Plasminogen Activator Inhibitor-1 Is Critical in Alcohol-Enhanced Acute Lung Injury in Mice

Lauren G. Poole^{1,2}, Veronica L. Massey^{1,2}, Deanna L. Siow^{1,2}, Edilson Torres-González³, Nikole L. Warner⁴, James P. Luyendyk⁵, Jeffrey D. Ritzenthaler³, Jesse Roman³, and Gavin E. Arteel^{1,2}

¹Department of Pharmacology and Toxicology, ³Division of Pulmonary, Critical Care and Sleep Disorders Medicine, Department of Medicine, and ⁴Department of Microbiology and Immunology, University of Louisville Health Sciences Center, Louisville, Kentucky; ²Alcohol Research Center, University of Louisville, Louisville, Kentucky; and ⁵Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan

Abstract

Chronic alcohol exposure is a clinically important risk factor for the development of acute respiratory distress syndrome, the most severe form of acute lung injury (ALI). However, the mechanisms by which alcohol sensitizes the lung to development of this disease are poorly understood. We determined the role of the antifibrinolytic protein plasminogen activator inhibitor-1 (PAI-1) in alcohol enhancement of experimental endotoxin-induced ALI. Wild-type, PAI-1^{-/-}, and integrin β_3 ^{-/-} mice were fed ethanol-containing Lieber-DeCarli liquid or a control diet for 6 weeks, followed by systemic LPS challenge. LPS administration triggered coagulation cascade activation as evidenced by increased plasma thrombin-antithrombin levels and pulmonary fibrin deposition. Ethanol-exposed animals showed enhanced PAI-1 expression and pulmonary fibrin deposition with coincident exaggeration of pulmonary inflammatory edematous injury. PAI-1 deficiency markedly reduced pulmonary fibrin deposition and greatly reduced inflammation and injury without impacting upstream coagulation. Interestingly, pulmonary platelet accumulation was effectively abolished by PAI-1 deficiency in ethanol/LPS-challenged

mice. Moreover, mice lacking integrin $\alpha_{IIb}\beta_3$, the primary platelet receptor for fibrinogen, displayed a dramatic reduction in early inflammatory changes after ethanol/LPS challenge. These results indicate that the mechanism whereby alcohol exaggerates LPS-induced lung injury requires PAI-1-mediated pulmonary fibrin accumulation, and suggest a novel mechanism whereby alcohol contributes to inflammatory ALI by enhancing fibrinogen-platelet engagement.

Keywords: alcohol; acute lung injury; inflammation; Serpine1

Clinical Relevance

Chronic alcohol abuse is an independent risk factor for the development of acute respiratory distress syndrome. The mechanisms by which alcohol sensitizes the lung to damage are unclear. The results of this study suggest a novel mechanism whereby alcohol contributes to inflammatory acute lung injury by enhancing fibrinogen-platelet engagement.

Alcohol consumption is a common custom worldwide. In the United States alone, 87.6% of adults report consuming alcohol at some point in their lives (1). This widespread consumption of alcohol continues despite the fact that the detrimental health effects

of alcohol abuse are well established. In fact, alcohol is known to contribute to the development of more than 200 disease states (2), making alcohol the fifth leading risk factor for premature death and disability worldwide (3). The lung is

recognized as a target of chronic alcohol abuse, and alcohol-related lung injury is estimated to account for tens of thousands of deaths in the United States each year (4). Although chronic alcohol consumption is not directly linked to the development of

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Correspondence and requests for reprints should be addressed to Gavin E. Arteel, Ph.D., Department of Pharmacology and Toxicology, University of Louisville Health Sciences Center, 505 South Hancock Street, CTRB, Room 552, Louisville, KY 40292. E-mail: gavin.arteel@louisville.edu

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lung disease *per se*, it appears to sensitize the lung to damage from other causes. For example, acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), occurs 3.7 times more often in people who meet the diagnostic criteria for alcohol-use disorders, and these patients have a worse prognosis than those with no history of alcohol abuse (5–7).

