Presence of Active and Latent Type 1 Plasminogen Activator Inhibitor Associated With Porcine Platelets

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Data from a number of laboratories indicate that human platelets contain type I plasminogen activator inhibitor (PAI-1) primarily in a latent form; however, one report (Biochemistry 28:5773, 1989) indicated that it is predominantly the active form of PAI-1 that is present in and can be purified from an ammonium sulfate precipitate of porcine platelets. To clarify this situation, we investigated and compared the status of PAI-1 in porcine and human platelets. Immunologic analysis of the activity of PAI-1 to form complexes with immobilized t-PA indicated that porcine and human platelets contained 3.7 ± 0.4 and 1.7 ± 0.3 U of PAI activity per 10^9 platelets (n = 6; ± SD), respectively; sodium dodecyl sulfate (SDS) activation of the lysates increased PAI-1 activity to 10.8 ± 3.0 and 3.8 ± 0.5 U per 10^9 platelets. Platelet lysates were also treated with an excess of soluble t-PA, which formed complexes with active PAI-1, whereas the latent form was detected by SDS-polyacrylamide gel electrophoresis and reverse fibrin autography. Furthermore, immobilized t-PA was able to deplete active PAI-1 from the platelet extracts, and the latent form remaining in the absorbed extract could be quantitated by activation with 4 mol/L guanidine. To investigate the differences between our observations and the published data, porcine platelets were extracted, and PAI-1 was purified as described in the literature. For quantitative analysis, porcine platelet PAI-1 was also purified to homogeneity using standard chromatographic procedures optimized in our laboratory for endothelial PAI-1, and the purified protein was used to develop an enzyme-linked immunosorbent assay for porcine PAI-1 antigen. Our results indicate that: (1) latent PAI-1 in concentrated ammonium sulfate precipitates of porcine platelet lysates cannot be detected unless the precipitates are diluted before treatment with denaturants; and (2) active and latent porcine platelet PAI-1 can be separated by gel filtration over molecular sieving columns. In summary, this report documents that PAI-1 in porcine platelets is present in both an active and a latent form.

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ABNORMAL thrombus formation and dissolution are associated with several cardiovascular diseases including atherosclerosis and both thromboembolic and hemorrhagic conditions. Platelets play a critical role in these processes at early times during the initial formation of the platelet/fibrin hemostatic plug, and at later times in which they help to maintain the integrity of the thrombus through the release of fast-acting plasminogen activator inhibitors (PAlAs).1 The primary PAI released by platelets is immunologically identical to the inhibitor produced by endothelial cells2-6 and is routinely referred to as type-1 PAI.7-9 This molecule has been cloned and classified in the serine protease inhibitor (Serpin) superfAMILY.7,8 PAI-1 reacts rapidly against urokinase and both forms of tissue-type plasminogen activator (ie, single- and two-chain t-PA) with rate constants greater than 10^7 [mol/L]-1 sec^-1, which results in the high molecular weight, inactive PAI/PAI-1 complexes.7-8 The importance of PAI-1 as a key regulator of vascular fibrinolysis is provided by numerous reports associating elevated levels of PAI-1 activity and the increased tendency for thrombosis, as well as the correlation of isolated bleeding disorders with states of defective PAI-1 activity.9

Current data indicate that PAI-1 is produced in an active conformation; however, this inhibitor is relatively unstable and is rapidly converted at 37°C into an inactive inhibitor.7-10 A variety of denaturants (eg, guanidine, urea) are able to convert this inactive form into an active inhibitor,11 hence the term “latent” is commonly used to denote this denaturant-activatable form of PAI-1.5,11 The observation that latent PAI-1 can be reactivated in vivo12 indicates that natural mechanisms exist to mediate the conversion of the latent form of PAI-1 into an active molecule. For example, negatively charged phospholipid vesicles have been found to be capable of activating latent PAI-1,13 suggesting that negatively charged platelet surfaces, which are exposed during the aggregation of platelets, play a role in this process. Although human platelets contain PAI-1 primarily in a latent form,5,6,14,15 one group reported that it is predominantly the active form of PAI-1 that is present in and can be purified from an ammonium sulfate precipitate of porcine platelets.16 These latter data have raised the possibility that porcine platelets may represent a unique opportunity for delineating the mechanism(s) that mediate the conversion of latent PAI-1 into a fully active inhibitor. In light of this possibility, we initiated an in-depth study of the status of PAI-1 in porcine platelet lysates.

