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Neutrophil-cytokine interactions in a rat model of sulindac-induced idiosyncratic liver injury

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Abstract

Previous studies indicated that lipopolysaccharide (LPS) interacts with the nonsteroidal anti-inflammatory drug sulindac (SLD) to produce liver injury in rats. In the present study, the mechanism of SLD/LPS-induced liver injury was further investigated. Accumulation of polymorphonuclear neutrophils (PMNs) in the liver was greater in SLD/LPS-cotreated rats compared to those treated with SLD or LPS alone. In addition, PMN activation occurred specifically in livers of rats cotreated with SLD/LPS. The hypothesis that PMNs and proteases released from them play critical roles in the hepatotoxicity was tested. SLD/LPS-induced liver injury was attenuated by prior depletion of PMNs or by treatment with the PMN protease inhibitor, eglin C. Previous studies suggested that tumor necrosis factor- α (TNF) and the hemostatic system play critical roles in the pathogenesis of liver injury induced by SLD/LPS. TNF and plasminogen activator inhibitor-1 (PAI-1) can contribute to hepatotoxicity by affecting PMN activation and fibrin deposition. Therefore, the role of TNF and PAI-1 in PMN activation and fibrin deposition in the SLD/LPS-induced liver injury model was tested. Neutralization of TNF or inhibition of PAI-1 attenuated PMN activation. TNF had no effect on PAI-1 production or fibrin deposition. In contrast, PAI-1 contributed to fibrin deposition in livers of rats treated with SLD/LPS. In summary, PMNs, TNF and PAI-1 contribute to the liver injury induced by SLD/LPS cotreatment. TNF and PAI-1 independently contributed to PMN activation, which is critical to the pathogenesis of liver injury. Moreover, PAI-1 contributed to liver injury by promoting fibrin deposition.

Keywords

Idiosyncratic drug-induced liver injury; Sulindac; Lipopolysaccharide; Tumor necrosis factor- α ; Plasminogen activator inhibitor-1; Polymorphonuclear neutrophils

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1. Introduction

Idiosyncratic drug-induced liver injury (IDILI) has been a major concern for pharmaceutical companies because of its typically rare occurrence and unpredictable nature. Since predictive animal models for idiosyncratic drug-induced liver injury are lacking, this adverse effect typically manifests itself in late phase clinical trials or postmarketing. Accordingly, IDILI is a frequent reason for drug withdrawal from the market (Kaplowitz, 2005).

Sulindac is a nonsteroidal anti-inflammatory drug that has been used in the treatment of inflammation and pain. A retrospective analysis indicated that SLD has the greatest propensity to cause idiosyncratic liver injury among all currently marketed nonsteroidal anti-inflammatory drugs (Walker, 1997). Although the exact mechanism of SLD-induced idiosyncratic liver injury is not completely understood, the hypothesis has been raised that an inflammatory episode occurring during drug therapy might precipitate idiosyncratic liver injury (Roth et al., 2003; Ganey et al., 2004). Based on this hypothesis, a model of SLD-induced idiosyncratic liver injury was developed by cotreating rats with SLD and LPS (Zou et al., 2009b). Neither SLD nor LPS at the doses used was hepatotoxic, whereas cotreatment caused significant liver injury (Zou et al., 2009b). SLD enhanced the LPS-induced elevation in tumor necrosis factor- α (TNF) and plasminogen activator inhibitor-1 (PAI-1) in the plasma of rats (Zou et al., 2009b). TNF neutralization protected against liver injury in this model, suggesting that TNF plays an important role in the pathogenesis (Zou et al., 2009a). Fibrin deposition in liver sinusoids resulted from cotreatment and also contributed to SLD/LPS-induced hepatotoxicity (Zou et al., 2009b).

Understanding factors and mechanisms involved in the pathogenesis of IDILI could lead to the development of more effective preclinical screens to predict it. TNF and PAI-1 participate in other liver injury models by causing activation of PMNs and deposition of fibrin in liver sinusoids (Deng et al., 2008). Activated PMNs release proteases such as elastase and cathepsin G that can cause tissue injury. Accordingly, in this study the contribution of PMNs to SLD/LPS-induced liver injury was evaluated, and the role of TNF and PAI-1 in mediating PMN accumulation and activation as well as fibrin deposition in livers of SLD/LPS-treated rats was investigated.

