The Role of Hepatic Type 1 Plasminogen Activator Inhibitor (PAI-1) During Murine Hemorrhagic Shock

Claudio E. Lagoa,1 Yoram Vodovotz,1 Donna B. Stolz,2 Franck Lhuillier,1 Carol McCloskey,1 David Gallo,1 Runkuan Yang,3 Elena Ustinova,4 Mitchell P. Fink,3 Timothy R. Billiar,1 and Wendy M. Mars4

Hemorrhagic shock (HS) followed by resuscitation (HS-R) is characterized by profound physiological changes. Even if the patient survives the initial blood loss, these poorly understood changes can lead to morbidity. One of the tissues most often affected is liver. We sought to recognize specific hepatic changes induced by this stressor to identify targets for therapeutic intervention. Gene array analyses using mouse liver mRNAs were used to identify candidate genes that contribute to hepatic damage. To verify the role of one of the genes identified using the arrays, mice were subjected to HS-R, and multiple parameters were analyzed. A profound increase in plasminogen activator inhibitor type 1 (PAI-1) mRNA was observed using hepatic mRNAs from C57BL/6 mice after HS, both with and without resuscitation. Constitutive loss of PAI-1 resulted in notable tissue preservation and lower (P < .05) alanine aminotransferase (ALT) levels. Fibrin degradation products (FDPs) and interleukins 6 and 10 (IL-6 and IL-10) were unaffected by loss of PAI-1; however, enhanced urokinase activity, an elevation of active hepatocyte growth factor (HGF), an increase in unprocessed transforming growth factor-β1 (TGF-β1), and retention of ERK phosphorylation after HS-R were associated with improved hepatic function. In conclusion, PAI-1 protein is a negative effector of hepatic damage after HS-R through its influence on classic regulators of hepatic growth, as opposed to its role in fibrinolysis. (HEPATOLOGY 2005;42:390-399.)

Hemorrhagic shock (HS) is characterized by a diverse range of physiological changes.1,2 The body’s exact response to shock depends on the type and severity of the original insult, combined with the host’s genetic factors. These act in concert to determine the sequence of inflammatory events, degree of end-organ damage, and patient fate. Unfortunately, despite successful resuscitation and surgical intervention, HS often results in complications, leading to increased mortality.3 We have previously demonstrated the upregulation of inducible nitric oxide synthase during shock. This increase precedes significant pro-inflammatory events such as nuclear factor-kappaB activation, cytokine expression, and neutrophil infiltration.4 We also have demonstrated the importance of interleukin-6 (IL-6) in posthemorrhage inflammation and organ damage/dysfunction.5,6 Nevertheless, knowledge regarding the pathogenesis of HS is limited.

DNA microarray analysis provides a rapid, efficient method for identifying global changes in mRNA transcription.7 We carried out DNA microarray analyses on mouse livers using an array capable of interrogating ~12,000 genes. In accordance with previous findings in rat,8 examination of hepatic mRNAs from mice subjected to HS or HS followed by resuscitation (HS-R) indicated a potential role for plasminogen activator type 1 (PAI-1), an appealing candidate for therapeutic intervention after shock. Hence, we designed studies to verify the role of PAI-1 after HS-R.

PAI-1 inhibits plasmin generation, playing a role in a diverse range of proteolysis-associated processes.4 In this...
regard, the protein functions by preventing the tissue- and urokinase-type plasminogen activators (t-PA and u-PA) from processing plasminogen to plasmin. Additionally, PAI-1 has other roles that are independent of its ability to inhibit plasmin formation, including regulating cellular migration and adhesion to vitronectin and generation of active hepatocyte growth factor (HGF).9-12 Various publications have shown that PAI-1 is induced after hemorrhage.8,13-15 Although never tested, it was assumed that PAI-1 functions by inhibiting fibrinolysis, because fibrin buildup leads to endothelial damage. We now report that, unexpectedly, despite apparent endothelial (and hepato-cellular) damage, hepatic PAI-1 functions in a manner that appears to be independent of fibrin deposition. Instead, PAI-1 affects established pathways already known to regulate hepatic growth.

Materials and Methods

Animals. This study was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and conforms to National Institute of Health guidelines for the care and use of laboratory animals. C57Bl/6 male mice were purchased from Charles River Laboratories (Southbridge, MA). All other mice were bred in a pathogen-free facility, provided standard food and water ad libitum, and used between 8 and 12 weeks of age. Derivation of PAI-1 knockout (KO) mice has been described.16 PAI-1 animals were originally obtained in a mixed genetic background (87.5% C57Bl/6, 12.5% C129), back-crossed twice into C57Bl/6, and 8 pairs of heterozygotes were then inter-bred to obtain matched male siblings for study.

