ml/min. To simplify treatment nomenclature for the remainder of the paper, the following designations have been applied: Saline/PBS (Veh/Veh), LPS/PBS (LPS/Veh), Saline/RAN (Veh/RAN), and LPS/RAN. Three or 6 h after RAN administration, rats were anesthetized with sodium pentobarbital (75 mg/kg, i.p.). Plasma was collected by drawing two ml of blood from the vena cava into a syringe containing sodium citrate (final concentration, 0.38%), and serum was collected by drawing blood from the dorsal aorta using a separate syringe. Blood collected from the aorta was allowed to clot at room temperature, and serum was collected and stored at -80˚ C until use. Representative slices (3-4

mm thick) of the ventral portion of the left lateral liver lobe were collected and fixed in 10% neutral buffered formalin.

Anticoagulation and PMN depletion. Inhibition of coagulation system activation was achieved by administration of heparin. Rats were treated with LPS/RAN as above, but 1 h before RAN treatment, heparin (3000 U/kg, s.c.) or sterile saline was administered. Rats were killed 3 or 6 h after RAN administration, and serum and liver samples were taken.

PMN depletion was accomplished by administration of a PMN antiserum before treatment with LPS/RAN. Rats were given 0.25 ml of either normal rabbit serum (normal serum) or rabbit anti-rat PMN serum (anti-PMN serum, Inter-cell Technologies, Jupiter, Fl) diluted 1:1 in sterile saline (total injection volume per rat, 0.5 ml, iv) 18 h before administration of LPS. Previous studies in which anti-PMN serum was administered to rats demonstrated a selective and complete depletion of PMNs (Snipes et al., 1995). Rats were killed 6 h after treatment with RAN, and citrated plasma was collected. Total blood leukocyte count was quantified using a Unopette white blood cell (WBC) determination kit (Becton-Dickinson, Franklin Lakes, NJ) and a hemacytometer. Slides were prepared from whole blood, stained using the Hema 3® Staining System (Fisher Scientific, Middletown, VA) and differential counting performed.

Hepatotoxicity assessment. Hepatic parenchymal cell injury was estimated as an increase in serum alanine aminotransferase (ALT) activity. ALT activity was determined spectrophotometrically using Infinity-ALT reagent from Thermo Electron Corp. (Louisville, CO). Formalin-fixed liver samples (3-4 samples/rat) were embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and examined by light microscopy. All tissue sections were coded and examined without knowledge of treatment.

Fibrin immunohistochemistry and quantification. Fibrin immunohistochemistry and quantification were performed as described previously (Copple et al., 2002). This protocol solubilizes all fibrinogen and fibrin except for cross-linked fibrin (Schnitt et al., 1993); therefore, only cross-linked fibrin stains in sections of liver.

Evaluation of liver hypoxia. Liver hypoxia was evaluated by immunohistochemical staining for pimonidazole (PIM) adducts. PIM is a 2-nitroimidazole marker of hypoxia and has been used to identify regions of hypoxia in liver (Arteel et al., 1995; Arteel et al., 1998). Rats were given 120 mg/kg Hypoxyprobe™-1 (PIM hydrochloride; Chemicon International Inc., Temecula, CA) i.p. two hours before they were killed. PIM-adduct immunostaining was performed as described previously (Copple et al., 2004). Quantification of PIM immunostaining was performed using Scion Image Beta 4.0.2 (Scion Corp., Frederick, MD). Background was set to be the average pixel intensity in periportal regions of Veh/Veh-treated livers (i.e., an area where no hypoxia occurs; Arteel et al., 1995). An increase in positive immunostaining for PIM-modified proteins indicates hypoxia in the liver tissue.

Evaluation of hepatic PMN accumulation and serum CINC-1 concentration. PMN immunohistochemistry was performed on formalin-fixed liver sections as described previously (Yee et al., 2003a). Hepatic PMN accumulation was evaluated by identifying the average number of PMNs counted in 10-20 randomly selected, high-power fields (HPF, 400X). Qualitative differences in liver PMNs were assessed by segregating PMNs into 3 groups characterized by: 1) segmented nucleus, various sizes, distinct borders, 2) donut-shaped nucleus (>75% complete ring), somewhat larger, distinct borders and 3) whole or fragmented nuclei, no distinct borders, smudgy PMN-positive staining, various sizes. These groups were designated as segmented PMNs, band cells, and degenerate PMNs, respectively. Slides were coded and the evaluator was

unaware of treatment. Serum CINC-1 concentration was evaluated using a commercially available ELISA (Assay Designs, Inc., Ann Arbor, MI).

Evaluation of coagulation system activation. Plasma fibrinogen was determined from thrombin clotting time of diluted samples using a fibrometer and a commercially available kit (B4233) from Dade-Behring Inc. (Deerfield, IL). Plasma thrombin-antithrombin (TAT) concentration was determined by ELISA using kit #OWMG15 from Dade-Behring.

HPC isolation. HPCs were isolated using the Gibco HPC Product Line (Invitrogen, Carlsbad, CA) including liver perfusion medium, liver digest medium, and HPC wash buffer (Cat. Nos 17701, 17703, 17704). All reagents were warmed to 37 °C before perfusion. Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and the portal vein was cannulated. The liver was initially perfused with 150 ml perfusion medium at a rate of 12 ml/min, with excess medium draining from the severed vena cava. This was followed immediately by perfusion with 100 ml of liver digestion medium at a rate of 12 ml/min. The liver was transferred carefully to a culture dish containing HPC wash medium and gently scraped to separate cells. The resulting liver digest was passed through sterile gauze and spun briefly at 50xg to pellet HPCs. The resulting pellet was washed two times with 50 ml volumes of HPC wash medium. HPCs were then resuspended at a density of 2.5 X 10^5 cells/ml in Williams' Medium E (Invitrogen, Carlsbad, CA) containing 5% Cosmic Calf Serum (CCS, Atlanta Biologicals, Norcross, GA) and plated in 12-well cell culture plates (Fisher Scientific Co., Pittsburgh, PA) at 0.80 ml/well. Cells were allowed to attach for 2-3 h before treatment.

Effect of hypoxia on elastase-induced cytotoxicity. Serum-containing medium was removed, and fresh Williams' Medium E containing various concentrations of human PMN elastase (Molecular Innovations, Southfield, MI) were added to HPCs. PMN elastase activity

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was determined using the colorometric PMN elastase substrate, MeOSuc-Ala-Ala-Pro-Val-pNA (Calbiochem, San Diego, CA). One unit of PMN elastase activity was defined as the amount of enzyme required to cause a change of absorbance of 1.0 at 410 nm in 10 min at 37° C. PMN elastase-treated cells were immediately transferred to incubators containing either 20% or 5% O_2, with CO_2 controlled at 5%. Two or 8 h later, the medium was collected, and the remaining attached cells were lysed with an equal volume of 1% triton X-100. Media and lysates were centrifuged at 600xg for 5 min. Cytotoxicity was assessed by measuring ALT release into the medium using Infinity-ALT reagent from Thermo Electron Corp. (Louisville, CO). ALT activity in the medium was expressed as a percent of total ALT activity (i.e., medium activity plus lysate activity).

Statistical analysis. Two-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons was used for analysis of PMN accumulation and serum CINC-1 at 3 h and for all measures in the PMN depletion study. One-way ANOVA with Tukey's test was used for all measures in the heparin experiment. Two-way repeated measures ANOVA with Tukey's test was used for *in vitro* studies. The criterion for significance for all studies was $p<0.05$.

Results

Effect of LPS/RAN-treatment on serum CINC-1 concentration and liver PMN accumulation. Serum CINC-1 concentration and liver PMN accumulation were evaluated at a time near the onset of hepatotoxicity (3 h; Luyendyk et al., 2003b) in rats treated with LPS/RAN. Treatment with RAN alone caused a modest (~8-fold) but statistically significant increase in serum CINC-1 (Fig. 1A). By contrast, rats treated with either LPS alone or LPS/RAN had a large increase (~700-fold) in serum CINC-1 (Fig. 1A). PMN accumulation occurred in livers of rats treated with LPS; the degree of accumulation was slightly less in rats cotreated with RAN (Fig. 1B). The distribution of PMNs was panlobular in rats given either LPS/Veh or LPS/RAN, with occasional clusters of PMNs in midzonal areas of necrosis in LPS/RAN-treated rats (Fig 1C and D).