2. Materials and Methods

2.1. Materials

LPS (Lot 075K4038) derived from *Escherichia coli* serotype O55:B5 with an activity of 3.3×10^6 endotoxin units (EU)/mg as well as SLD and its metabolites were purchased from Sigma-Aldrich (St. Louis, MO). Eglin C was provided by Novartis Pharm AG (Basel, Switzerland). PAI039 was purchased from Axon Medchem BV (Groningen, Netherlands).

2.2. Animals

Male, Sprague-Dawley rats (CrI:CD(SD)IGS BR; Charles River, Portage, MI) weighing 250 to 370 g were used. 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 hr light/dark cycle before use in experiments. All procedures were approved by the MSU Committee on Animal Use and Care and complied with "Guide for the Care and Use of Laboratory Animals" published by the National Academy of Sciences.

2.3. Animal Model and Sample Collection

The SLD/LPS-induced liver injury model was described previously (Zou et al., 2009b). Briefly, food was removed, and rats were given the first administration of SLD (50 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) 16 hr before a second administration of the same dose. LPS (8.25×10^5 EU/kg, i.v.) or its vehicle (saline) was administered half an hour before the second administration of SLD. Rats were anesthetized at various times after the second administration of SLD. Serum and plasma were prepared from blood withdrawn from the vena cava. Liver tissue from the left lateral lobe was collected and fixed in 10% buffered formalin for PMN staining. A portion of the left medial lobe of the liver was flash-frozen in isopentane for determination of hypochlorous acid (HOCl)-protein adduct staining as well as for fibrin deposition analysis.

2.4. Anti-PMN Serum, Eglin C, PAI039 and Etanercept Treatment Protocols

In PMN depletion experiments, rabbit anti-PMN serum or normal rabbit serum control was diluted 1:1 (v/v) in sterile saline and given to rats (0.5 ml per rat, i.v.) half an hour before the first administration of SLD. The efficacy of the anti-PMN serum in depleting PMNs has been demonstrated in previous studies (Deng et al., 2007). Eglin C is a potent and selective inhibitor of elastase and cathepsin G released by activated PMNs (Schnebli et al., 1985; Braun et al., 1987). Initial studies indicated that PMN activation began between 4 and 8 hrs after the second administration of SLD (see below). Accordingly, Eglin C (8 mg/kg, i.v.) or its saline vehicle was administered to rats 4, 6 and 8 h after the second administration of SLD, ie, prior to PMN activation. Multiple administrations were required due to the short half life of eglin C. A PAI-1 inhibitor, PAI039 [{1-benzyl-5-[4-(trifluoromethoxy)phenyl]-1H-indol-3-yl}(oxo)acetic acid] (6 mg/kg, p.o.) or its vehicle (0.5% methyl cellulose) was administered to rats 1 hr after the second administration of SLD. Etanercept (8 mg/kg) or vehicle (sterile water) was given to rats subcutaneously one hour before LPS or its saline vehicle. A previous study indicated that etanercept reduces LPS-induced increases in TNF activity in serum of rats using this treatment protocol (Tukov et al., 2007).

2.5. Evaluation of Hepatotoxicity

The activity of alanine aminotransferase (ALT) in serum was used as a marker to assess injury to hepatic parenchymal cells. The assay was performed using a diagnostic kit from Thermo Fisher Scientific (Waltham, MA). Three liver slices were fixed in 10% buffered formalin and stained with hematoxylin and eosin, and histological evaluation was performed.

2.6. Determination of CINC-1, MIP-1 α and PAI-1 Concentrations in Plasma

The concentrations of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and macrophage inflammatory protein-1 α (MIP-1 α) in plasma were estimated by multiplex ELISA. Specific antibody-coupled beads were purchased from Millipore Corp. (Billerica, MA). Functionally active PAI-1 was measured by ELISA using a commercially available kit from Molecular Innovations, Inc (Southfield, MI).