Hemorrhagic Shock Protocol. Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Both femoral arteries were cannulated, one for continuous blood pressure monitoring and the other for blood withdrawal or fluid administration. Cannulae, syringes, and tubing were flushed with sodium heparin (1,000 U/mL) before use. Blood was withdrawn (2.25 mL/100 g body weight) over 10 minutes to achieve a mean arterial pressure (MAP) of 25 mmHg. MAP was maintained at 25 to 30 mmHg for 2.5 hours with continuous monitoring of blood pressure, withdrawing and returning blood as needed. For resuscitation, animals were restored to a MAP of ≥80 mmHg by administration of the remaining shed blood plus intrararterial injection of lactated Ringer’s (2-fold the volume of shed blood) over 30 minutes. In some experiments, PAI-1 KO mice received a stable form of PAI-1 (10 μg/kg body weight in 50 μL phosphate-buffered saline [PBS], MPAL-191L, Molecular Innovations, Southfield, MI) or vehicle control, 1 hour before initiating HS-R. Mice were anesthetized with isoflurane and then killed by exsanguination. Time-matched sham-operated animals underwent anesthesia and cannulation but were not bled or resuscitated. There were 4 to 19 animals per group. No physiological difference in response to HS-R was observed between phenotypes.

Harvesting of Liver Tissue. After killing, the portal vein was clamped, and the left and median left lobes were removed and snap-frozen in liquid nitrogen. Remaining lobes were perfused with 2% paraformaldehyde in PBS, stored 4 hours in fixative, and then immersed overnight in 30% sucrose in PBS. Fixed specimens were placed in plastic molds, embedded using OCT Compound (TissueTek, Sakura Finetek U.S.A., Inc., Torrance, CA), frozen in liquid nitrogen, and stored at −80°C.

Gene Array Analyses of Livers Subjected to HS and HS-R. Total RNA samples were prepared from livers. Three or four samples from each condition were pooled and subjected to DNA microarray analyses (GeneChip u74a oligonucleotide array, Affymetrix, Santa Clara, CA, ~12,000 genes) by the University of Pittsburgh, Department of Pathology microarray facility. DNA microarray data were analyzed using Microarray Suite 4.0 software (Affymetrix) and considered significant when pair-wise baseline analysis had a P value of <.05 and at least 2-fold variation. To assure accuracy, RNA samples were routinely spiked with control bacterial cRNAs.

Reverse Transcriptase Polymerase Chain Reaction. Total RNA was isolated, treated with DNase, and subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using SuperScript III One Step RT-PCR (Invitrogen, Carlsbad, CA). Sequences for actin primers amplify a 103-bp fragment and are as follows: Forward: 5′-GTGACGTTGACCATCCGTAAGACCTC-3′, Reverse: 5′-GCACTAATCCTCCTTCTGATCCTGTC-3′. PAI-1 primers were as reported previously.17

Immunohistochemical Staining. OCT-embedded tissue was sectioned (5 μm), fixed for 5 minutes with 2% paraformaldehyde in PBS, washed with PBS then PBS containing 0.5% bovine serum albumin/0.15% glycine (PBSG), blocked with 20% goat serum in PBSG, and then washed with PBSG. Slides were incubated with 100 μg/mL primary antibody (rabbit anti-rat PAI-1, American Diagnostica Inc., Greenwich, CT) in PBSG (2 hours, room temperature), washed with PBSG, and Cy3-conjugated donkey anti-rabbit secondary was applied. After rinsing with PBSG, nuclei were stained for 30 seconds with Hoechst’s dye (1 mg/100 mL bisBenzimide), slides were washed with PBS and then coverslipped using gelvatol before visualizing with an Olympus (Melville, NY) Provis epi-fluorescence microscope connected to a Sony (Tokyo,
postfixed in aqueous 1% OsO4, 1% K3Fe(CN)6 for 1 hour. Heparinized blood (500 μL) was obtained by cardiac puncture. Samples were centrifuged at 15,000 g for 10 minutes, and plasma was collected and assayed for alanine aminotransferase (ALT) using an automated assay system (Vitros Chemistry Products, Ortho-Clinical Diagnosis, Raritan, NJ) in the University of Pittsburgh, Department of Pathology, Division of Laboratory Medicine.