Effect of PMN depletion on LPS/RAN-induced liver injury. Although LPS/RAN administration is hepatotoxic, given alone the doses of LPS and RAN are not hepatotoxic within 24 h after administration (Luyendyk et al., 2003b). LPS/RAN-treated rats were pretreated with either normal serum or anti-PMN serum and were killed 6 h after RAN administration (i.e., time of peak injury; Luyendyk et al., 2003b) to determine the role of PMNs in the liver injury. LPS/RAN-treatment caused a pronounced neutrophilia (Table 1) and lymphocytopenia (NS/Veh/Veh-treated rats, 4900 ± 112 lymphocytes/μL; NS/LPS/RAN-treated rats, 2566 ± 260 lymphocytes/μL) compared to Veh/Veh-treatment. Whereas most of the LPS/RAN-induced increase in blood PMNs comprised mature cells (with segmented nuclei), immature PMNs (band cells) were also elevated. Anti-PMN serum pretreatment significantly reduced total blood leukocytes and caused a pronounced decrease (~95%) in blood PMNs in LPS/RAN-treated rats

(Table 1); Lymphocyte, monocyte, eosinophil and basophil numbers were unaffected (data not shown).

To confirm that depletion of circulating PMNs resulted in reduced accumulation of these cells in liver, PMNs were enumerated in livers from LPS/RAN-treated rats. Few PMNs were observed in livers of rats given either normal serum/Veh/Veh or anti-PMN serum/Veh/Veh, and these were characterized by segmented nuclei. Normal serum/LPS/RAN-treatment caused significant hepatic PMN accumulation (Fig. 2A) that was reduced by 50% in anti-PMN serum/LPS/RAN-treated rats (Fig. 2B and C). In addition to a decrease in PMN accumulation, anti-PMN serum caused a qualitative change in hepatic PMNs. Compared to normal serum/LPS/RAN-treated rats, fewer segmented (mature) and degenerate PMNs were found in livers of anti-PMN serum/LPS/RAN-treated rats (Table 2). In contrast, a slight but significant increase in accumulated band cells (immature PMNs) occurred in anti-PMN serum/LPS/RAN-treated rats compared to those animals given normal serum (Table 2).

A significant increase in serum ALT activity was observed in normal serum/LPS/RAN-treated rats at 6 h, and this was markedly reduced (~80%) by anti-PMN serum pretreatment (Fig. 2D). Distribution of PMNs was panlobular in LPS/RAN-treated rats given either normal serum or anti-PMN serum. However, clustering of PMNs, many of which were categorized as degenerate, occurred in midzonal and subserosal areas of oncotic necrosis in normal serum/LPS/RAN-treated rats (Fig. 2A). Consistent with the decrease in serum ALT activity, the frequency and size of lesions in LPS/RAN-treated rats was markedly reduced by anti-PMN serum pretreatment (Fig. 2B).

Effect of PMN depletion on coagulation system activation after LPS/RAN treatment. Coagulation system activation was estimated in LPS/RAN-treated rats given either

normal serum or anti-PMN serum 6 h after RAN administration by a decrease in plasma fibrinogen and an increase in plasma TAT concentrations. LPS/RAN-cotreatment significantly decreased plasma fibrinogen concentration, and this decrease was not significantly affected by administration of anti-PMN serum (Fig. 3A). Plasma TAT concentration increased after LPS/RAN-treatment and was not significantly reduced by anti-PMN serum administration (Fig. 3B).

Effect of heparin on hepatotoxicity after LPS/RAN treatment. LPS/RAN-treatment caused a significant increase in serum ALT activity that was modest at 3 h and progressed through 6 h after RAN administration (Fig. 4A). Lesions in livers of LPS/RAN-treated rats have been described previously (Luyendyk et al., 2003b) and were characterized by midzonal and subserosal hepatocellular oncotic necrosis and neutrophilic infiltrate (data not shown). Previous studies showed that the coagulation system is a critical mediator of LPS/RAN-induced liver injury (Luyendyk et al., 2004a). Confirming these results, serum ALT activity was significantly attenuated by heparin at 6 h (Fig. 4A), a time associated with maximal injury in LPS/RAN-treated rats (Luyendyk et al., 2003b). Furthermore, heparin prevented the slight increase in ALT activity at 3 h observed in this study (Fig. 4A). The reduction in serum ALT activity by heparin was associated with a marked reduction in midzonal oncotic necrosis, though infrequent subserosal lesions were observed in livers of some LPS/heparin/RAN-treated rats (data not shown). Consistent with a previously demonstrated role for fibrin deposition in this model, the efficacy as anticoagulant and hepatoprotective effect of heparin were confirmed by reductions in LPS/RAN-induced hepatic fibrin deposition and in serum ALT activity at 3 and 6 h, respectively (Fig. 4B).

Effect of heparin on serum CINC-1 concentration and liver PMN accumulation after LPS/RAN treatment. Heparin did not alter serum CINC-1 concentration at either 3 or 6 h in LPS/RAN-treated rats (Fig. 5A). However, CINC-1 decreased between 3 h and 6 h significantly (by approximately 65%) in rats treated either with LPS/Veh/RAN or with LPS/heparin/RAN. Heparin did not reduce hepatic PMN accumulation at either time (Fig. 5B).

Effect of heparin on liver hypoxia after LPS/RAN treatment. One consequence of sinusoidal fibrin deposition, i.e., liver hypoxia, occurs at a time near the onset of liver injury in LPS/RAN-treated rats (Luyendyk et al., 2004a). We tested whether the reduction in hepatocellular injury by heparin was associated with prevention of tissue hypoxia. Little pimonidazole (PIM)-adduct staining was noted in livers of Veh/Veh-treated rats (Fig. 6A). As described previously (Luyendyk et al., 2004a), hepatocellular PIM-adduct staining increased in livers of LPS/RAN-treated rats at 3 h, and this staining persisted to 6 h (Fig. 6B and 6D). At 3 h, PIM staining was panlobular, with darker areas of staining localized to areas of liver destined to become damaged (i.e., midzonal areas; Fig 6B). Heparin coadministration reduced this markedly at both 3 and 6 h (Fig. 6C and 6D).

Effect of hypoxia on PMN elastase induced killing of rat HPCs. The observation that either PMN depletion (Fig. 2) or anticoagulation (Fig. 4) almost abolished hepatocellular injury suggested an interdependence of hemostasis-mediated hypoxia and PMNs in LPS/RAN-induced liver injury. Since in other models of PMN-dependent liver injury, toxic PMN proteases are important mediators of hepatocellular killing both *in vitro* and *in vivo* (Ho et al., 1996; Ishii et al., 2002), we explored the potential for interaction between hypoxia and PMN elastase in HPC killing. Using isolated HPCs, preliminary experiments were conducted to identify a reduction in chamber O_2 concentration to which HPCs could be exposed without evidence of significant

cytotoxicity, as evaluated by release of ALT into culture medium. Incubation of HPCs for 2 or 8 h in 5% O_2 did not lead to ALT release (Fig. 7), whereas incubation in concentrations less than 3% O_2 did (data not shown). By 8 h in oxygen replete atmosphere (20% O_2), PMN elastase caused significant ALT release at the largest concentration tested (8.8 U/ml; Fig. 7B), confirming previous results (Ganey et al., 1994). Exposure to 5% O_2 resulted in a leftward shift in the concentration-response relationship for PMN elastase-induced cytotoxicity at 8 h, indicating increased potency of PMN elastase in HPC killing. At a shorter incubation time of 2 h, PMN elastase failed to cause HPC cytotoxicity in an O_2-replete atmosphere, whereas it was considerably cytotoxic in hypoxic HPCs (Fig. 7A).