MATERIALS AND METHODS

Fibrinolytic proteins. t-PA purified from Bowes melanoma cell line was obtained from American Diagnostica Inc (Greenwich, CT). The specific activity of sc-PA used was 500,000 IU/mg when compared in a chromogenic assay against the t-PA International standard (National Institute for Biological Standards and Controls [NIBSC], London, Great Britain; Ref No. 83/517). Human PAI-1 was purified from the media conditioned by a transformed human lung fibroblast cell line (SVAg W138 VA132 RA), and antiserum to purified PAI-1 was raised in New Zealand rabbits according to procedures described previously.17 Affinity purification of the rabbit antihuman PAI-1 antibody was performed as described.18 In

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brief, PAI-1 was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) according to the manufacturer’s instructions. The IgG fraction of rabbit antisera to PAI-1 was isolated, and 100 mg of IgG was circulated (16 hours, 4°C) through the PAI-1-Sepharose column at 10 mL/h. Protein was determined by the BCA assay (Pierce, Rockford, IL). For the biotinylation of the affinity purified antibody, ImmunoPure NHS-LC-Biotin (Pierce) was used according to the manufacturer’s instructions.

Preparation of platelets. Porcine blood was collected from two groups of pigs. In the first group, whole blood from anesthetized, untreated, healthy domestic pigs (800 mL/pig; n = 10) was collected into acid citrate dextrose (ACD) (0.025 mol/L citric acid, 0.85 mol/L sodium citrate, 2% dextrose; one part ACD, five parts whole blood) using an axillary catheter. Following withdrawal of the PRP was aspirated without agitating the buffy coat and centrifuged (200g for 10 minutes). The supernatant was brought to 40% saturation with ammonium sulfate, stirred and photographed in indirect light.

Preparation of PAI-1 from porcine platelets. The procedures described by Fay and Owen16 were modified to include: (1) the use of fresh (ie, not frozen and thawed) platelets; and (2) a modification of volume scale to permit the partial purification of porcine PAI-1 from porcine platelets. In the first group, whole blood from anesthetized, untreated, healthy domestic pigs (800 mL/pig; n = 10) was collected into acid citrate dextrose (ACD) (0.025 mol/L citric acid, 0.85 mol/L sodium citrate, 2% dextrose; one part ACD, five parts whole blood) using an axillary catheter. Following withdrawal of the blood, this group of pigs would serve in a separate study as organ donors for the recovery of pulmonary macrophages. In the second group, blood (20 to 30 mL, n = 20) was withdrawn from lightly restrained pigs via an axillary vein catheter before injection of a compound. This latter group of animals was to be subsequently used in a study evaluating the hemodynamic effects of a series of plasma expanders. Platelet-rich plasma (PRP) was prepared by centrifugation of anticoagulated whole blood (200g x 15 minutes). The PRP was aspirated without agitation of the buffy coat and centrifuged (2,500g x 15 minutes). The platelet pellet was washed twice with 0.2 mol/L Tris-HCl, 0.15 mol/L NaCl, and 2.5 mmol/L EDTA, pH 7.5. Unless otherwise indicated, the resultant pellet was resuspended to a final concentration of 109 platelets/mL and frozen at −70°C until used. The platelets were lysed either with 0.2% Triton X-100 (Sigma Chemical Co, St Louis, MO) or by repeated freezing and thawing cycles. Alternatively, to rule out possible effects of repeated freezing and thawing on the activity status of PAI-1, purification experiments were repeated using fresh platelets immediately after their harvest from the PRP. In a separate series of experiments, platelets were diluted into 50 mmol/L arginine-HCl, pH 7.4,19 and frozen at −80°C until used.