**Plasma Alanine Aminotransferase Measurement.** Heparinized blood (500 μL) was obtained by cardiac puncture. Samples were centrifuged at 15,000 g for 2 days at 4°C. Samples were processed for transmission electron microscopy as described previously. Briefly, several 1-mm3 cubes were harvested, washed in PBS, and postfixed in aqueous 1% OsO4, 1% K3Fe(CN)6 for 1 hour. After several PBS washes, pellets were dehydrated through a graded series of 30% to 100% ethanol, 100% propylene oxide, and infiltrated for 1 hour in a 1:1 mixture of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA). After several changes of 100% resin over 24 hours, pellets were embedded in molds, cured at 37°C overnight, and then hardened for 2 days at 65°C. Ultrathin (60 nm) sections were collected onto 200-mesh copper grids, stained with 2% uranyl acetate in 50% methanol for 10 minutes, and 1% lead citrate for 7 minutes. Samples were photographed with a JEOL JEM 1210 transmission electron microscope (Peabody, MA) at 80 kV using electron microscope film (Kodak, ESTAR base solution). Slices were mounted onto aluminum stubs, sputter coated with gold/palladium (Cressington Auto 108, Cressington, UK), and viewed in a JEOL JSM-6330F scanning electron microscope (Peabody, MA) at 10-15 kV.

**Ultrastructural Analyses.** Livers were cleared by perfusion with PBS (1 mL/min) through the inferior vena cava, perfusion fixed with PBS (1 mL/min) through the inferior vena cava, perfusion fixed with 3 mL 2.5% glutaraldehyde in PBS, removed, and immersed in 2.5% glutaraldehyde for 2 days at 4°C. Samples were processed for transmission electron microscopy as described previously. Briefly, several 1-mm3 cubes were harvested, washed in PBS, and postfixed in aqueous 1% OsO4, 1% K3Fe(CN)6 for 1 hour. After several PBS washes, pellets were dehydrated through a graded series of 30% to 100% ethanol, 100% propylene oxide, and infiltrated for 1 hour in a 1:1 mixture of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA). After several changes of 100% resin over 24 hours, pellets were embedded in molds, cured at 37°C overnight, and then hardened for 2 days at 65°C. Ultrathin (60 nm) sections were collected onto 200-mesh copper grids, stained with 2% uranyl acetate in 50% methanol for 10 minutes, and 1% lead citrate for 7 minutes. Samples were photographed with a JEOL JEM 1210 transmission electron microscope (Peabody, MA) at 80 kV using electron microscope film (Kodak, ESTAR base solution). Slices were mounted onto aluminum stubs, sputter coated with gold/palladium (Cressington Auto 108, Cressington, UK), and viewed in a JEOL JSM-6330F scanning electron microscope (Peabody, MA) at 10-15 kV.

**Analysis of D-Dimers and Cytokines.** Plasma levels of D-dimer were measured per the manufacturer’s instructions using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

**Western Blot Analyses.** Western blot analyses were performed essentially as described. Snap-frozen livers were homogenized in detergent-free buffer (10 mmol/L Tris-HCl, pH 7.6) with inhibitors (Sigma-Aldrich, St. Louis, MO; catalog nos. P8340, P2714, P2850 and P75726), AEBSF (50 μg/mL), and sodium amiloride (1 mmol/L) (Sigma-Aldrich). Lysates were centrifuged at 26,800 g for 3 hours at 4°C to separate the cytosolic-enriched supernatant from the membrane-enriched pellets. Pellets were solubilized in 1% sodium dodecyl sulfate (SDS) in 10 mmol/L Tris-HCl buffer (pH 7.6, with inhibitors). Protein concentrations were determined using the bicinecinonic acid assay (Pierce, Rockford, IL). Samples were mixed with SDS sample loading buffer, with or without 100 mmol/L dithiothreitol, heated to 65°C for 15 minutes, separated by electrophoresis through SDS-polyacrylamide gel electrophoresis gels and transferred to Immobilon-PVDF (Millipore, Bedford, MA) (50 mmol/L Tris, 95 mmol/L glycine, 0.005% SDS, 10% methanol). Even transfer was confirmed by staining with 0.2% Ponceau Red S in 3% trichloroacetic acid. Membranes were blocked with either 5% milk or fish gelatin in base solution (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween 20), washed with either 1% milk or gelatin in base solution, and then incubated with horseradish peroxidase–conjugated secondary antibodies in wash buffer. Blots were further washed with base solution and developed with ECL (Amersham, Arlington Heights, IL) before visualizing using X-ray film (Dupont, Boston, MA). Antibodies were as follows: mouse anti-transforming growth factor (TGF)-β1 (Pro-mega, Madison, WI), goat anti-fibrinogen (MP Biomedicals, Irvine, CA), goat anti-HGF (R&D Systems), and mouse anti-p-ERK (Santa Cruz Biologicals, Inc., Santa Cruz, CA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). After probing, even loading was verified a second time by stripping the blots with ImmunoPure IgG elution buffer (Pierce) and re-probing the filters with mouse anti-actin (Chemicon, Temecula, CA). Bands from tiff files of the scanned films were quantified using NIH Image 1.58.