Discussion

Previous experiments demonstrated that LPS/RAN-cotreatment caused hepatotoxicity in rats beginning about 3 h after RAN administration (Luyendyk et al., 2003b). Evaluation of H&E-stained liver sections from LPS/RAN-treated rats revealed neutrophilic infiltration in areas of liver damage in this model. Using immunohistochemical staining of PMNs, we quantified PMN accumulation in livers of rats given either LPS and/or RAN. At 3 h, liver PMN accumulation occurred to a similar degree in LPS-treated rats, irrespective of RAN administration (Fig. 1A). Consistent with CINC-1 gene expression in this model (Luyendyk et al., 2004b; D11445exon#1-4_s_at), serum CINC-1 concentration was increased in LPS/Veh and LPS/RAN-treated rats to a similar degree. This suggests that increased CINC-1 is not sufficient to cause liver injury. In addition to its effect on accumulation of PMNs in liver, LPS/RAN-cotreatment caused a decrease in blood lymphocytes and an increase in blood PMNs in the absence of a change in total leukocyte count (Table 1). Similar changes in blood leukocyte concentration were observed in rats treated with a large, hepatotoxic dose of LPS (Hewett et al., 1992). These results suggest that the effect of LPS/RAN-treatment on circulating and hepatic PMNs is driven largely by LPS.

Liver injury in this model depends on an activated hemostatic system and the formation of fibrin clots (Luyendyk et al., 2004a). Confirming this result, heparin administration prevented or delayed hepatic fibrin deposition and hepatocellular injury in LPS/RAN-treated rats (Fig 4). In another model of liver damage involving hypoxia (i.e., ischemia-reperfusion), coagulation system activation favors chemokine production and PMN accumulation (Yamaguchi et al., 1997). Inasmuch as hemostasis-mediated hepatocellular hypoxia occurs in LPS/RAN-treated rats, we considered the possibility that coagulation system activation is important for the production of CINC-1 and liver PMN accumulation. This possibility seems unlikely, since

coagulation system activation occurs only in LPS/RAN-treated rats (Luyendyk et al., 2004a), yet PMN accumulation was similar in LPS-treated and LPS/RAN-treated rats (Fig. 1). Furthermore, heparin did not significantly reduce either liver PMN accumulation or serum CINC-1 concentration (Fig. 5). Overall, these results suggest that the coagulation system is not required for PMN accumulation in LPS/RAN-treated rats.

The observation that liver PMN accumulation was similar after treatment with LPS only or LPS/RAN indicates that accumulation of those cells is not injurious by itself, since LPS given alone at the dose used did not cause liver injury. Nevertheless, since there could be treatment-related differences in PMN activation, we tested the hypothesis that PMNs are critical for LPS/RAN-induced liver injury. LPS/RAN-treated rats given anti-PMN serum demonstrated attenuated hepatic accumulation of mature, segmented PMNs, evidenced by a 50% reduction in total PMN accumulation (Fig. 2C) and a qualitative shift towards the accumulation of immature (band) PMNs (Table 2). This reduction in accumulated PMNs substantially reduced hepatocellular injury, suggesting a non-linear relationship between the number of accumulated PMNs and hepatocellular injury (Fig. 2). Accordingly, RAN cotreatment probably triggers signals secondary to PMN accumulation that are required for the toxic action of PMNs on HPCs. Indeed, to cause hepatocellular injury, PMNs require chemotactic signals, adhesion molecule interactions and cell activation, and these events can be independent of or secondary to signals required for their accumulation (Jaeschke and Smith, 1997; Maher et al., 1997). This raises the question of what factor(s) permit accumulated PMNs to become cytotoxic to HPCs in LPS/RAN-cotreated rats. Additional studies will be needed to address this.

Either depletion of PMNs or anticoagulation reduced liver injury in this model by more than 80%, suggesting a requirement for interaction between PMNs and the coagulation system,

as opposed to each contributing independently to liver damage. One possibility is that PMNs contribute to LPS/RAN-induced liver injury by participating in coagulation system activation. PMN depletion was without effect on LPS/RAN-induced changes in plasma biomarkers of coagulation system activation (Fig. 3), suggesting that PMNs do not play a major role in coagulation in this model; However, we cannot rule out the possibility that liver specific effects might not be reflected in systemic markers of coagulation system activation. Another possibility is that an activated coagulation system is required for full activation of PMNs in LPS/RAN-treated rats. This could occur in several ways. For example, thrombin contributes to PMN activation by activating protease activated receptor-1 (PAR-1) in rats treated with a large dose of LPS (Copple et al., 2003). This receptor is expressed by Kupffer cells, sinusoidal endothelial cells (SECs) and hepatic stellate cells, but not by rat PMNs (Copple et al., 2003; Marra et al., 1998), suggesting that thrombin-mediated PAR-1 activation activates PMNs by indirect pathways. Another possibility is that fibrin clot formation causes liver hypoxia that can enhance PMN activation. Hypoxia might promote PMN activation by direct or indirect mechanisms, including altered expression of adhesion molecules on SECs (Arnould et al., 1994; Tamura et al., 2002). Consistent with the hypothesis that coagulation system activation causes liver hypoxia and injury, heparin reduced hepatic fibrin deposition, hypoxia, and HPC injury in LPS/RAN-treated rats (Figs. 4 and 6). Accordingly, coagulation system activation might promote activation of PMNs secondary to their accumulation by causing liver hypoxia. However, the contributions of either PAR-1 activation or hypoxia to PMN activation in this model are not yet known.

If severe enough, hypoxia is sufficient to cause hepatocellular injury in rats (Fassoulaki et al., 1984) and in isolated, perfused livers (Lemasters et al., 1981), and it is capable of inciting cell death in cultured HPCs (Khan and O'Brien, 1997). Less severe hypoxia can render the liver

and isolated HPCs sensitive to secondary insult from hepatotoxicants and some drugs (Bacon et al., 1996; McGirr et al., 1990; Shen et al., 1982; Silva et al., 1992). That is, otherwise noninjurious degrees of hypoxia can lower the threshold for xenobiotic-induced hepatocellular injury. Furthermore, histopathologic changes in liver caused by LPS exposure are worsened by coexposure to an otherwise noninjurious reduction in inspired O_2 (Shibayama, 1987), suggesting that hypoxia increases either the degree of LPS-induced inflammation or the sensitivity of hepatocytes to the cytotoxic effects of inflammatory mediators. Interplay between hypoxia and inflammatory factors such as macrophages, cyclooxygenase-2 and PMNs, has been reported (Hannah et al., 1995; Lahat et al., 2003; Tamura et al., 2002; Zhong et al., 2004).

Activated PMNs release several cytotoxic factors including the proteases such as PMN elastase, which can kill rat HPCs *in vitro* (Ho et al., 1996). Few studies have examined the relationship between hypoxia and cellular sensitivity to toxic proteases. In one *in vitro* model of hypoxia/reoxygenation, cardiac myocytes were rendered more sensitive to PMN elastase cytototoxicity during the reoxygenation phase (Buerke et al., 1994). However, the relative roles of hypoxia and reoxygenation phases in enhancing sensitivity to PMN elastase were not separated. As the data in Fig. 7 indicate, HPCs cultured under hypoxic conditions are rendered susceptible to killing by normally nontoxic concentrations of PMN elastase. Interestingly, the timecourse over which HPC killing occurred during hypoxia resembles the development of injury in LPS/RAN-treated rats (Luyendyk et al., 2003b). By contrast, the killing of HPCs by PMN elastase in an oxygen-replete atmosphere (20% O_2) requires 8-16 h to reach statistical significance (Ganey et al., 1994). This result indicates that hypoxia can increase sensitivity to PMN elastase cytotoxicity as well as accelerate PMN elastase-induced cell killing. The applicability of this observation likely extends outside the LPS/RAN-model, insofar as

neutrophils are probably often activated in a hypoxic environment in tissue. Indeed, hypoxia-induced hypersensitivity of HPCs to the toxic effects of PMN elastase might be important in liver transplantation, hepatic ischemia-reperfusion, alcoholic liver disease and other conditions in which both PMN activation and liver hypoxia occur.