Human PRP was prepared by collecting blood from the antecubital vein of six healthy individuals into 5 vol ACD followed by centrifugation (200g x 15 minutes). Platelets were isolated from the PRP and frozen at −80°C as described above.

Partial purification of PAI-1 from porcine platelets. The procedures described by Fay and Owen16 were modified to include: (1) the use of fresh (ie, not frozen and thawed) platelets; and (2) a modification of volume scale to permit the partial purification of porcine platelet PAI-1 to be performed on an analytical scale. Basically, fresh porcine platelets (109 platelets/mL, 25 mL) were harvested and immediately diluted with 4 vol of 0.02 mol/L Tris-HCl, 0.065% β-mercaptoethanol (BME), 0.01% Tween 80, 0.2 mmol/L EDTA, and 50 KIU/mL aprotinin (Calbiochem, Inc, La Jolla, CA), pH 8.0. This and all subsequent steps were performed at 4°C. The mixture was stirred slowly for 12 hours and the suspension was centrifuged (10,000g x 20 minutes). The supernatant was brought to 40% saturation with ammonium sulfate, stirred for 30 minutes, and centrifuged (13,000g x 20 minutes). The supernatant was brought to 65% ammonium sulfate saturation, stirred for 30 minutes, and centrifuged as before. The pellet was dissolved in a minimal volume (2 mL) of 0.02 mol/L Tris-HCl, 0.5 mol/L NaCl, 0.01% Tween 80, 0.05% BME, 0.2 mmol/L EDTA, and 50 KIU/mL aprotinin, pH 8.0 (gel filtration buffer). This crude ammonium sulfate platelet extract was incubated with 0.1% dithiothreitol for 2 hours and clarified by centrifugation (20,000g x 10 minutes). The supernatant was fractionated on a column of Sephacryl S-200 (1.8 x 120 cm; Pharmacia) that was eluted at 13.5 mL/h with gel filtration buffer. Fractions (2.1 mL) were collected and assayed for active and denaturant-activatable PAI-1 as described below. Blue Dextran 2000 (2 x 106 daltons), bovine serum albumin (BSA) (67,000 daltons), and ribonuclease A (13,700 daltons) (all obtained from Pharmacia) were used to calibrate the column. Experiments were also performed with pools of porcine platelets that were frozen and thawed (5 x 109 platelets/mL, 8 mL), yielding similar results as obtained with fresh porcine platelets.

Purification of porcine PAI-1. To obtain data on the specific activity of porcine PAI-1, it was purified from porcine platelets using a procedure that had been optimized for the purification of PAI-1 from bovine and human endothelial cell-conditioned medium. In brief, platelets were lysed by repeated freezing and thawing cycles (approximately 20 mL of 109 platelets/mL), centrifuged, and mixed overnight (4°C) with 20 mL of concanavalin A Sepharose that had been previously equilibrated with TBS (0.01 mol/L Tris, pH 8.1, 0.14 mol/L NaCl, 0.01% Tween 80, 1 mmol/L CaCl2, 1 mmol/L MgCl2). The slurry was poured into a column (25 x 1 cm), washed with TBS and eluted with high salt buffer (0.01 mol/L Tris, pH 8.1, 0.01% Tween 80, 1 mmol/L CaCl2, 1 mmol/L MgCl2, 0.5 mol/L α-methyl-d-mannoside, 1 mol/L NaCl). The eluate was passed over a lysine-Sepharose column (10 x 1 cm) that had been equilibrated overnight with TBS and absorbed onto a hydroxylapatite column. Protein was eluted from the hydroxylapatite column with a stepwise gradient of sodium phosphate (0.01 mol/L NaPO4 to 0.13 mol/L NaPO4), and the PAI-1-containing fractions were pooled and concentrated on an Amicon 10 YM10 (Amicon Corp, Danvers, MA) 25-mm filtration membrane. The concentrate was further purified by gel filtration on a Sephacryl S-200 column (120 x 1.8 cm; Pharmacia) in 0.1 mol/L ammonium bicarbonate buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the final product showed a single molecular weight (Mw) 46-Kd band when analyzed either by silver staining or by immunoblotting with rabbit antihuman PAI-1 using previously described protocols.17 Purified porcine PAI-1 was used as a calibration standard in the analysis of porcine platelets using the functional immunoassay for PAI-1 activity and the enzyme-linked immunosorbent assay for PAI-1 antigen.