The exact mechanisms by which alcohol sensitizes the lung to ALI are incompletely understood. Although alcohol does not appear to directly cause overt histologic injury to the lung, experimental alcohol exposure induces expression of proinflammatory cytokines (8–10), activates tissue remodeling (11, 12), and increases oxidative stress in rodent lungs (13). These factors are hypothesized to contribute to the development of an “alcoholic lung” phenotype, which enhances the host’s susceptibility to serious lung diseases (4). A better understanding of this complex process could enable investigators to identify potential therapeutic targets to treat or prevent alcohol-related lung dysfunction, including enhanced susceptibility to ALI and ARDS.

Plasminogen activator inhibitor-1 (PAI-1) is a key regulator of the fibrinolytic system. PAI-1 is an acute-phase protein that is normally expressed by adipocytes and endothelial cells but is inducible in other cell types, including macrophages, during times of inflammation and/or stress (14). PAI-1 is readily induced by almost all forms of stress signaling, including hypoxia, oxidative stress, and inflammatory cytokines (15). PAI-1 is the primary physiological inhibitor of fibrinolysis. PAI-1 inhibits tissue-type plasminogen activator and urokinase-type plasminogen activator, enzymes that convert plasminogen to plasmin, which degrades fibrin. Our group has demonstrated that PAI-1 is critical in experimental inflammatory liver injury caused by alcohol (16, 17). PAI-1 also plays a critical role in experimental ALI (18), and is suspected to contribute to the incidence and severity of ARDS in humans (19, 20). However, the contribution of PAI-1 to alcohol-enhanced ALI has not been determined. The purpose of this study was to determine the role of PAI-1 in alcohol-enhanced inflammatory lung injury caused by lipopolysaccharide (LPS). Some of the preliminary results of

this study have been previously reported in the form of an abstract (21).

Materials and Methods

Animals and Treatment

Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the University of Louisville’s Institutional Animal Care and Use Committee. Eight-week-old male C57Bl/6J, PAI-1 knockout (B6.129S2-Serpine1^{tm1Mlg/J}; PAI-1^{−/−}), and integrin β_3 (B6.129S2-Itgb3^{tm1Hyn/J}SemJ; β_3 ^{−/−}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Knockout mice were backcrossed at least 10 times onto C57Bl/6J, avoiding concerns regarding differences between the strains at nonspecific loci (22). The mice were fed either an ethanol-containing or iso-caloric control Lieber-DeCarli diet (Dyets, Inc., Bethlehem, PA) for 6 weeks (23, 24). At the end of the exposure period, the animals were separated into two additional groups to receive either LPS (*Escherichia coli*, serotype 055:B5; Sigma, St. Louis, MO; 10 mg/kg intraperitoneally) or vehicle (saline), and were killed 4 or 24 hours after injection (24).

Before the animals were killed, they were anesthetized with ketamine/xylazine (100/15 mg/kg intraperitoneally). Blood was collected from the vena cava just before the mice were killed by exsanguination, and citrated plasma was stored for further analysis. Lung isolation and collection of bronchoalveolar lavage fluid (BALF) were performed as previously described (24). Total RNA was immediately extracted from fresh lung tissue using RNA-stat (Tel-Test, Austin, TX) and chloroform/phenol separation.

Histology and Clinical Chemistry

Formalin-fixed, paraffin-embedded lung tissue was cut at 5 μ m, mounted on glass slides, stained with hematoxylin and eosin or chloracetate esterase, and scored as described previously (24). Lung myeloperoxidase (MPO) activity and plasma thrombin-antithrombin (TAT) levels were measured as previously described (17, 25). PAI-1 protein was measured by ELISA (Molecular Innovations, Novi, MI).

Immunofluorescent detection of fibrin accumulation in lung tissue with rabbit polyclonal anti-fibrinogen (A0080; Dako, Santa Clara, CA) has been described previously (17). Immunohistochemistry for CD41 in lung tissue was performed using rabbit polyclonal anti-CD41/integrin α_{IIb} (ab83961; Abcam, Cambridge, MA). For additional details on immunofluorescence and immunohistochemistry, *see* the online supplement.