In summary, rats cotreated with LPS/RAN develop liver hypoxia with a timecourse similar to that of hepatocellular injury (Luyendyk et al., 2003b). Anticoagulation with heparin markedly attenuated hepatic fibrin deposition, hypoxia, and hepatocellular injury, suggesting a connection among these three events. However, anticoagulation was without effect on CINC-1 and hepatic PMN accumulation. PMN accumulation occurred in livers of rats treated with LPS/RAN, and depletion of PMNs almost abolished liver injury but was without effect on coagulation system activation. Overall, the results are consistent with LPS/RAN-induced hepatocellular injury occurring by two independent arms, culminating in increased hepatocellular sensitivity to PMN-derived cytotoxic factors caused by hemostasis-mediated hypoxia (Fig. 8). Hypoxia rendered HPCs sensitive to killing by PMN elastase, so that fibrin-mediated liver hypoxia might be an important determinant of hepatocellular sensitivity to cytotoxic effects of PMNs in this and other models.

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Footnotes:

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Legends for figures

Figure 1: Effect of LPS/RAN-treatment on serum CINC-1 concentration and liver PMN accumulation. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then two h later with 30 mg/kg RAN or its Veh (iv). Serum CINC-1 concentration (A) and hepatic PMN accumulation (B) were evaluated 3 h after drug treatment. Data are expressed as mean ± SEM. n=3-4 rats per group. *Significantly different from respective group not given LPS $^{\#}$Significantly different from respective group not given RAN ($p<0.05$). (C) Representative liver section (100X) from a LPS/Veh-treated rat 3 h after Veh treatment showing panlobular PMN accumulation. (D) Representative liver section (100X) from a LPS/RAN-treated rat showing panlobular PMN accumulation and early foci of PMN accumulation in midzonal areas (asterisk). PP, periportal. CL, centrilobular.

Figure 2: Effect of PMN depletion on hepatotoxicity in LPS/RAN-treated rats. Rats were pretreated (iv) with either 0.25 ml of normal rabbit serum (NS) or rabbit anti-rat PMN serum (NAS). 16 h later, they were given 44.4 X 10^6 EU/kg LPS or its Veh (iv), then 2 h later 30 mg/kg RAN or its Veh was administered (iv). (A) Representative liver section from a normal serum/LPS/RAN-treated rat 6 h after RAN treatment showing PMNs (purple), especially concentrated in a focus of oncotic necrosis. (B) Representative liver section from a NAS/LPS/RAN-treated rat 6 h after RAN treatment showing fewer PMNs and no necrotic focus (C) Liver PMN accumulation was evaluated in 10-20, randomly selected, 400X fields 6 h after drug administration as described in *Materials and Methods.* (D) Hepatic parenchymal cell injury was estimated 6 h after drug administration by increases in serum ALT activity. Data are expressed as mean ± SEM. n=4-6 rats per group. *Significantly different from respective

Veh/Veh-treated group [#]Significantly different from respective normal serum-treated group ($p<0.05$).

Figure 3: Effect of PMN depletion on coagulation system activation in LPS/RAN-treated rats. Rats were pretreated (iv) with either 0.25 ml of normal rabbit serum (NS) or rabbit anti-rat PMN serum (NAS). 16 h later, they were given 44.4 X 10^6 EU/kg LPS or its Veh (iv), then 2 h later 30 mg/kg RAN or its Veh was administered (iv). Activation of the coagulation system was estimated as decreased plasma fibrinogen concentration (A) and increased plasma thrombin-antithrombin (TAT) concentration (B) 6 h after RAN treatment. Data are expressed as mean ± SEM. n=4-6 rats per group. *Significantly different from respective Veh/Veh-treated group.

Figure 4: Effect of heparin on hepatotoxicity and hepatic fibrin deposition after LPS/RAN treatment. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then one h later with 3000 U/kg heparin or its Veh (s.c.). Two h after LPS, 30 mg/kg RAN or its Veh was administered (iv). For (A), hepatic parenchymal cell injury was estimated 3 or 6 h after RAN administration by increases in serum ALT activity. For (B), livers were removed 3 or 6 h after RAN treatment and processed for fibrin immunohistochemistry, and the area of positive fibrin staining was determined morphometrically in 10, randomly chosen, 100X fields per tissue. Data are expressed as mean ± SEM. n=6-9 in each group. *Significantly different from Veh/Veh/Veh-treated rats at that time. #Significantly different from LPS/Veh/RAN-treated rats at that time. [a]Significantly different from the same treatment at 3 h. ($p<0.05$).

Figure 5: Effect of heparin on serum CINC-1 concentration and PMN accumulation in LPS/RAN-treated rats. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then one h later with 3000 U/kg heparin or its Veh (s.c.). Two h after LPS, 30 mg/kg RAN or its Veh was administered (iv). Serum CINC-1 concentration (A) and hepatic PMN accumulation (B) were evaluated 6 h after RAN treatment. Data are expressed as mean ± SEM. n=4-9 rats per group. *Significantly different from Veh/Veh/Veh-treated rats at that time. #Significantly different from LPS/Veh/RAN-treated rats. aSignificantly different from the same treatment at 3 h. ($p<0.05$). Values for Veh/Veh/Veh-treated rats were too small to be apparent in (B).

Figure 6: Effect of heparin on liver hypoxia in LPS/RAN-treated rats. Rats were treated with 44.4 X 10^6 EU/kg LPS or its Veh (iv), then one h later with 3000 U/kg heparin or its Veh (s.c.). Two h after LPS, 30 mg/kg RAN or its Veh was administered (iv). Two h before rats were killed, 120 mg/kg PIM was given (i.p.). Livers were removed 3 or 6 h after RAN treatment and processed for PIM-adduct immunohistochemistry as described in *Materials and Methods*. Representative photomicrograph (100X) showing (A) little PIM-adduct staining in a liver from a Veh/Veh/Veh-treated rat, (B) marked panlobular PIM-adduct staining in a liver from a LPS/Veh/RAN-treated rat and (C) reduced staining intensity in a liver from a rat treated with LPS/heparin/RAN. (D) The area of PIM-adduct staining in 10 randomly chosen, 100X fields per tissue was determined morphometrically as described in *Materials and Methods*. Data are expressed as mean ± SEM. n=6-9 rats per group. *Significantly different from Veh/Veh/Veh-treated rats at that time. #Significantly different from LPS/Veh/RAN-treated rats. Values for Veh/Veh/Veh-treated rats were too small to be apparent in (D).

Figure 7: Effect of hypoxia on PMN elastase-induced killing of rat HPCs. Rat HPCs were cultured in serum-free medium containing various concentrations of human PMN elastase and incubated in either 5% or 20% O_2. Cytotoxicity was evaluated as ALT released into culture medium 2 h (A) or 8 h (B) later. Data are expressed as mean ± SEM. n=3 separate HPC isolations. *Significantly different from respective treatment with 0 U/ml PMN elastase. #Significantly different from the respective treatment incubated in 20% O_2 ($p<0.05$).

Figure 8: Increased susceptibility to PMN-mediated hepatocellular injury by fibrin-mediated hypoxia in LPS/RAN-treated rats. LPS/RAN-cotreatment caused activation of the coagulation system, increased antifibrinolytic PAI-1 expression (Luyendyk et al., 2004b), hepatic fibrin deposition, and liver hypoxia (Luyendyk *et al.*, 2004a). Anticoagulation with heparin significantly reduced fibrin deposition, hypoxia, and hepatocellular injury (Figs. 4 and 6). LPS alone caused accumulation of PMNs in liver (Fig. 1) but at the dose used did not cause hepatocellular injury (Luyendyk *et al.*, 2003b). In LPS/RAN-treated rats, hepatocellular injury was dependent on PMNs (Fig. 2), suggesting that RAN contributes to PMN activation and release of cytotoxic factors including proteases (e.g., elastase). Depletion of PMNs was without effect on coagulation system activation (Fig. 3) and anticoagulation did not influence hepatic PMN accumulation (Fig. 5), suggesting that PMN accumulation/activation and coagulation system activation occur independently. I*n vitro*, hypoxia rendered hepatocytes (HPCs) sensitive to killing by PMN elastase (Fig. 7), suggesting liver hypoxia (*e.g.*, caused by fibrin deposition) increases susceptibility to PMN elastase -induced hepatocellular injury.