SDS-PAGE. SDS-polyacrylamide slab gels and buffers were prepared as described by Laemmli et al.18 Samples were applied to gels composed of 10 cm resolving gels of 9% acrylamide and 2 cm stacking gels of 4% acrylamide, and subjected to electrophoresis at near-constant current for 16 hours or until the dye reached the bottom of the gel. Direct and reverse fibrin autography. Fibrin autography was performed as previously described. Briefly, fibrin-agar indicator films were prepared containing 1% agarose, fibrinogen (2.4 mg/mL), human plasminogen (25 μg/mL), and α-thrombin (0.5 U/mL) (all final concentrations). The solution was mixed and poured onto prewarmed glass plates. After solidification of the fibrin-agar film, the SDS-gel that had been soaked for 1.5 hours in 2.5% Triton X-100 (Sigma) was layered onto the film and incubated in a humid chamber. Plates were read several times within the following hours of incubation at 37°C. For reverse fibrin autography, fibrin-agar indicator films were prepared as described above with the modification that the indicator films were supplemented with 0.35 U/mL urokinase, which converts plasminogen to plasmin, thus hydrolyzing the fibrin. Development of opaque, lysis-resistant zones in the otherwise clear indicator film showed PAI activity. The gel and indicator were incubated in a moist chamber for 2 hours at 37°C and photographed in indirect light.

Quantitation of PAI-1 activity. PAI-1 activity was quantitated as previously described17,21 by using immobilized t-PA to bind active
Porcine platelet PAI-1

PAI-1 in a sample and the bound PAI-1 immunologically detected by incubation with affinity-purified rabbit anti-PAI-1 (10 μg/mL) followed by 125I-goat antirabbit IgG (50,000 cpm/well). Two denaturants were used for activation of latent PAI-1. Samples were incubated in 0.1% SDS (1 hour, 37°C) followed by neutralization with a 50-fold excess of Triton X-100. Alternatively, samples (500 μL) were dialyzed against 4 mol/L guanidine-HCl (4 hours, 37°C), followed by dialysis against PBS. Platelet lysates were also depleted of active PAI-1 before activation with 4 mol/L guanidine. In this case, flat-bottom microtiter plates were precoated overnight at 4°C with 150 μL t-PA (5 μg/mL in PBS). After a blocking step and two washes with PBS containing 0.01% Tween 20, platelet lysates (108 platelets/mL) were incubated in the wells (1 hour, 4°C). This process was repeated four times with each sample. Control samples were incubated simultaneously in wells that had been precoated overnight at 4°C with BSA (5 μg/mL).

Amidolytic assay for activity determination of purified porcine and human PAI-1. For standardizing PA-inhibitory activity of purified human and porcine PAI-1, an amidolytic assay was performed. In brief, increasing amounts of the purified inhibitor were added to a constant known amount of sc-PA. A standard curve was generated by the addition of increasing amounts of sc-PA to a buffer control. Residual PA activity was determined by reaction of the sample mixture with cyanogen bromide digest fragments of fibrinogen (American Diagnostica Inc), glu-plasminogen, and the chromogenic substrate, p-Val-Leu-Lys pNA (S-2251; Kabi Vitrum Inc, Franklin, OH). One unit of PAI-1 activity is defined as that amount needed to interact with and neutralize 1 IU t-PA activity in a standard plasminogen-based chromogenic assay.