2.7. Evaluation of Liver PMN Accumulation and Activation

Paraffin-embedded liver tissue was cut into 6 μ m sections on which PMN immunohistochemistry was performed as described previously (Yee et al., 2003). Briefly, paraffin was removed with xylene, and liver sections were incubated with polyclonal rabbit anti-PMN IgG as first antibody, and then incubated with biotinylated goat anti-rabbit IgG, avidin-conjugated alkaline phosphatase, and Vector Red substrate to stain PMNs. The

numbers of PMNs enumerated in 10 randomly selected, 400 X high power fields were averaged to assess PMN accumulation in the liver.

Upon activation of PMNs, the potent oxidant HOCl is generated from hydrogen peroxide by myeloperoxidase in the presence of physiological chloride concentrations and reacts with proteins to form reactive chloramines. These HOCl-protein adducts can be used as fingerprints (Malle et al., 2006) to assess activation of PMNs in tissue (Hasegawa et al., 2005; Deng et al., 2007). Frozen liver sections fixed in 4% formalin for 10 min at room temperature were washed with phosphate-buffered saline (PBS) 3 times for 5 min each. The sections were blocked for 1 hr at room temperature with 3% [v/v] goat serum (Molecular Probes, Carlsbad, CA) in PBS, and then incubated for 2 hr at room temperature with a monoclonal antibody (clone 2D10G9, subtype IgG2bk; diluted 1:1 in 3% [v/v] goat serum) specific for HOCl-modified protein adducts generated *in vivo* (Malle et al., 2006) and *in vitro* (Malle et al., 1995). After another 3 washes with PBS, slides were incubated with Alexa Fluor 488-labeled goat anti-mouse secondary antibody (diluted 1:500 in 3% [v/v] goat serum, Molecular Probes, Carlsbad, CA). Ten pictures were taken of 200X, randomly selected fields using a fluorescence microscope, and the fraction of positive pixels was averaged for each slide (Deng et al., 2008).

2.8. Assessment of Fibrin Deposition in Liver

Immunohistochemistry for cross-linked fibrin in liver was performed as described previously (Zou et al., 2009b). Fibrin monomer is solubilized in the protocol, and only cross-linked fibrin stains immunochemically. Quantification of stained fibrin was performed using Scion Image (Scion Corporation, Frederick, MD). For each experiment, the threshold for staining was set so that few positive pixels were present in liver sections of Vehicle-treated rats. The same threshold value was used to quantify fibrin immunofluorescence from all treatment groups. The fraction of positive pixels averaged from 10 randomly chosen microscopic fields was determined for each animal.

2.9. Statistical Analyses

Results are presented as means $\pm$ SEM. Student's t-test was performed on fibrin deposition data. For the rest of the studies, one way or two way analysis of variance (ANOVA) was applied for data analysis, as appropriate, and Student-Newman-Keuls test was used as a *post hoc* test to compare means. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Evaluation of PMN accumulation and activation in livers

A previous study revealed that SLD administered 15.5 hr before and 0.5 hr after LPS resulted in significant hepatocellular injury. Liver injury induced by SLD/LPS occurred between 4 and 8 hr after the second administration of SLD, and ALT activity in rats increased significantly by 12 hr (Zou et al., 2009b). Therefore, the factors (PMNs, PAI-1 and fibrin deposition) potentially contributing to liver injury were assessed between 4 and 8 hr, whereas the effect of those factors on liver injury was investigated at 12 hr. PMN accumulation was assessed in livers collected at 4 hr, a time before the onset of hepatocellular injury induced by SLD/LPS. SLD given alone had no significant effect on hepatic PMN accumulation (Fig. 1A). An increase in PMN number was observed in livers of rats treated with LPS, and PMN accumulation was significantly greater in livers of rats cotreated with LPS and SLD compared to those treated with LPS alone.