**Casein Zymography for Plasminogen Activators.** Zymography was performed essentially as described.

Liver lysates were prepared in ice-cold 5 mmol/L HEPES, 250 mmol/L sucrose buffer (pH 6.0) without inhibitors, resuspended in SDS-loading buffer, heated, and subjected to electrophoresis through a 10% SDS-PAGE containing 1% casein and 12.5 μg/mL (~0.03 U/mL) plasminogen (Sigma-Aldrich). Gels were washed with 2.5% Triton X-100, further washed with incubation...
buffer (50 mmol/L Tris-HCl; 100 mmol/L NaCl; pH 7.5), then incubated at 37°C for 40 to 48 hours. Subsequently, gels were stained with 0.125% Coomassie Brilliant Blue in 50% methanol/10% acetic acid and then destained with 50% methanol/10% acetic acid. Control gels contained casein but no plasminogen.

Statistical Analyses. Data were analyzed using one-way ANOVA (SigmaStat or InStat from GraphPad, San Diego, CA). When indicated, either Fisher’s or Dunn’s least significant difference test was used for post-analyses. Significance was determined at the 95% confidence level (P < .05). Data are reported as the mean ± SEM.

Results

PAI-1 mRNA Is Induced by HS and HS-R. To identify genes that contribute to HS and HS-R in mice, we examined changes in the expression of hepatic mRNAs from wild-type (WT) animals subjected to HS, HS-R, or sham operation, using a microarray that assays approximately 12,000 genes. Among the most dramatic changes in response to HS and HS-R were an approximately 8- and 45-fold increase in mRNA levels, respectively, for PAI-1 (Table 1). Elevation of PAI-1 message after HS-R was confirmed using RT-PCR (Fig. 1). Results varied from animal to animal, with elevations ranging from 1.5- to 64-fold.

Immunohistochemical Localization of PAI-1. To confirm induction of PAI-1 protein after HS-R and determine its location, we stained for PAI-1 antigen in WT animals (Fig. 2). As cytokines play a prominent role in reperfusion injury, studies focused on the resuscitation time point when induction of cytokine mRNAs is maximal (4 hours). PAI-1 was heterogeneous in the livers of resting and sham-operated mice (Fig. 2A and B, respectively), with lighter staining noted in the hepatocytes of sham-operated animals. After HS-R, localization shifted,
with the PAI-1 disappearing from within the hepatocytes and relocating to the basolateral region of the hepatic sinusoids where endothelia reside (Fig. 2C). No staining was observed in PAI-1 KO animals, indicating the specificity of our antibody (data not shown).

**Loss of PAI-1 Protects Livers From Damage After HS-R.** To determine whether PAI-1 induction affects hepatic damage, we subjected PAI-1 WT and KO mice to HS-R. As shown in Fig. 3, plasma ALT levels were significantly lower in PAI-1 KOs when compared with their WT counterparts (P < .05). Histological evaluation of livers confirmed that loss of PAI-1 is protective. Figure 4A and B depicts thick sections of livers harvested after HS-R and stained with 0.5% toluidine blue. Whereas little or no hepatocellular injury was observed in PAI-1 KO mice, their WT counterparts displayed prominent damage between the periportal and pericentral regions (zone 2). Electron microscopy also identified changes induced by PAI-1. Endothelial cells lining the sinusoids of PAI-1 WT mice showed consistently enlarged fenestrae (Fig. 4C). In contrast, PAI-1 KO animals displayed normal fenestrae and little to no endothelial cell damage (Fig. 4D).