Analysis of PAI-1 antigen. Porcine PAI-1 antigen was quantitated using a double antibody immunoassay. Flat-bottom microtiter plates were precoated overnight (4°C) with affinity-purified rabbit antihuman PAI-1 (10 μg/mL/well). At this and each subsequent step, the plates were washed with 0.01 mol/L TBS supplemented with 0.1% Triton X-100. The wells were then incubated with blotto (5% wt/vol skimmed milk powder in 10 mmol/L Tris, pH 7.5; 200 μL/well) for 2 hours to block any remaining reactive sites on the plastic. Test samples and standard curves of purified porcine PAI-1 were prepared in dilution buffer (blotto containing 0.5% Triton X-100). The diluted samples (100 μL) were incubated on the wells that had been precoated with antibody (1.5 hours, 37°C). Bound PAI-1 was detected by incubating the washed wells (1 hour, 37°C) with biotinylated affinity-purified rabbit antiserum to human PAI-1 (5 μg/mL, 100 μL/well) followed by incubation (30 minutes, 23°C) with streptavidine alkaline-phosphatase conjugate (Zymed Laboratories Inc, San Francisco, CA; 1:1000 dilution in blotto, 100 μL/well). After addition of the substrate para-nitrophenylphosphate, the resulting color change was measured at 405 nm over 10 minutes. The sensitivity and linear range of the calibration curve for porcine PAI-1 antigen was reduced by a factor of four in comparison with PAI-1 activity in porcine platelets.

Immunoprecipitation procedures. Protein A-Sepharose CL-4B (Pharmacia) was rehydrated in 0.14 mol/L NaCl, 10 mmol/L sodium phosphate buffer containing 0.1% BSA, 0.05% Tween 20, and 0.02% sodium azide (immunoprecipitation buffer, pH 7.4) according to Erickson et al.5 Rabbit antiserum against human PAI-1, human t-PA, and nonimmune rabbit serum (40 μL, respectively) were incubated (15 minutes, 23°C) with the washed beads (30 μL packed beads representing 8 μg of Protein A-Sepharose). The beads were washed three times with immunoprecipitation buffer containing 0.5 mol/L NaCl, and platelet lysates (100 μL, representing 6 × 108 platelets) were added. The mixtures were incubated (1 hour, 23°C), centrifuged, and the resultant pellets were extracted with sample buffer. Both immunosupernatants and the extracts from the immunoprecipitates were fractionated by SDS-PAGE and analyzed by fibrin autography.

RESULTS

Active and denaturant-reactivatable PAI-1 in porcine platelets. To demonstrate that human t-PA reacts with porcine platelet PAI-1, we incubated human t-PA with porcine platelet lysates and subsequently analyzed the mixtures for the presence of high MI t-PA/inhibitor complexes by direct fibrin autography. Figure 1A indicates that human t-PA (M, 60 Kd, lane 1) interacts with a molecule in the porcine platelet lysates and forms high MI, lytic zones (M, 110 Kd, lane 3). Moreover, these high MI, lytic zones can be immunodepleted from the t-PA/platelet lysate incubation mixtures using antibodies to either human PAI-1 or human t-PA (lanes 4 and 5, respectively) and the high MI, lytic zones can be recovered in the respective immunoprecipitates (lanes 7 and 8). The ability of human t-PA to form complexes with porcine PAI-1 coupled with the observation that our rabbit antihuman PAI-1 preparation was able to recognize these complexes suggested that we should be able to quantitate the activity of porcine PAI-1 using a previously described functional immunoassay. This assay uses immobilized human t-PA to complex with solution phase PAI-1 and the bound PAI-1 molecules are subsequently quantitated using polyclonal antibodies to this inhibitor. Figure 1B indicates a comparison of the activity of PAI-1 in human platelets with the PAI-1 activity in porcine platelets using this system. Porcine platelet lysates exhibited approximately twofold more PAI-1 activity than human platelet lysates (3.7 ± 0.4 and 1.7 ± 0.3 U per 108 platelets, respectively).