Quantitative RT-PCR

The mRNA expression of selected genes in whole-lung homogenate was detected by qRT-PCR, which is routine for our group (17). PCR primers and probes were designed using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge, MA) or purchased from Applied Biosystems (Foster City, CA) as kits. Primers were designed to cross-introns, ensuring that only cDNA and not genomic DNA was amplified. Amplification reactions were performed using a StepOnePlus machine and software (Applied Biosystems). The comparative CT method was used to determine the fold changes in mRNA expression compared with an endogenous reference gene (β -actin).

Statistical Analysis

Results are reported as means $\pm$ standard error mean (SEM; $n = 4$ –6). ANOVA with Bonferroni’s post hoc test was used to determine statistical significance among treatment groups, using SigmaPlot (version 11.0). A P value < 0.05 was selected before the study as the level of significance (^a $P < 0.05$ compared with pair-fed control, ^b $P < 0.05$ compared with LPS alone, ^c $P < 0.05$ compared with wild-type [WT] animals).

Results

Chronic Ethanol Feeding Enhances Pulmonary PAI-1 Expression and Fibrin Accumulation Caused by LPS

PAI-1 has been proposed to play a role in models of ALI in the absence of alcohol (18, 26). Furthermore, PAI-1 is critically involved in alcohol-induced liver injury (27). Therefore, the effects of ethanol and LPS on pulmonary PAI-1 expression were determined (Figure 1A). LPS administration robustly increased the

Figure 1. Effect of ethanol on LPS-induced pulmonary plasminogen activator inhibitor-1 (PAI-1) expression and pulmonary fibrin accumulation. (A) Pulmonary PAI-1 mRNA expression measured by qRT-PCR, and PAI-1 protein levels in bronchoalveolar lavage fluid. (B) Representative photomicrographs ($\times 400$) of pulmonary fibrin deposition detected immunofluorescently (green) in paraffin-embedded tissues 24 hours after LPS injection. ^a $P < 0.05$ compared with pair-fed control, ^b $P < 0.05$ compared with LPS alone. BAL, bronchoalveolar lavage.

expression of PAI-1 mRNA ($\sim 1,000$ -fold, $P < 0.05$) in the lungs. Although ethanol feeding alone did not affect PAI-1 expression, it significantly enhanced the increase in PAI-1 expression caused by LPS (by ~ 2 -fold compared with LPS alone).

PAI-1 protein levels in the BALF (24 h after LPS) paralleled the pattern of mRNA expression (Figure 1A).

As the canonical inhibitor of urokinase-type plasminogen activator and tissue-type plasminogen activator, PAI-1 prevents the

degradation of fibrin by plasmin. Therefore, fibrin accumulation in lung tissue was also measured. Figure 1B shows representative photomicrographs of lung tissue stained immunofluorescently for fibrin. LPS administration caused fibrin to accumulate in both vascular and extravascular tissues in the lung 24 hours after LPS. There was no detectable effect of LPS on this variable at the 4-hour time point (not shown). In contrast, ethanol feeding alone did not affect pulmonary fibrin deposition; however, it enhanced fibrin accumulation caused by LPS administration (Figure 1B).

PAI-1 Deficiency Blocks Alcohol-Enhanced Pulmonary Fibrin Deposition and LPS-Induced Pulmonary Platelet Accumulation

Fibrin may accumulate at sites of injury via enhanced activation of the coagulation cascade (i.e., thrombin activation) or by impaired fibrinolysis (i.e., PAI-1 induction). Therefore, the effect of PAI-1 deficiency on activation of the coagulation cascade was determined. In the current study, ethanol pre-exposure enhanced PAI-1 expression in the lung after LPS exposure, and this enhanced PAI-1 expression correlated with increased deposition of fibrin in lung tissue (Figures 1A and 1B). LPS administration significantly increased plasma TAT (4 h after injection) by 7-fold, indicating activation of the coagulation cascade. Ethanol feeding alone did not significantly enhance plasma TAT; however, ethanol significantly enhanced the increase caused by LPS administration, by ~ 13 -fold compared with control. Interestingly, PAI-1 deficiency dramatically attenuated pulmonary fibrin deposition (Figure 2B) even though plasma TAT was unchanged in the knockout animals (Figure 2A).