**PAI-1 Protein Causes Acute Damage to Endothelia and Hepatocytes.** To determine whether acute induction of PAI-1 can cause damage, KO mice were treated with either a stable form of PAI-1 protein or vehicle control, 1 hour before HS-R. Livers from mice pre-treated with stable PAI-1 antigen developed mild hepatocellular damage (compare Figs. 4A and 5A) and a more significant endothelial injury (Fig. 5C). Damage was absent in KO mice that received PBS (Fig. 5B,D). Separate culture assays showed that PAI-1 preparation was free of contaminating toxins.

**Levels of Fibrin Degradation Products.** PAI-1 can inhibit plasmin generation and, hence, fibrin breakdown. Because excess fibrin can damage endothelia, we assayed circulating plasma levels of D-dimer (a specific fibrin degradation product [FDP]) to determine whether loss of PAI-1 provides protection by allowing increased breakdown of fibrin. As shown in Fig. 6A, after HS-R, circulating D-dimer levels rose significantly in both KO and WT mice. No statistical differences were detected be-
between the 2 populations. Hepatic lysates were also subjected to Western blot analyses under non-reducing conditions. As seen in Fig. 6B, levels of both fibrin/fibrinogen aggregates and FDPs were the same in WT and KO animals.

**Plasma Concentrations of Interleukins 6 and 10.** The absence of fibrinolytic changes was unexpected. Because activation of the coagulation cascade often occurs in response to inflammatory stimuli, we next assayed cytokine levels. IL-6 is both central to inflammatory responses and an accepted modulator of hepatic regeneration, whereas IL-10 suppresses the tumor necrosis factor alpha (TNF-α) surge and confers postischemic protection by enhancing fibrinolysis. As shown in Fig. 7, significant differences were noted for IL-10, but not IL-6, levels in both sets of animals after HS-R. Although IL-10 was reduced in PAI-1 KOs, relative to WT, the difference was not statistically significant.

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**Fig. 4.** Loss of PAI-1 reduces hepatic damage induced by HS-R. After HS-R, thick sections (300 nm) of glutaraldehyde sections were stained with 0.5% toluidine blue (panel A, WT; and panel B, KO). Hepatocellular damage is indicated by the lighter color of stain. Scanning electron microscopy (panel C, WT; and panel D, KO) confirmed damage is also present in the endothelia lining the sinusoids (S) of PAI-1 WT but not KO mice. Arrowheads indicate platelets within the sinusoid. Small arrows point to abnormal fenestrations in the endothelia. Original magnification ×200 (A-B) BC and H represent bile canaliculi and hepatocytes, respectively. PAI-1, plasminogen activator inhibitor type 1; HS-R, hemorrhagic shock with resuscitation; WT, wild-type; KO, knockout.

**Fig. 5.** Active PAI-1 antigen restores endothelial cell damage in KO mice. Either PAI-1 or vehicle was administered to animals 1 hour before HS-R. Light (arrows) versus dark staining (arrowheads) hepatocytes indicate dying and healthy cells, respectively, as seen with toluidine blue staining (A-B) and electron microscopy (C-D) when active PAI-1 (A,C) versus vehicle (B,D) is administered. Swollen mitochondria (asterisks) seen with transmission electron microscopy verify hepatocellular damage. Dead endothelia (E) being engulfed by infiltrating white cells (W) indicate this is not a processing artifact. PAI-1, plasminogen activator inhibitor type 1; KO, knockout; HS-R, hemorrhagic shock with resuscitation.
Active u-PA, HGF, and Unprocessed TGF-β1 Are Increased in KO Animals. Given the unanticipated results with fibrin/fibrinogen and cytokines, we tested for changes in other hepatic proteins regulated by PAI-1. HGF is a positive effector of liver growth because of its ability to affect both hepatocyte and endothelial cell proliferation.29,30 Generation of mature HGF is impaired when PAI-1 is present in hepatocyte culture,12 likely because the PAI-1 inhibits u-PA activity.31 To assess functional u-PA, we used zymography studies. As shown in Fig. 8A, loss of PAI-1 was associated with a modestly augmented u-PA activity in KO mice after HS-R (approximately 25% increase, P < .05). Control gels without added plasminogen, but with the t-PA control lane (1 ng), were completely negative for clearing (not shown). (B) Western blot analyses show a moderate elevation in active HGF (tHGF) in the cytosols of KO animals (approximately 25% increase, P < .05), concurrent with the loss of latent HGF (sHGF) (approximately 63% of WT, P < .0001). (C) NIH Image analyses of Western blots probed for TGF-β1 (n = 5) shows loss of PAI-1 is associated with a significant increase in unprocessed TGF-β1 in the matrices of HS-R mice. Processed TGF-β1 was undetectable after HS-R (see Results). Error bars represent SEM. (D) Western blot analyses show a moderate elevation of phosphorylated ERK-1/2 (p-ERK) in the nuclei of KO animals (2-fold increase, P < .05). u-PA, urokinase-type plasminogen activator; HGF, hepatocyte growth factor; TGF, transforming growth factor; PAI-1, plasminogen activator inhibitor type 1; t-PA, tissue-type plasminogen activator; HS-R, hemorrhagic shock with resuscitation.