PMNs can accumulate in tissue without becoming activated to release proteases and reactive oxygen species. To assess the activation of PMNs in livers of rats treated with SLD and LPS

or their vehicles, HOCl-protein adducts in the liver were evaluated. HOCl-protein adducts are generated by the myeloperoxidase -hydrogen peroxide-chloride system of activated PMNs (Malle et al., 2006). Adducts were not elevated in liver sections at 4 hr (not shown). However, at 8 hr pronounced formation of HOCl-modified epitopes was found in livers of rats treated with SLD/LPS, but not in rats treated with either SLD or LPS alone (Fig. 1B). This result indicates that the myeloperoxidase-hydrogen peroxide-halide system of PMNs was activated between 4 and 8 hr in the livers of rats treated with SLD/LPS.

3.2. Time course of changes in CINC-1 and MIP-1 α concentrations in plasma

Plasma was collected from rats euthanized at various times (1, 4 and 12 hr), and the concentrations of the PMN chemokines, CINC-1 and MIP-1 α , were measured. LPS increased CINC-1 and MIP-1 α concentrations at 1 and 4 hr (Fig. 2). The concentrations of both chemokines had returned to baseline by 12 hr. SLD treatment had no effect on CINC-1 or MIP-1 α concentrations in vehicle- or LPS-cotreated rats.

3.3. Effect of PMN depletion and PMN protease inhibition on SLD/LPS-induced liver injury

To assess the role of PMNs in SLD/LPS-induced liver injury, rabbit anti-PMN serum or normal serum was given to rats. In a previous study, anti-PMN serum selectively reduced PMNs without affecting other leukocyte numbers in blood (Deng et al., 2007). Blood PMN number in the anti-PMN serum/SLD/LPS group (499 ± 23 per μL) was significantly smaller than that in the normal serum/SLD/LPS group (2726 ± 144 per μL) at 12 hr. PMN number in livers of rats treated with anti-PMN serum/SLD/LPS (10.7 ± 1.4 per high power fields) was also significantly smaller than in livers of rats treated with normal serum/SLD/LPS (44.7 ± 3.8 per high power fields). Cotreatment with normal serum/SLD/LPS led to increased serum ALT activity (Fig. 3). Pretreatment with anti-PMN serum abolished the SLD/LPS-induced increase in ALT activity.

Similarly, neutrophil protease inhibition using eglin C had no effect on ALT activity in serum of rats treated with vehicle but attenuated the elevation in serum ALT activity of rats treated with SLD/LPS (Fig. 4).

3.4. Effect of TNF on PMN accumulation and activation

As noted above, cotreatment with SLD/LPS caused accumulation of PMNs in liver (Fig. 1A). Etanercept, which neutralizes TNF and inhibits its biological effects, did not affect PMN numbers in livers of rats treated with SLD/LPS (Fig. 5A). In contrast, etanercept prevented the elevation in SLD/LPS-induced panlobular formation of HOCl-protein adducts (Fig. 5B). These results suggest that TNF contributes to the release of cytotoxic factors from PMNs but not to PMN accumulation in the liver.

3.5. Role of PAI-1 in liver injury and accumulation and activation of PMNs

A previous study indicated that PAI-1 was selectively increased in the plasma of SLD/LPS-cotreated rats (Zou et al., 2009b); however, its role in liver injury was not investigated. The PAI-1 inhibitor, PAI039, attenuated liver injury from SLD/LPS cotreatment, as marked by serum ALT activity (Fig. 6A). Histological evaluation confirmed that PAI039 attenuated midzonal lesions induced by SLD/LPS cotreatment (Fig. 7). PAI039 also reduced PMN activation (Fig. 6C) but not PMN accumulation at 8 hr (Fig. 6B).

3.6. Effect of TNF on plasma PAI-1 concentration

In a previous study, TNF was increased significantly as early as 1 hr by SLD/LPS cotreatment, and PAI-1 was increased by 8 hr (Zou et al., 2009b). To evaluate whether TNF regulates the production of PAI-1, plasma concentration of PAI-1 was evaluated in rats

cotreated with etanercept. Etanercept given at a dose that protected against liver injury in this model (Zou et al., 2009a) had no effect on the increase in plasma PAI-1 concentration caused by SLD/LPS (Fig .8).