One potential mechanism by which fibrin matrices can be proinflammatory is by contributing to platelet aggregation. Fibrin can drive platelet aggregation at sites of injury, and in turn, the platelets themselves may propagate injury (28). Therefore, we determined platelet accumulation in lung tissue immunohistochemically by detecting the platelet-specific integrin $\alpha_{IIb}\beta_3$ and subsequently performing quantitative image analysis (Figures 2C and 2D). Ethanol feeding alone had no significant effect on platelet accumulation (CD41-positive staining), and LPS administration significantly enhanced platelet accumulation in lung tissue. LPS-induced platelet

Figure 2. Effect of PAI-1 deficiency on pulmonary fibrin accumulation and platelet accumulation. (A) Plasma thrombin-antithrombin (TAT) levels. (B) Representative photomicrographs ($\times 400$) of pulmonary fibrin deposition detected immunofluorescently (green) in paraffin-embedded tissues 24 hours after LPS injection. (C) Representative photomicrographs ($\times 400$) of platelets detected immunohistochemically in paraffin-embedded sections via integrin α_{IIb} (CD41) 4 hours after LPS injection. (D) Quantitative image analysis of CD41-positive staining. ^a $P < 0.05$ compared with pair-fed control, ^b $P < 0.05$ compared with LPS alone, ^c $P < 0.05$ compared with wild-type [WT] animals.

accumulation was not affected by ethanol pre-exposure. PAI-1 deficiency prevented platelet accumulation in lung tissue after LPS administration.

PAI-1-Deficient Mice Are Protected from Alcohol-Enhanced Edematous Lung Injury Caused by LPS

The results described in Figures 1 and 2 suggest that ethanol feeding enhances LPS-induced fibrin deposition at least in part by superinducing PAI-1 expression (Figures 1A and 2B). Additionally, Figure 2C indicates that PAI-1 is critical for LPS-induced platelet aggregation in lung tissue. Previous studies indicated that PAI-1 contributes to inflammatory damage to the lungs (18, 26), as well as other organs (e.g., the liver) (17), and that platelet aggregation is involved in the development of ALI (29). It is also known that chronic ethanol feeding sensitizes the lung to endotoxemia-induced ALI and enhances edematous lung injury (13, 24). Therefore, the effect of PAI-1 deficiency on alcohol-enhanced experimental ALI was determined.

There was no significant effect of diet or genotype on the growth of the animals over the 6-week feeding period (not shown). Additionally, neither ethanol pre-exposure nor genotype affected attrition after LPS injection. As expected based on previous results (24), extrathoracic LPS administration caused pulmonary injury and inflammation, as indicated by extravasation of erythrocytes (4 h after LPS; data not shown) and inflammatory cells (4 h after LPS) (Figures 3A (insets) and 3B). At the 4-hour time point, there was no discernible effect of ethanol pre-exposure on LPS-induced injury. Additionally, ethanol feeding was associated with pulmonary edema 24 hours after LPS administration, as indicated by an increase in BALF total protein content (Figure 3C) and enhanced thickening of the alveolar septa (Figure 3D). The increase in BALF protein and in septal thickening caused by the interaction of ethanol and LPS was completely attenuated in PAI-1^{-/-} mice compared with the WT (Figures 3A [bottom panels], 3C, and 3D). LPS administration increased the number of pulmonary neutrophils, as indicated by an increase in MPO activity (Figure 3B). Ethanol exposure did not significantly alter the increase in MPO activity caused by LPS.

Figure 3. Effect of PAI-1 deficiency on ethanol-enhanced acute lung injury. (A) Representative photomicrographs ($\times 400$, hematoxylin and eosin) of formalin-fixed paraffin-embedded lung tissues 24 hours after LPS and chloracetate esterase staining (inset, $\times 800$). Neutrophils are shown as *bright pink cells* (4 h after LPS). (B) Pulmonary myeloperoxidase activity 4 hours after LPS. (C) BAL fluid total protein content 24 hours after LPS. (D) Quantification of alveolar septal thickening, as described in MATERIALS AND METHODS, 24 hours after LPS. ^a $P < 0.05$ compared with pair-fed control, ^b $P < 0.05$ compared with LPS alone, ^c $P < 0.05$ compared with WT animals. MPO, myeloperoxidase.