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Table 1
Effect of PMN-depleting antiserum on circulating leukocyte concentrations in LPS/RAN-treated rats

Serum Pretreatment	Treatment	Leukocytes	Segmented PMNs	Band Cells
NS	Veh/Veh	5663 ± 382	834 ± 140	0 ± 0
NAS	Veh/Veh	3392 ± 184#	45 ± 17#	40 ± 10#
NS	LPS/RAN	5460 ± 542	2370 ± 296*	306 ± 49*
NAS	LPS/RAN	3242 ± 424#	43 ± 20#	200 ± 52*

Rats were pretreated with either 0.25 ml of normal rabbit serum (NS) or rabbit anti-rat PMN serum (NAS) diluted 1:1 in sterile saline (iv). 16 h later, they were given 44.4 X 10^6 EU/kg LPS or its Veh (iv), then 2 h later 30 mg/kg RAN or its Veh was administered (iv). Total leukocyte and differential counts were performed 6 h after RAN administration as described in *Materials and Methods.* All values are cells per μL blood *Significantly different from respective Veh/Veh-treated group #Significantly different from respective normal serum-treated group ($p<0.05$).

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Table 2
Effect of PMN-depleting antiserum on types of PMNs accumulated in livers of LPS/RAN-treated rats

		Average # of PMNs/ 400X HPF		
Serum Pretreatment	**Treatment**	**Segmented**	**Band**	**Degenerate**
NS	**Veh/Veh**	**0.4 ± 0.05**	**0**	**0**
NAS	**Veh/Veh**	**0.2 ± 0.05**	**0.1 ± 0.04**	**0**
NS	**LPS/RAN**	**16.6 ± 2.1***	**1.4 ± 0.4***	**6.5 ± 1.0***
NAS	**LPS/RAN**	**8.9 ± 0.4*#**	**2.2 ± 0.3*#**	**2.0 ± 0.3*#**

Rats were pretreated with either 0.25 ml of normal rabbit serum (NS) or rabbit anti-rat PMN serum (NAS) diluted 1:1 in sterile saline (iv). 16 h later, they were given 44.4 X 10^6 EU/kg LPS or its Veh (iv), then 2 h later 30 mg/kg RAN or its Veh was administered (iv). PMN immunohistochemistry and classification and enumeration of liver PMNs were performed 6 h after RAN administration as described in *Materials and Methods.* *Significantly different from respective Veh/Veh-treated group. #Significantly different from respective normal serum-treated group ($p<0.05$).

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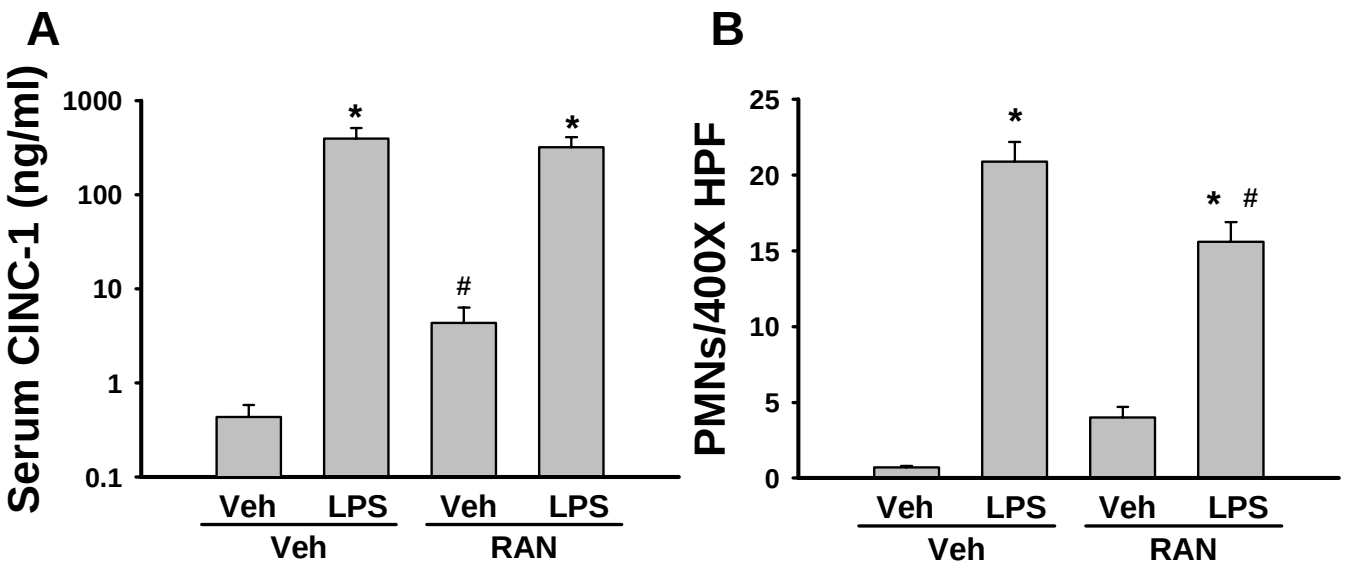
Figure 1



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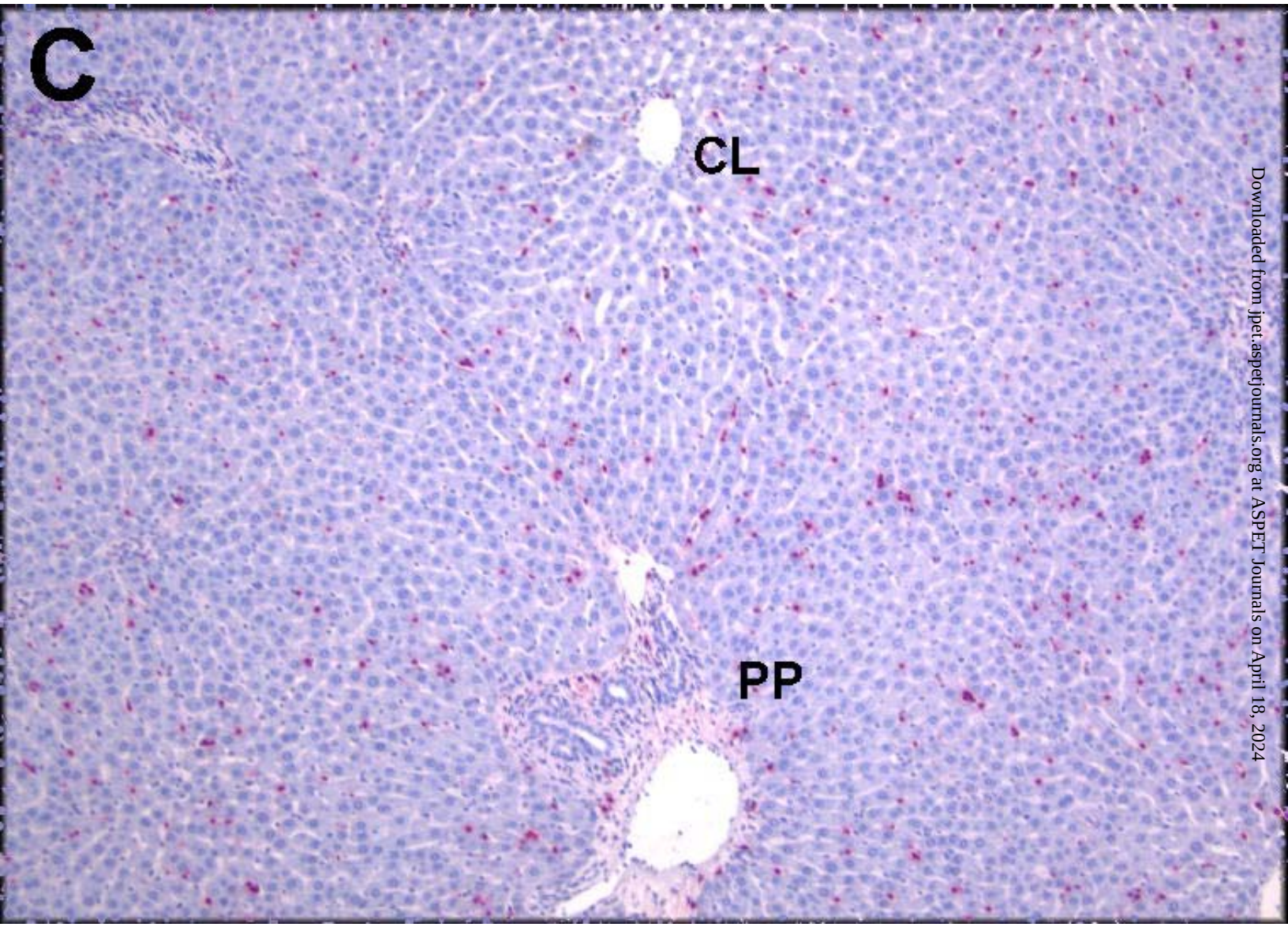