Fig. 8A, loss of PAI-1 was associated with a modestly augmented u-PA activity in KO mice after HS-R (approximately 25% increase, P < .05). Control zymography, in which the plasminogen is omitted from the gels, was completely negative, indicating no other casein-cleaving proteases (e.g., plasmin or trypsin) were present (not shown). (B) Western blot analyses show a moderate elevation in active HGF (tHGF) in the cytosols of KO animals (approximately 25% increase, P < .05), concurrent with the loss of latent HGF (sHGF) (approximately 63% of WT, P < .0001). (C) NIH Image analyses of Western blots probed for TGF-β1 (n = 5) shows loss of PAI-1 is associated with a significant increase in unprocessed TGF-β1 in the matrices of HS-R mice. Processed TGF-β1 was undetectable after HS-R (see Results). Error bars represent SEM. (D) Western blot analyses show a moderate elevation of phosphorylated ERK-1/2 (p-ERK) in the nuclei of KO animals (2-fold increase, P < .05). u-PA, urokinase-type plasminogen activator; HGF, hepatocyte growth factor; TGF, transforming growth factor; PAI-1, plasminogen activator inhibitor type 1; KO, knockout; WT, wild-type; t-PA, tissue-type plasminogen activator; HS-R, hemorrhagic shock with resuscitation.

Plasmin generation can lead to enhanced processing of TGF-β1, a negative regulator of liver expansion.32,33 Western blot analyses were performed to monitor the expression of TGF-β1 in the livers of control and HS-R mice. Processed (i.e., active) TGF-β1 was present in the
resting livers from WT animals at approximately 58% of the quantity found in KO siblings \( (n = 4 \text{ each}, P < .003) \); however, at 4 hours post–HS-R, the protein was completely undetectable. Conversely, unprocessed (i.e., inactive) TGF-β1 (approximately 55 kd) was abundant in the matrix-enriched fraction of PAI-1 KO mice relative to their WT counterparts (Fig. 8C). Levels of nuclear phosphorylated ERK were significantly elevated (approximately a 2-fold increase, \( P < .05 \)) in nuclei of PAI-1 KO animals versus their WT counterparts after HS-R (Fig. 8D), indicating a biological response from these changes.

**Discussion**

This study demonstrates an important function for PAI-1 in exacerbating hepatic injury after HS-R, a paradigm for systemic ischemia/reperfusion injury. Among approximately 12,000 genes analyzed, PAI-1 was the mRNA most dramatically increased by HS-R in the livers of WT mice (approximately 45-fold increase), with an approximately 8-fold in the livers of animals after HS alone. Although elevated PAI-1 mRNA in response to shock has previously been reported in rat liver,\(^{8,13,14}\) it’s role has never been elucidated. As elevated PAI-1 antigen is negatively associated with outcome in many models of ischemic and inflammatory disease, because of promotion of thrombosis with subsequent tissue damage,\(^{13,24,34-37}\) we fully anticipated that PAI-1 would function in a similar capacity in our model of HS-R. Instead, the data indicate a role for PAI-1 in inducing damage to both hepatocytes and to endothelia, independent of fibrin deposition, supporting the idea that the function of PAI-1 is to alter the balance of positive and negative hepatic growth regulators.