Alcohol Enhances LPS-Induced Cytokine/Chemokine Expression; Effect of PAI-1 or Integrin β_3 Deficiency

The results of the current study indicate that PAI-1 deficiency attenuates alcohol-enhanced pulmonary fibrin deposits and

platelet accumulation, and also protects against alcohol-enhanced ALI. Although the number of infiltrating neutrophils is unaffected by ethanol pre-exposure or PAI-1 deficiency, the phenotype of recruited inflammatory cells may be influenced by how these factors affect inflammatory

cytokine and chemokine expression (30). Therefore, the mRNA expression of key inflammatory mediators was measured in whole-lung homogenate (Figure 3). Protein levels of cytokines and chemokines relative to mRNA expression have been validated previously (24). Ethanol alone did not affect

expression of any of the tested cytokines (TNF- α , IL-6, and IL-1 β ; Figure 3A), chemokines (KC and MIP-2; Figure 3B), or adhesion molecules (ICAM-1, VCAM-1, and PECAM; Figure 3C). LPS administration induced expression of all variables, with the exception of PECAM, which was significantly decreased by LPS administration (Figure 3C). As demonstrated previously (24), ethanol feeding enhanced the induction of IL-6, KC, MIP-2, ICAM-1, and VCAM-1 expression caused by LPS. With the exception of IL-6, these effects of ethanol were significantly attenuated in PAI-1^{-/-} mice, with values for the knockout strain being similar to those obtained for WT mice that received LPS in the absence of ethanol feeding. For example, the expression of MIP-2 caused by ethanol/LPS was ~3-fold lower in PAI-1^{-/-} compared with WT mice. In the absence of ethanol pre-exposure, PAI-1 deficiency did not significantly affect LPS-induced expression of any mediators measured compared with WT animals (Figure 4A).

The CXC chemokines MIP-2 (CXCL1) and KC (CXCL2) are murine homologs of IL-8. In experimental studies, these chemokines have been shown to be critical for the development of ALI (31). In the current study, ethanol feeding enhanced expression of the chemokines MIP-2 and KC, and this effect was significantly attenuated in PAI-1-deficient mice (Figure 3B). One potential mechanism that may drive expression of these CXC chemokines is aggregation of platelets in the pulmonary vasculature (32). Results in Figure 2C indicate that PAI-1 deficiency attenuates LPS-induced platelet aggregation in the lung, which is mediated, at least in part, by integrin $\alpha_{IIb}\beta_3$ binding to fibrin. Therefore, the effect of β_3 integrin deficiency on ethanol-enhanced expression of the chemokines MIP-2 and KC in whole-lung homogenate were determined (Figure 4B). The enhanced induction of pulmonary MIP-2 and KC caused by the interaction of ethanol and LPS exposure was completely attenuated by β_3 deficiency. Interestingly, the superinduction of PAI-1 caused by this interaction was also completely attenuated (Figure 4B). Markers of other integrin β_3 -expressing cells, such as Th17 cells (e.g., IL-22 and IL-23), were also measured and found to be unaffected by diet or genotype (data not shown). Moreover, because the surface expression

Figure 4. Effect of PAI-1 deficiency and integrin β_3 deficiency on LPS-induced cytokine and chemokine expression. (A) Pulmonary mRNA expression in whole-lung homogenate of cytokines, chemokines, and vascular adhesion molecules measured by qRT-PCR in WT and PAI-1 knockout mice treated with ethanol and/or LPS. (B) Pulmonary mRNA expression of select chemokines in wild-type and integrin β_3 knockout mice, measured by qRT-PCR. ^a P < 0.05 compared with pair-fed control, ^b P < 0.05 compared with LPS alone, ^c P < 0.05 compared with WT animals. ICAM-1, intercellular adhesion molecule 1; KC, keratinocyte chemoattractant; MIP-2, macrophage inflammatory protein-2; PECAM, platelet endothelial cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

of CD41 is altered by β_3 deficiency (33), quantitation of CD41 staining via image analysis was not feasible. However, morphologic assessment of CD41 staining in β_3 knockout mice suggested that β_3 deficiency blocks LPS-induced platelet accumulation in the lung after ethanol administration (not shown), similar to findings in PAI-1-deficient mice (Figure 2).