3.7. Effect of TNF and PAI-1 on fibrin deposition

Fibrin clots form in the sinusoids of livers of SLD/LPS-cotreated rats and result in hepatic hypoxia (Zou et al., 2009b). Both fibrin deposition and hypoxia were reduced by anticoagulant treatment, which protected against liver injury. Accordingly, we evaluated whether TNF or PAI-1 exerts its injurious effect by causing fibrin deposition in the liver. Etanercept had no effect on fibrin deposition caused by SLD/LPS cotreatment, whereas PAI039 reduced it slightly but significantly (Fig. 9).

4. Discussion

PMNs are a double-edged sword in the innate immune response to microbial infection and tissue trauma (Butterfield et al., 2006). Stimulated by inflammatory signals, they attach to endothelial cells via adhesion molecules and transmigrate to the site of infection/trauma, where they become activated to release cytotoxic factors. PMNs can be beneficial by removing invading organisms and stimulating tissue repair. However, excessive PMN activation causes tissue injury in many animal models (Jaeschke et al., 1990; Hewett et al., 1992). PMNs are involved in several models of drug-induced liver injury and in some drug-LPS interaction models of IDILI (Deng et al., 2006; Deng et al., 2007; Ramaiah and Jaeschke, 2007; Shaw et al., 2009b).

As has been reported, LPS caused PMNs to accumulate in liver. Although SLD mildly inhibited the adhesion of PMNs to nylon-wool columns in vitro (Venezio et al., 1985), it increased the LPS-induced PMN accumulation before the onset of liver injury (Fig. 1A). Two PMN chemokines, MIP-1 α and CINC-1, are potent inducers of PMN recruitment and extravasation. A neutralizing antibody to either MIP-1 α or CINC-1 attenuated neutrophil sequestration in LPS-treated rodents (Standiford et al., 1995; Zhang et al., 1995). The concentrations of both chemokines were significantly increased in plasma by LPS, whereas SLD had no effect (Fig. 2). Thus, both chemokines might contribute to PMN accumulation in livers of LPS-treated rats, but if they contribute to liver injury other factors must be involved since injury occurred only after SLD/LPS cotreatment. In other words, the presence of these chemokines was not sufficient to cause PMN activation or liver injury in this model. How SLD enhanced PMN accumulation is unknown, but some possibilities arise from previous results. SLD/LPS cotreatment caused fibrin deposition in the liver (Zou et al., 2009b). It is possible that the meshwork of sinusoidal fibrin entrapped these cells. In addition, as a result of fibrin clots in sinusoids hypoxia occurred in livers of cotreated rats (Zou et al., 2009b). Hypoxia can enhance the adherence of PMNs to human endothelial cells in vitro (Milhoan et al., 1992), and such an effect might contribute to the SLD-related increase in PMN accumulation.

Generally, PMNs that sequester in the liver are not injurious unless extravasation of them into the parenchyma and activation occur (Chosay et al., 1997). Although the small dose of LPS by itself caused PMNs to accumulate in livers (Fig. 1B), staining for HOCl-modified proteins suggested no activation of these cells. In contrast, SLD/LPS cotreatment increased HOCl-protein adducts, indicating that the myeloperoxidase-hydrogen peroxide-chloride system of PMNs became activated between 4 and 8 hr, when the onset of liver injury occurred. Our observations parallel recent findings in patients with steatohepatitis in which liver chemokine expression was greater in patients with myeloperoxidase-mediated oxidation products and correlated with hepatic neutrophil sequestration (Rensen et al., 2009). The role of PMNs in SLD/LPS-induced liver damage was further tested using anti-

PMN serum, which markedly reduced PMNs in the circulation and liver. The protection by anti-PMN serum showed that PMNs are critical to the development of SLD/LPS-induced liver injury (Fig. 4).