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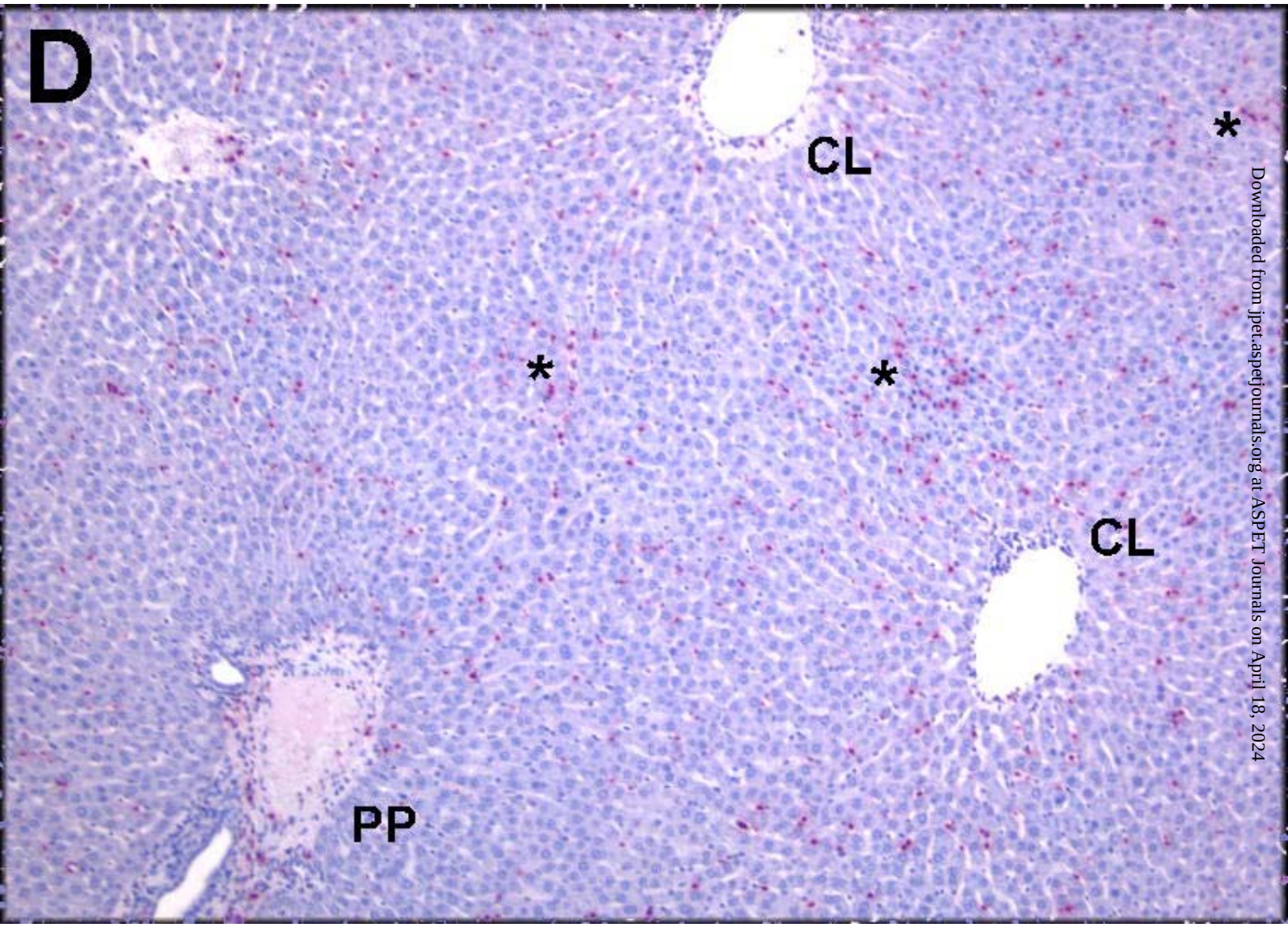