Because SDS has been used by a number of investigators to activate the latent form of PAI-1,5-11 we used this assay to compare the ability of SDS to increase the activity of PAI-1 in porcine platelet lysates with the known effect of this denaturant to increase the activity of PAI-1 associated with human platelet lysates. Following a 1-hour treatment with 0.1% SDS, both porcine and human platelet lysates exhibited an approximately threefold increase in PAI-1 activity (10.8 ± 3.0 and 3.8 ± 0.5 U per 108 platelets). To control for the effect of SDS in the functional assay, samples with SDS were first neutralized with a 50-fold excess of Triton X-100 and then mixed with the platelet lysate samples. PAI-1 activity in platelet lysates treated in this manner was found to be comparable with PAI-1 activity in untreated platelet lysates (Fig 1B). In a separate series of experiments, freshly prepared porcine and human platelets were diluted to 108 platelets/mL into a buffer containing 50 mmol/L arginine, pH 7.4, a known stabilizer of PAI-1 activity.19 Latent PAI-1 activity in these samples could still be detected following SDS-treatment similar to results shown in Fig 1B.
Fig 1. Detection of active and denaturant-activatable PAI-1 in porcine and human platelet lysates. (A) Freeze-thawed porcine platelets (6 x 10^9/mL) were incubated (10 minutes, 37°C) in the absence (lane 2) or presence (lanes 3 through 9) of 4 IU t-PA. The mixtures were incubated with Sepharose-Protein A beads that had been preincubated with rabbit antisera to either human PAI-1 (lanes 4 and 7), human t-PA (lanes 5 and 8), or nonimmune rabbit serum (lanes 6 and 9). Both the immunosupernatants (lanes 4 through 6) and the immunoprecipitates (lanes 7 through 9) were fractionated by SDS-PAGE followed by fibrin autography. The positions of M, markers are indicated, and lane 1 contained 2 IU of t-PA. (B) Freeze-thawed porcine and human platelets (10^9/mL) were assayed for active PAI-1 either immediately (open bars) or following treatment with 0.1% SDS (1 hour, 37°C) (hatched bars). Samples containing SDS were neutralized by treatment with a 50-fold excess of Triton X-100 (30 minutes, 22°C). Solid bars represent controls in which the SDS was neutralized before the sample was added. PAI-1 activity was quantitated in the functional immunoassay. (C) Freeze-thawed porcine (lanes 1 through 3, 10^9/lane) and human (lanes 4 through 6, 10^9/lane) platelets were incubated in the absence (lanes 1 and 4) or presence (lanes 2 and 5) of t-PA (125 U, 10 minutes, 37°C). To account for the presence of t-PA, lanes 3 and 6 are controls in which the SDS sample buffer was added to the respective platelet samples before the addition of t-PA. Samples were subjected to SDS-PAGE and analyzed by reverse fibrin autography. The positions of the M, markers are indicated, and lane 7 contains 100 ng of purified PAI-1. (D) Freeze-thawed porcine and human platelets (10^9/lane) were incubated either in BSA-coated wells (light shaded bars) or t-PA-coated wells (dark shaded bars) as described in Materials and Methods. The samples were dialyzed either against PBS alone (28 hours, 4°C) (open bars) or against 4 mol/L guanidine (4 hours, 37°C) (hatched bars) followed by dialysis against PBS (24 hours, 4°C). Dialyzed samples were assayed for PAI-1 activity in the functional immunologic assay.