After HS-R, staining was prominent in the hepatic sinusoid, where the basolateral surface of the hepatocyte is located and where endothelia reside. Because we have now demonstrated that the presence of PAI-1 is associated with endothelial changes after HS-R, it seems probable that the PAI-1 staining we observed is at least partly endothelial. Still, this assertion remains to be proven. Endothelia have been shown to produce PAI-1 mRNA after HS, and these data may reflect protein that is subsequently translated.\(^{14}\) Conversely, the mRNA has also been localized to hepatocytes during the reperfusion phase after acute ischemia/reperfusion, so this has potential to be hepatocellular as well.\(^{38}\)

Localization of PAI-1 to the luminal surface of endothelial cells can lead to reduced fibrinolytic activity, promoting formation of microvascular thrombi with ensuing endothelial damage.\(^{21}\) Thus, we anticipated that the improved outcome observed in PAI-1 KO mice would be related to an increased generation of plasmin and enhanced fibrinolysis. Despite the location of PAI-1 in WT, and the obvious endothelial damage we observed, the data do not support this hypothesis. In both sets of animals, plasmin was undetectable, and the quantities of fibrin/fibrinogen/FDPs were similar. Furthermore, microscopic evaluation showed few visible microthrombi (not shown). The easiest rationalization for this discrepancy is that the time point we selected for study is beyond the time when fibrin-mediated damage occurs and that the microthrombi have already been cleared. Although this explanation seems logical and merits testing, there are 2 reasons to believe it may not be so. First, we did not observe large numbers of overt microthrombi after HS alone, likely because of the model of ischemia we used. Okajima et al.\(^{39}\) recently showed that microthrombi formation in liver is dependent on the degree of ischemic insult and that not all models of ischemia invoke development of microthrombi. Second, even if PAI-1 did lead to an elevation of fibrin at an earlier point, it does not necessarily mean that fibrin deposition led to the observed endothelial changes. In at least one documented model of hepatic insult, damage to endothelia and fibrin deposition were shown to be simultaneous, yet independent, events. Thus, even when present, endothelial damage is not necessarily related to hepatic fibrin.\(^{30}\)

Urokinase-type plasminogen activator regulates conversion of inactive single-chain HGF to the mature, two-chain,\(^{31,41}\) and can lead to the generation of plasmin. Both HGF and plasmin are positive effectors of hepatic regeneration\(^{23,31,42,43}\); however, plasmin also has the potential to be a negative effector because it is capable of regulating maturation of unprocessed TGF-β1.\(^{32,33}\) After HS-R, we observed an increase in active HGF in the liver extracts of PAI-1 KO mice, consistent with the increase of u-PA activity seen in the livers of KO mice. Conversely, zymography was unable to detect plasmin in hepatic extracts from either WT or KO animals, corresponding with our inability to detect processed TGF-β1. Instead, for reasons that are unclear, levels of unprocessed (hence inactive) TGF-β1 were increased in the matrices of PAI-1 KO mice (Fig. 8). Taken together, the combined evidence suggests that constitutive loss of PAI-1 during HS-R can protect the liver via pathways involving HGF (increase of a positive regulator) and TGF-β1 (loss of a negative regulator). These results concur with those of Shimizu et al.,\(^{44}\) who showed that generation of mature HGF, regulated by u-PA and PAI-1, controls the extent of hepatic injury mediated by Fas.\(^{44}\) Our findings also corroborate studies demonstrating that mature HGF can confer protection against hepatic ischemia/reperfusion injury.\(^{45}\) This protection is likely to involve signaling through the ERK-1/2 pathway, as our data show the KO animals have a relative
increase in phosphorylated ERK-1/2 after HS-R. In liver, signaling though ERK-1/2 is associated with both regeneration and survival. Microarray analyses using hepatic mRNAs from WT and KO animals after HS-R also suggest selected cytokines may be the downstream targets of HGF or TGF-β1 signaling (data not shown).

Clinically, these results may be very relevant because plasma PAI-1 levels are a good prognostic indicator of whether patients will progress to multiple organ dysfunction after shock. Furthermore, a genetic polymorphism in the PAI-1 promoter that results in elevated PAI-1 levels in the PAI-1 promoter in HS-R also has been negatively linked with prognosis after severe trauma. Thus, controlling PAI-1 production or the consequences of its elevation may be critical for designing strategies to ensure better patient recovery.

In conclusion, the absence of PAI-1 after HS-R contributes to hepatic protection by a variety of mechanisms, including increased u-PA activity and alterations in hepatic growth regulators such as TGF-β1 and HGF, but apparently not by gross changes in the fibrinolytic system. Thus, hepatic protection after injury induced by HS-R is at least partially under the control of some of the less-studied regulatory functions of PAI-1 protein.

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