Discussion

Endotoxemia in the setting of sepsis is one of the top causes of ALI. As mentioned above, chronic alcohol exposure is one of the most clinically important susceptibility factors for this deadly disorder in at-risk individuals. The goal of the current study was to investigate the mechanisms responsible for these events in mice chronically exposed to ethanol (6 wk), followed by induction of endotoxemia. Furthermore, the role of antifibrinolytic PAI-1 and β_3 integrins was explored with the use of genetically engineered (i.e., knockout) animals.

These studies yielded important and novel observations. First, it was observed that extrathoracic LPS induced the deposition of fibrin in the lung, which was associated with elevated PAI-1 levels (Figure 1). These effects were also associated with the accumulation of platelets in the lung (Figure 2). LPS caused inflammatory injury in the lung, as indicated by the accumulation of neutrophils and extravasation of erythrocytes (Figure 3), and induced expression of several proinflammatory mediators (Figure 4). Second, although exposure to ethanol alone did not affect these variables, ethanol enhanced LPS-induced PAI-1 expression (Figure 1A), pulmonary fibrin deposition (Figure 1B), and plasma TAT (Figure 2A); indices of transient lung damage (Figure 3); and expression of proinflammatory mediators as described above (Figure 4). As such, these studies further strengthen available data implicating alcohol as a major susceptibility factor in ALI. Third, PAI-1 deficiency dramatically blunted pulmonary fibrin deposition and platelet accumulation, and attenuated alcohol-enhanced ALI. PAI-1 deficiency also significantly protected against the alcohol-enhanced expression of inflammatory mediators (Figures 2, 3, and 4). Finally, mice lacking $\alpha_{IIb}\beta_3$, the primary platelet receptor for fibrinogen, displayed a

dramatic reduction in early inflammatory changes after alcohol/LPS challenge (Figure 4).

In the current study, alcohol enhanced the activation of the coagulation cascade, as well as induction of PAI-1 (Figures 1 and 2), both which are capable of enhancing fibrin extracellular matrix (ECM) deposition (Figure 1). Changes in the amount and composition of the ECM are well-recognized events in the pathology of end-stage diseases (e.g., hepatic cirrhosis and pulmonary fibrosis). However, the ECM is a dynamic and responsive entity, and subtle alterations to the ECM may be involved in inflammatory/prefibrotic stages of disease (34). The term “transitional tissue remodeling” describes qualitative and quantitative changes of matrix proteins in response to insults that do not alter the overall architecture of an organ. Recent studies suggest that transitional tissue remodeling contributes to damage caused/enhanced by alcohol in several organs, including the liver and lung (35). Our group has demonstrated that the hepatic fibrin ECM responds dynamically to alcohol exposure, sensitizing the liver to LPS-induced inflammatory damage (17). Similarly, chronic alcohol exposure alters the expression and degradation of the pulmonary ECM, favoring fibronectin deposition (36). Activation of the coagulation cascade is a key shared response to acute organ injury that transiently alters the ECM. Although these changes often revert without causing any prolonged tissue damage, they have the potential to alter the immune/inflammatory response to stress.

The finding that knocking out PAI-1 almost completely attenuated alcohol-enhanced fibrin deposition in the lung (Figure 2B), even though it had no effect on plasma TAT (Figure 2A), indicates that PAI-1 induction is critical under these conditions, regardless of activation of the coagulation cascade. These results are in line with previous investigations into fibrin and PAI-1 in mouse liver (17), and suggest that the half-life of fibrin is regulated predominantly at the level of fibrinolysis, rather than at the level of deposition. Interestingly, ethanol did not impact the increase in neutrophil recruitment caused by LPS (Figure 3B), but nevertheless increased injury (Figures 3A, 3C, and 3D) and proinflammatory chemokine expression (Figure 4). These results

suggest that PAI-1/fibrin mediates the inflammatory injury rather than inflammatory cell recruitment *per se*. Fibrin matrices contribute to inflammation in many models through several potential mechanisms (see Ref. 15 for review). For example, fibrin matrices provide a chemotactic substrate for monocytes and leukocytes, and induce cytokine expression (37).