To exclude the possibility that the SDS-induced increase in the activity of porcine platelet PAI-1 was mediated by an increase in the surface expression of certain epitopes on PAI-1, we attempted to confirm the presence of latent PAI-1 in porcine platelets by using an antibody-independent technique. Figure 1C indicates that both porcine (lane 1) and human (lane 4) platelets exhibit an M, 50-Kd fibrinolytic inhibitory zone following fractionation by SDS-PAGE and analysis by reverse fibrin autography. Because these lysis-resistant zones may represent the combined activity of both the active and latent form of a PAI, we pretreated the platelet lysates with an excess of t-PA to inactivate those PAIs in an active form. Subsequent analysis of the samples by SDS-PAGE is known to result in: (1) the separation of PAs, t-PA/PAI complexes, and PAIs based on molecular weight; and (2) the activation of latent PAIs.5 Figure 1C indicates that both porcine (lane 2) and human (lane 5) platelet lysates still exhibit prominent lysis-resistant zones in this technique, even though the samples had been pretreated with an excess of t-PA. In experiments not shown, preabsorption of the porcine and human platelet lysates with Sepharose Protein-A/anti-PAI-1 beads resulted in the loss of the M, 50-Kd lysis-resistant zones, thus indicating that these zones represented PAI-1 present in the platelet lysates.

Because Fay and Owen16 attempted to activate porcine platelet PAI-1 by using 4 mol/L guanidine, we complemented the above experiments by comparing the ability of this denaturant to activate the latent form of PAI-1 in both porcine and human platelets. Similar to the results obtained by treating porcine and human platelets with SDS (Fig 1B), a threefold to fivefold increase in the activity of PAI-1 was also observed by subsequent treatment with 4 mol/L guanidine (Fig 1D). Furthermore, immobilized t-PA was able to deplete active PAI-1 from the platelet extracts, and the latent form remaining in the absorbed extract could...
be shown by activation with 4 mol/L guanidine. Finally, freshly isolated porcine platelets were lysed either with Triton X-100 (0.2%) or by sonication, and assayed before and after activation with 4 mol/L guanidine, yielding data similar to those shown in Fig 1D.

Detection of active and denaturant-activatable PAI-1 during partial purification of PAI-1 from porcine platelets. To investigate the differences between our observations and those of Fay and Owen, we performed the initial two steps of the purification procedure on an analytical scale. However, analysis of the resuspended ammonium sulfate precipitate (40% to 65%) showed primarily active PAI-1 because only a minor (ie, 1.3-fold) increase in PAI-1 activity was detected following treatment with 4 mol/L guanidine. Because of the known inability to detect latent PAI-1 in certain biological samples (eg, serum) with a high protein content, we attempted to detect latent PAI-1 in these resuspended ammonium sulfate precipitates by diluting the samples 10-fold before treatment with denaturants. Table 1 indicates that a threefold to fivefold increase in PAI-1 activity could be detected in the concentrated platelet pool (10⁹ platelets/mL) and in the resuspended ammonium sulfate precipitates by using this protocol.

Chromatography of the resuspended ammonium sulfate precipitate over a molecular sieving column resulted in the elution of active PAI-1 before the elution of guanidine-activatable PAI-1 (Fig 2). Fracctions that represent the two peaks of PAI-1 activity were pooled separately (ie, fractions no. 49 to 51 and fractions no. 57 to 59; Fig 2) and assayed for PAI-1 activity before and after activation with 4 mol/L guanidine. PAI-1 activity in the active fractions (ie, no. 49 to 51) increased only about 1.3-fold following treatment with 4 mol/L guanidine, whereas little PAI-1 activity was detected in fractions no. 57 to 59 unless the samples were treated with a denaturant (Table 1). In addition, the partial purification of porcine PAI-1 was repeated using frozen and thawed platelet preparations, with similar results obtained with this material (data not shown).