Activated PMNs release various lysosomal hydrolases including serine proteases, among which elastase and cathepsin G have been identified as primary mediators of hepatocyte killing by PMNs in vitro (Ho et al., 1996). Eglin C is an inhibitor of these toxic proteases. Coadministration of eglin C attenuated SLD/LPS-induced liver injury (Fig. 4), suggesting that PMN proteases play a role in the pathogenesis. Compared to the complete protection by neutrophil depletion, eglin C substantially but incompletely reduced liver injury; thus, the proteases released from PMNs might not be the only PMN-derived mediators contributing to the pathogenesis. Antioxidants attenuated PMN-mediated hepatotoxicity in numerous inflammatory liver injury models (Liu et al., 1995; Jaeschke and Smith, 1997), including SLD/LPS-induced liver injury (Zou et al., 2010), supporting the possibility that both reactive oxygen species and proteases released by PMNs contribute to SLD/LPS-induced liver injury.

TNF neutralization significantly attenuated liver injury induced by SLD/LPS in vivo (Zou et al., 2009a). In addition, TNF potentiated the killing of hepatocytes in vitro by SLD sulfide, the toxic metabolite of SLD. TNF can also activate endothelial cells to promote PMN migration (Smart and Casale, 1994). In results presented here, the number of PMNs sequestered was not affected by TNF, but TNF neutralization reduced PMN activation in the liver (Fig. 5). These results suggest that TNF contributes to PMN activation, but not to hepatic accumulation of these cells.

Like PMN depletion, anticoagulation using heparin abolished the hepatotoxicity induced by SLD/LPS cotreatment of rats (Zou et al., 2009b), which raised the possibility that there is an interaction between PMNs and the hemostatic system in the pathogenesis. Hemostatic factors including thrombin and PAI-1 were increased in plasma by SLD/LPS cotreatment (Zou et al., 2009b). Interestingly, hemostatic factors can bind to PMNs and influence their accumulation and activation (Gillis et al., 1997). For example, thrombin can trigger rapid enzyme release from human PMNs and can promote PMN activation in perfused rat liver after LPS exposure (Baranes et al., 1986; Copple et al., 2003).

PAI-1 is an inhibitor of plasminogen activator and a key negative regulator of fibrinolysis. The PAI-1 inhibitor PAI039 significantly attenuated SLD/LPS-induced liver injury, suggesting that PAI-1 is a mediator of pathogenesis (Fig. 6A). PAI039 also decreased fibrin deposition in livers of SLD/LPS-treated rats (Fig. 9B), which suggests that PAI-1 contributes to fibrin deposition in this model.

In addition to inhibiting fibrinolysis, PAI-1 can regulate PMN migration and potentiate LPS-induced PMN activation through a c-Jun N-terminal kinase-mediated pathway (Kwak et al., 2006; Roelofs et al., 2009). Consistent with these findings, PAI-1 inhibition reduced HOCl-protein adduct staining in livers of SLD/LPS-cotreated rats (Fig. 6C), suggesting that PAI-1 is involved in PMN activation. Therefore, PAI-1 contributed to both PMN activation and fibrin deposition. It can also play a proinflammatory role by stimulating the production of cytokines and chemokines. For example, in a murine model of trovafloxacin/LPS-induced liver injury, PAI-1 knockout markedly decreased the plasma concentrations of interleukin-1 β , interleukin-10, keratinocyte chemoattractant and monocyte chemoattractant protein-1 (Shaw et al., 2009a). Whether PAI-1 similarly regulates the production of chemokines in SLD/LPS-treated rats and the role of these cytokines in PMN activation are topics for future investigation.

PMNs can exacerbate fibrin deposition indirectly by releasing proteases (Deng et al., 2007). For example, proteases from PMNs can release PAI-1 from endothelial cells and platelets and thereby inhibit fibrinolysis (Pintucci et al., 1992). Eglin C treatment significantly decreased active PAI-1 concentration and fibrin deposition in a model of ranitidine/LPS-induced liver injury (Deng et al., 2007). SLD/LPS cotreatment led to fibrin deposition at 4 hr, before the activation of PMNs at 8 hr. Therefore, PMNs do not contribute to the initial formation of fibrin, but proteases released by activated PMNs might prolong fibrin deposition.