In addition to physiochemical effects that may enhance inflammatory injury, fibrin may facilitate the recruitment of platelets to sites of injury. Indeed, the enhanced lung damage caused by the interaction between ethanol and LPS was associated with an increase in platelet

Working Hypothesis

Figure 5. Working hypothesis for the role of PAI-1 in alcohol-enhanced acute lung injury. It is hypothesized that excessive accumulation of fibrin after tissue injury and/or endotoxemia (lipopolysaccharide) can contribute to $\alpha_{IIb}\beta_3$ -mediated platelet accumulation and aggregation. Activated platelets can contribute to acute lung injury by many potential mechanisms, including the release of proinflammatory cytokines and chemokines from inflammatory cells, and ultimately, enhanced tissue injury. These effects are dramatically exacerbated by ethanol pre-exposure. Interventions designed to block this pathway, such as PAI-1 blockade and β_3 integrin inhibition, may stop the propagation of injury and promote wound healing.

recruitment and/or indices of platelet activators (i.e., TAT; Figure 4). Impairment of the ability of platelets to bind to fibrin via integrin $\alpha_{\text{IIb}}\beta_3$ (such as in the case of $\beta_3^{-/-}$ mice) also prevented injury. Platelets are known to be involved in many forms of lung injury (28), including allergic inflammation (38), cystic fibrosis (39), acid aspiration (40), and, importantly for the current study, endotoxemia (41). Platelets adhere to the pulmonary endothelium after LPS injection (41). After this initial adherence, platelets can become more tightly bound and aggregated by activation of integrin $\alpha_{\text{IIb}}\beta_3$ by binding to fibrin. Additionally, thrombin can then further activate platelets via the protease-activated receptors (42, 43). Adhered and activated platelets can promote inflammatory injury by releasing mediators (e.g., CD40L) that upregulate the expression of chemokines and vascular adhesion molecules in endothelial cells (32). In the current study, PAI-1-dependent platelet accumulation was associated with enhanced expression of the chemokines MIP-2 and KC, as well as the vascular adhesion molecules ICAM-1 and VCAM-1 (Figure 4). These effects were paralleled by

integrin β_3 deficiency (Figure 4), supporting the hypothesis that these are linked events. Interestingly, PAI-1 appeared to play a dominant role in mediating this effect: specifically, PAI-1-deficient mice were protected against injury and platelet accumulation even though plasma TAT levels did not decrease. These effects of ethanol/LPS represent a hypothesized pathway for accumulation of fibrin, platelet recruitment and activation, and progression of inflammatory injury in which both PAI-1 and platelet β_3 integrins are necessary (Figure 5).

PAI-1, like many acute-phase proteins, can act as a double-edged sword: it is required for injury and, in some cases, for wound healing and repair. For example, our group has demonstrated that PAI-1-deficient animals are protected against both acute and chronic alcohol-induced liver injury; importantly, injury in these models is inflammatory and relatively low grade. On the other hand, PAI-1-deficient mice show exacerbated damage in more robust models of liver injury, including acetaminophen overdose (44, 45), partial hepatectomy (46), and carbon tetrachloride-induced fibrosis (47).

Similarly, PAI-1 deficiency has been demonstrated to enhance ALI in severe models of ALI/ARDS, such as intratracheal LPS instillation (48). The current study demonstrates that PAI-1-deficient mice are protected against the enhancing effect of alcohol on extrathoracic endotoxemia-induced ALI, in which injury is less severe than that induced in the thoracic injection model. These data therefore indirectly suggest that PAI-1 may also play dual roles in lung injury, depending on the severity of the injury.

In summary, the current study demonstrates a critical role for PAI-1 in experimental alcohol-enhanced ALI. PAI-1-deficient mice were protected against alcohol-enhanced inflammatory lung injury. It is hypothesized that these effects are mediated, at least in part, by PAI-1-induced fibrin accumulation and subsequent platelet aggregation driven by integrin $\alpha_{\text{IIb}}\beta_3$. This study provides insight into novel targets for therapy to prevent the development and/or progression of ALI in at-risk patients. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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