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Figure 2

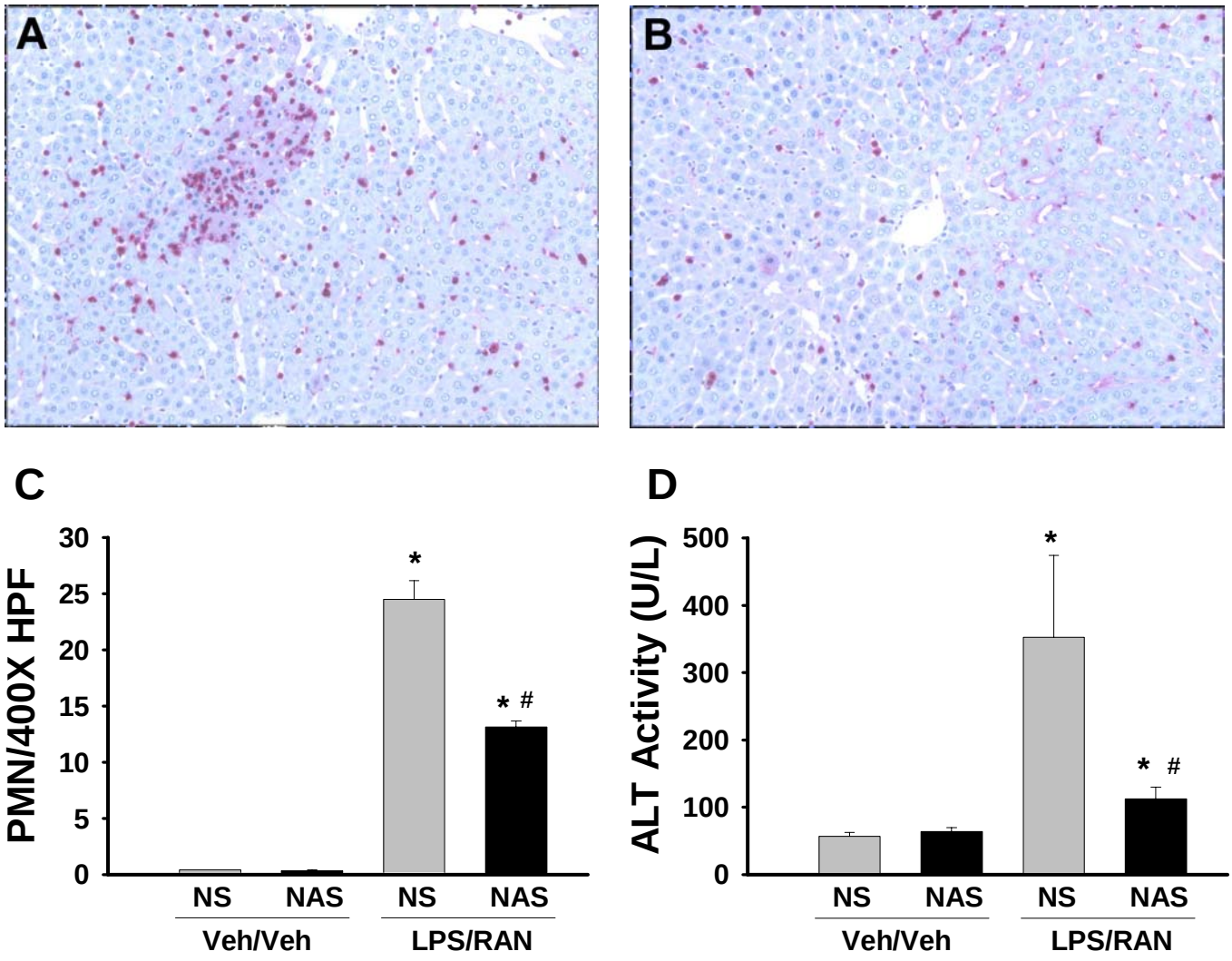

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Figure 3

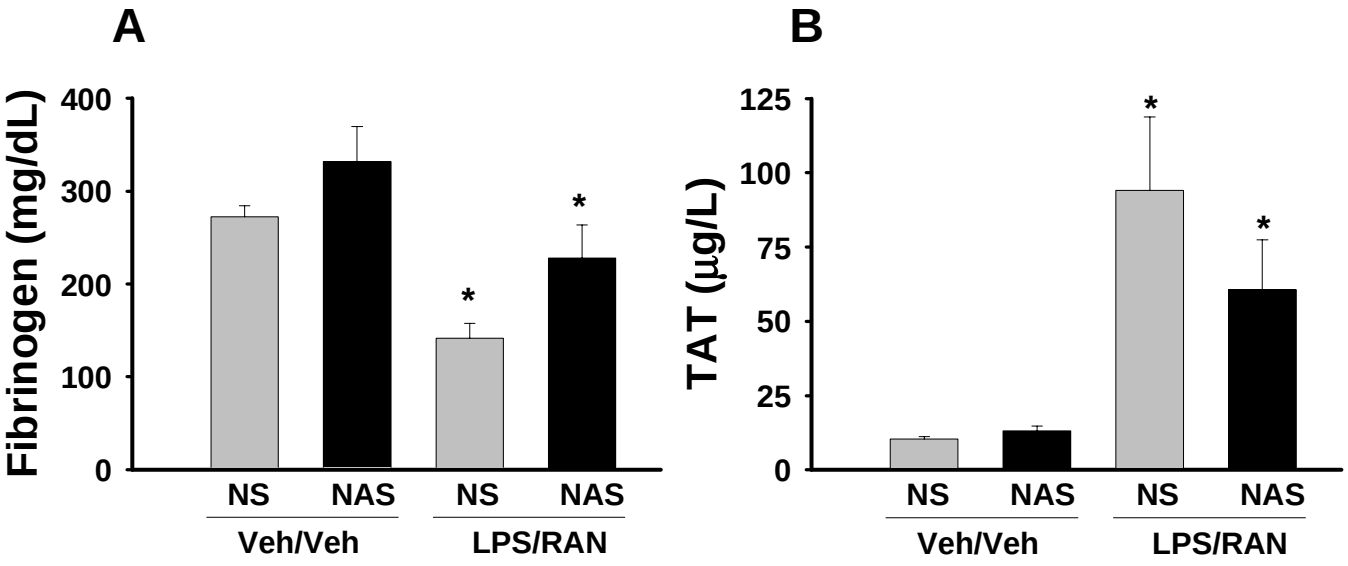

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Figure 4

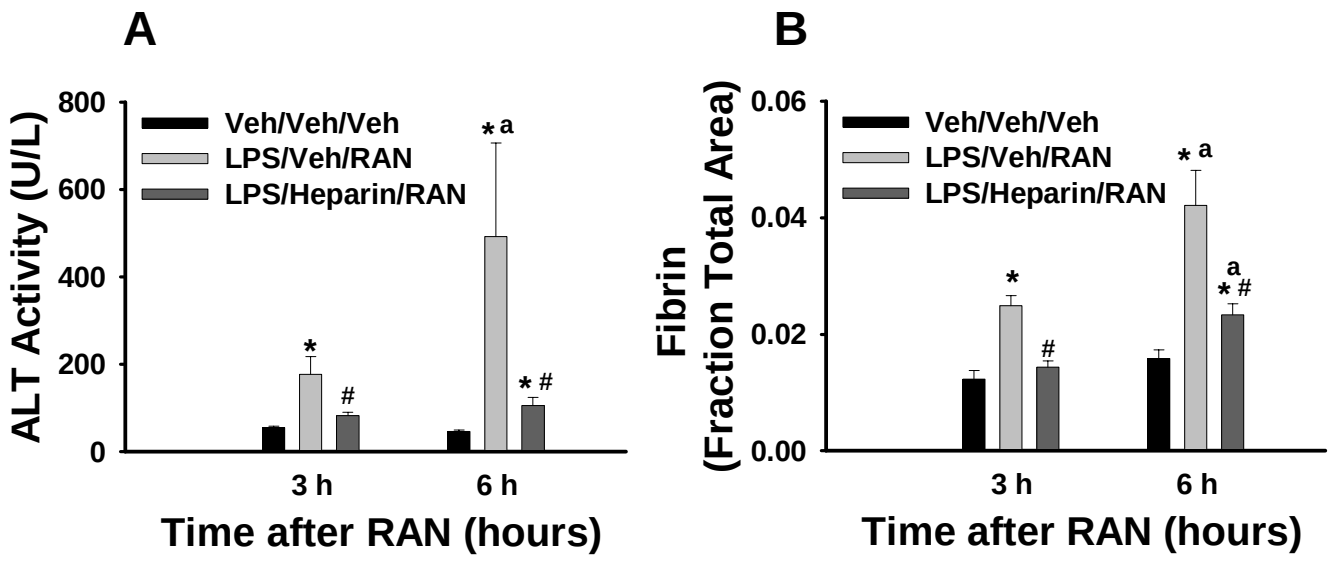