The concentrations of PAI-1 and TNF in blood were both significantly greater in SLD/LPS-treated rats than in rats treated with either LPS or SLD alone. According to the results shown above, both TNF and PAI-1 contribute to PMN activation in this SLD/LPS model. This suggests that SLD or its metabolites contribute to PMN activation in an indirect way through enhancing the production of PAI-1 and TNF induced by LPS. The peak of PAI-1 (4 hr) in plasma followed the peak of TNF production (i.e., 1 hr; Zou et al., 2009b). Although it has been reported that both TNF and LPS lead to PAI-1 release from endothelial cells in vitro (Riedo et al., 1990), inhibition of TNF did not decrease PAI-1 concentration in SLD/LPS-cotreated rats (Fig. 8). This indicates that PAI-1 production does not depend on TNF and that these two mediators contribute to PMN activation independently of each other in this model. Moreover, TNF did not influence fibrin deposition in liver (Fig. 9). Thus, the activation of hemostatic system is not mediated through TNF in this model. In contrast, TNF does mediate hemostatic system activation in ranitidine/LPS- and trovafloxacin/LPS-induced liver injury (Tukov et al., 2007; Shaw et al., 2009c). Therefore, these results suggest that TNF does not contribute to liver injury through the same mechanism in all drug-LPS interaction models.

From results of this and previous studies, mechanisms of liver injury induced by SLD/LPS cotreatment are summarized in Fig. 10. Various mediators including TNF, hypoxia caused by hemostatic system activation, and PMNs play critical roles in the pathogenesis of SLD/LPS-induced liver injury. SLD enhances the elevation in TNF induced by LPS. SLD/LPS cotreatment also leads to the production of hemostatic factors including thrombin and PAI-1, both of which contribute to fibrin clot formation in liver sinusoids (Zou et al., 2009b). Probably as a result of sinusoidal obstruction by fibrin, the liver becomes hypoxic. Production of SLD sulfide, the toxic metabolite of SLD is decreased by LPS administration, but enough is apparently produced to inflict damage in the context of other effects of LPS. Indeed, SLD sulfide kills hepatocytes synergistically with TNF and with the involvement of reactive oxygen species and hypoxia (Zou et al., 2009a; Zou et al., 2010). PMNs are another important contributor to SLD/LPS-induced liver injury (Fig. 3). PMN accumulation in the liver is primarily induced by LPS, and this effect is enhanced by SLD (Fig. 1A), but activation of PMNs occurs only in livers of rats cotreated with SLD/LPS (Fig. 1B). Both TNF and PAI-1 contribute to PMN activation independently (Figs. 5 to 8). When activated, PMNs release proteases which induce hepatocellular injury, and this effect is enhanced by hypoxia (Luyendyk et al., 2005). These studies advance our understanding of the roles of various mediators and their interaction in this model of SLD IDILI.

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Abbreviations

ALT alanine aminotransferase

CINC-1	cytokine-induced neutrophil chemoattractant-1
HOCl	hypochlorous acid
LPS	lipopolysaccharide
MIP-1α	macrophage inflammatory protein-1 α
PAI-1	plasminogen activator inhibitor-1
PMN	polymorphonuclear neutrophil
SLD	sulindac
TNF	tumor necrosis factor- α

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Fig. 1. PMN accumulation and activation in rat livers

Rats were treated with two administrations of SLD (50 mg/kg, p.o.) or its vehicle (Veh, 0.5% methyl cellulose) with a 16 hr interval. LPS (8.25×10^5 EU/kg, i.v.) or its saline vehicle was administered half an hour before the second administration of SLD. PMN staining was performed on livers collected 4 hr after the second administration of SLD. (A) PMN numbers in 400 X high power fields (HPF) were counted to evaluate PMN accumulation. (B) HOCl-protein adduct staining was performed on slides of frozen liver collected at 8 hr. Ten randomly chosen fields were photographed for each section, and the fraction of positive pixels was determined. *significantly different from respective group without LPS. #significantly different from Veh/LPS group. $P < 0.05$, $n = 4-5$.

Fig. 2. Concentrations of PMN chemokines in rat plasma

Rats were treated with SLD and LPS or their vehicles (Veh) as described in Fig. 1. At 1, 4 and 12 hr after the second administration of SLD, plasma was collected and concentrations of (A) cytokine-induced neutrophil chemoattractant-1 (CINC-1) and (B) macrophage inflammatory protein-1α (MIP-1α) were evaluated by multiplex ELISA. *significantly different from Veh/Veh group at the same time. $P < 0.05$, $n = 5$.

Fig.3. Effect of PMN depletion on SLD/LPS-induced liver injury

Rats were pretreated with either normal serum (NS) or rabbit anti-rat PMN serum (AS) half an hour before the first administration of SLD. Rats were euthanized 12 hr after the 2nd administration of SLD, and serum ALT activity was determined. Vehicle (Veh).

*significantly different from Veh/Veh/NS group, #significantly different from SLD/LPS/NS group. $P < 0.05$, $n = 3-6$.

Fig.4. Effect of PMN protease inhibition on SLD/LPS-induced liver injury

Rats were treated with SLD and LPS or their vehicles (Veh) as described in Fig. 1. Eglin C or its vehicle (Veh) was administered to rats 4, 6, and 8 hr after the 2nd administration of SLD. Rats were euthanized at 12 hr, and ALT activity in serum was determined.

*significantly different from Veh/Veh/Veh. #significantly different from Veh/Veh/Eglin C group. ^asignificantly different from SLD/LPS/Veh group. $P < 0.05$, $n = 3-6$.

Fig. 5. Effect of TNF inhibition on PMN accumulation and activation

Rats administered SLD/LPS were pretreated with etanercept(Etan) or its vehicle (Veh) 1 hr before LPS. (A) PMN staining was performed on livers collected at 8 hr. The accumulation of PMNs in livers was evaluated by averaging PMN numbers in 10, randomly chosen, 400 X fields. (B) Quantification of HOCl-protein adducts in the livers of rats at 8 hr. *significantly different from Veh/Veh/Veh group. #significantly different from Veh/SLD/LPS group. $P < 0.05$, $n = 3-6$.

Fig. 6. Effect of PAI-1 inhibition on liver injury and PMN accumulation and activation

Rats were treated with SLD/LPS as described in Fig.1. PAI-1 inhibitor, PAI039 (6 mg/kg, p.o.), or its vehicle (Veh, 0.5% methyl cellulose) was administered at 1 hr after the second administration of SLD. Rats were euthanized at 12 hr to measure ALT activity (A) or at 8 hr to assess PMN accumulation (B) and activation (C). * significantly different from Veh/Veh/Veh. # significantly different from SLD/LPS/Veh group. $P < 0.05$, $n = 4-16$.

Fig.7.

Effect of PAI-1 inhibition on liver histopathology in SLD/LPS-cotreated rats. Rats were treated with Veh/Veh/Veh (A), Veh/SLD/LPS (B) or PAI039/SLD/LPS (C) as described in Fig. 6. Liver sections collected at 12 h were stained with hematoxylin and eosin for histopathological evaluation.. Arrows point to areas of hepatocellular necrosis.

Fig.8. Effect of TNF inhibition on plasma PAI-1 concentration

Rats were treated with etanercept (Etan), SLD and LPS or their vehicles (Veh) as described in the legend to Fig. 5. Plasma active PAI-1 concentration was determined at 8 hr. * significantly different from Veh/Veh/Veh. $P < 0.05$, $n = 4$.

Fig. 9. Effect of TNF or PAI-1 inhibition on fibrin deposition in liver

Rats were treated with SLD/LPS and etanercept (Etan, A) or PAI-1 inhibitor (B) respectively. Fibrin deposition was evaluated at 8 hr. * significantly different from SLD/LPS/Vehicle (Veh). $P < 0.05$, $n = 4-7$.

Fig. 10. Mechanisms of SLD/LPS-induced liver injury

Solid lines indicate interactions observed in current research. Dotted lines indicate interactions that have been identified in previously published research. See text for details.