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Figure 5

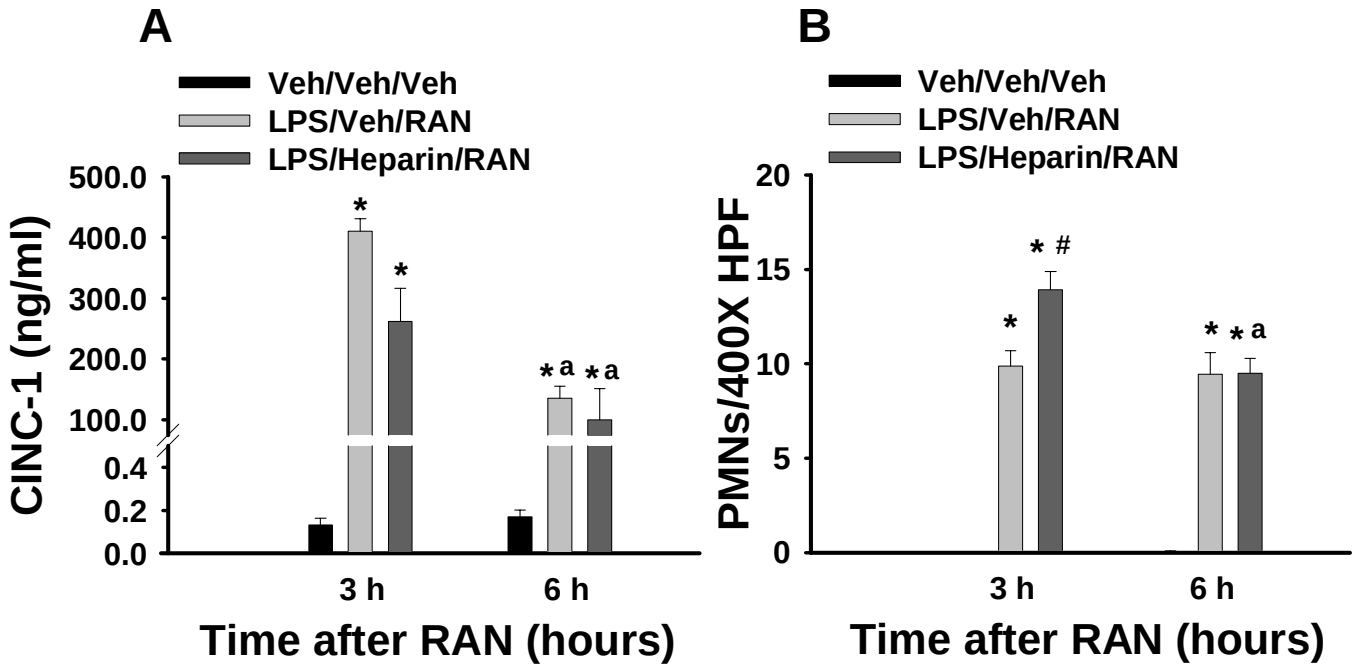

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Figure 6

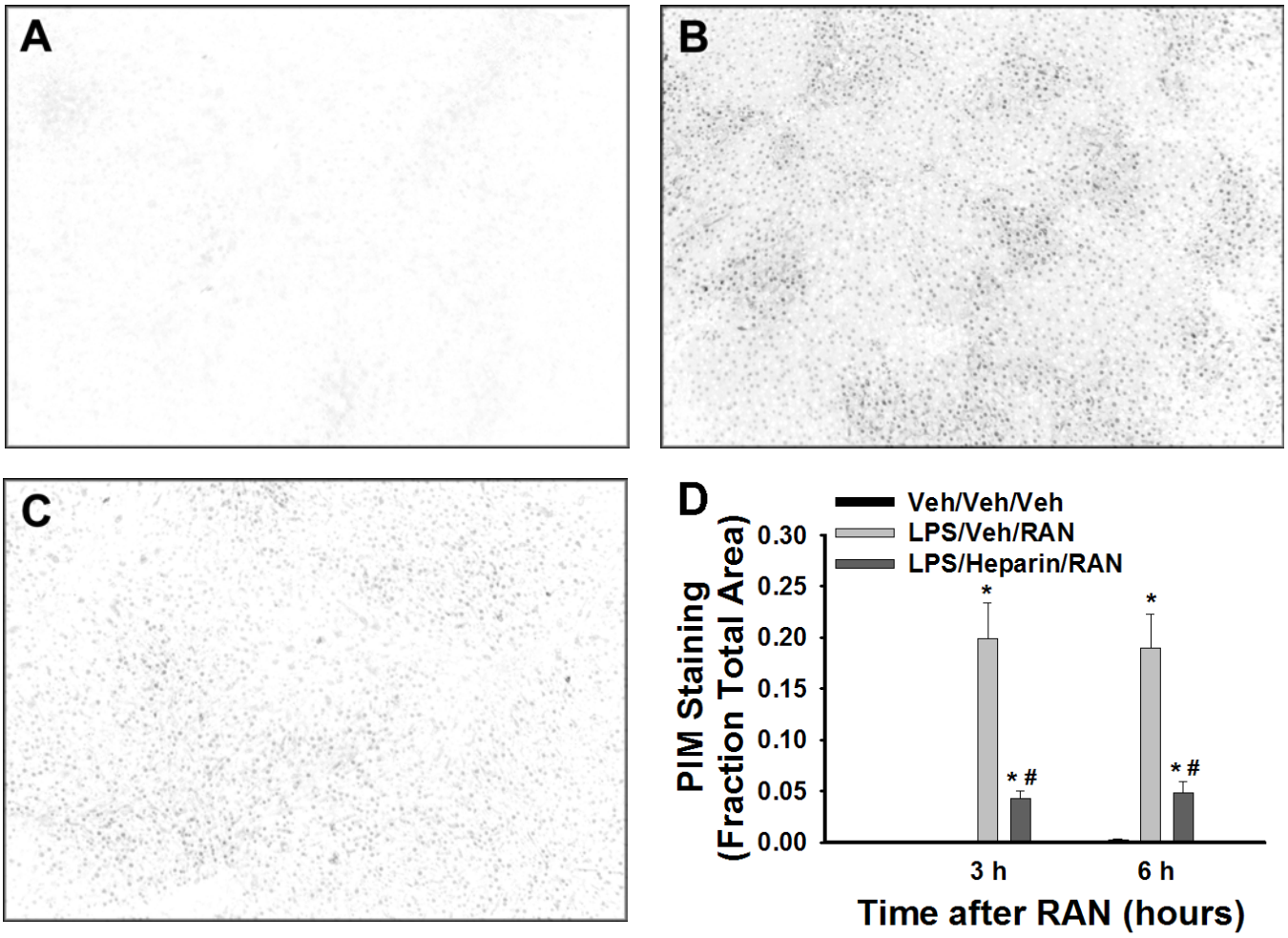

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Figure 7

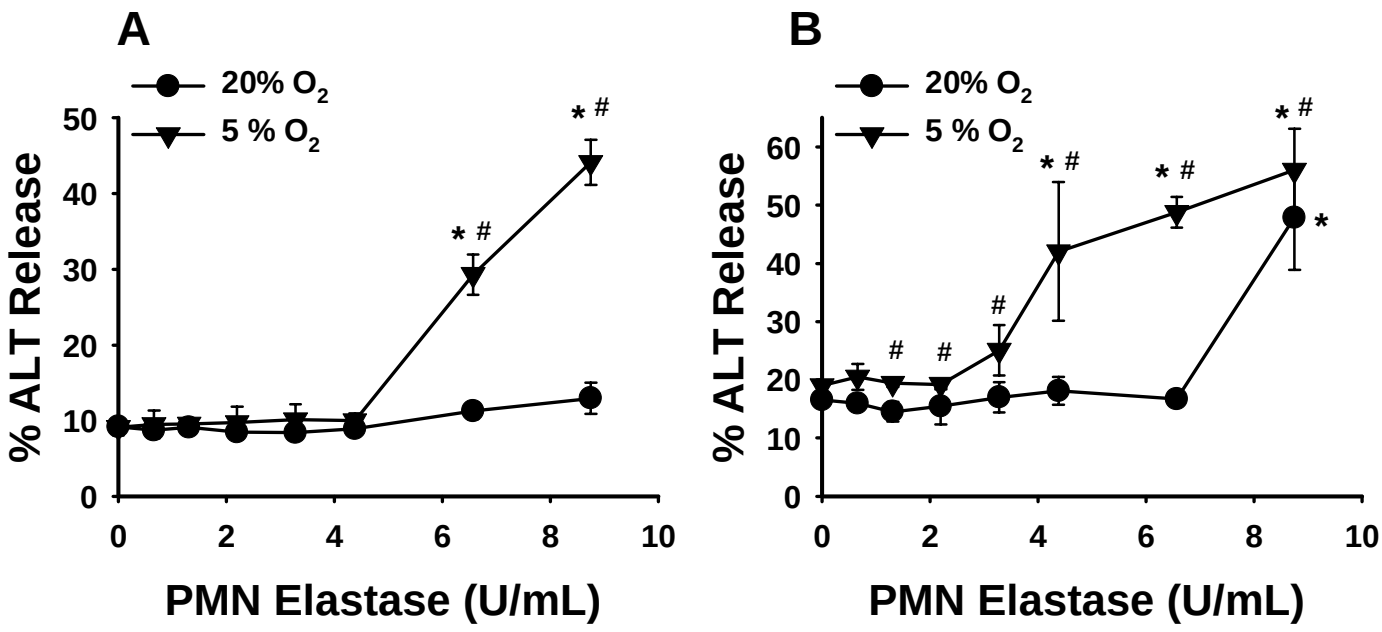

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Figure 8