**DISCUSSION**

In this report, we provide evidence that porcine platelets contain both active and latent PAI-1. Because platelet lysates/extracts represent a complex biological sample with the potential of containing a variety of protease inhibitors, an important feature of our analysis was the use of a functional immunoassay that uses antibodies to specifically quantitate the ability of PAI-1 to bind to and to form complexes with t-PA. Using this assay, porcine platelets demonstrated approximately twofold higher PAI-1 activity levels than human platelets, which may reflect differences in PAI-1 content of platelets obtained from different species. The results of the functional immunoassay were confirmed by a standard semiquantitative assay for PAIs, routinely referred to as reverse fibrin autography. Prominent lysis-resistant zones, which could be immunodepleted using polyclonal antibodies to PAI-1, were still detected following activation with SDS even in those samples pre-treated with a large excess of t-PA. Unfortunately, only small differences were detected between the t-PA-treated and untreated samples, suggesting that the activity of active

![Fig 2. Gel chromatography of porcine platelet extract on a molecular sieving column. Fresh porcine platelets were extracted with ammonium sulfate and passed over a Sephacryl S-200 column as described in Materials and Methods. PAI-1 was quantitated in the functional immunologic assay either before (open squares) or following treatment with guanidine (closed squares). The elution peaks of BSA and ribonuclease A are marked with arrows. Horizontal bars indicate the peak fractions that were pooled for analysis by treatment with 4 mol/L guanidine (Table 1).](https://www.bloodjournal.org)

| Table 1. Summary of Active and Guanidine-Activatable PAI-1 Activity During the Initial Purification Step of PAI-1 From Porcine Platelets |
|-----------------|-----------------|-----------------|------------------|------------------|
| Samples         | Volume (mL)     | Protein conc (µg/mL) | Total Protein (µg) | PAI-1 (U/mL) |
| Starting lysate (10⁹ platelets/mL mixture) | 25.0 | 5,150 | 128,750 | 35.9 | 45.9 | 145.9 | 8,653.5 | 4,148/16,861 |
| Resuspended ammonium sulfate | 2.0 | 5,035 | 10,070 | 158.7 | 154.2 | 659.9 | 32,150.0 | 4,936/20,525 |
| Active (fractions no. 49-51) | 6.3 | 110 | 693 | 9.8 | 11.2 | 12.5 | 170.2 | 57,579/73,443 |
| Latent (fractions no. 57-59) | 6.3 | 91 | 573 | 0.4 | 46.9 | 48.1 | 2,738.0 | 146/17,567 |

Porcine platelets were extracted and chromatographed on a Sephacryl S-200 column as described. Aliquots from the indicated steps were assayed for PAI-1 activity in the functional immunoassay either directly (active) or after treatment with 4 mol/L guanidine.

**Abbreviation:** conc, concentration.
PAI-1 may not be completely shown by this technique (ie, SDS-PAGE followed by reverse fibrin autography). In fact, the active form of human recombinant PAI-1 has been observed to be sensitive to SDS-induced denaturation (ie, a 65% decrease in active PAI-1 with 0.2% SDS-treatment), whereas detection of the latent form required activation with a denaturant.22

The differences between our observation and those of Fay and Owen16 were addressed by repeating the initial two steps of their purification procedure for porcine platelet PAI-1. A number of possibilities existed to account for the inability of these investigators16 to detect the latent inhibitor in the crude platelet extracts. For example, Franke et al23 observed that the latent form of human recombinant PAI-1 is selectively precipitated using 35% ammonium sulfate. Differences between Fay and Owen's data and those of Franke et al23 may reflect species differences or the absence of oligosaccharides on the recombinant molecule. Another explanation arose from the reported inability of denaturants to activate latent PAI-1 in biological samples with a high protein content.5 As observed for latent PAI-1 in serum samples,5 we have found that of a 10-fold dilution of the concentrated porcine platelet pool (10^6 platelets/mL) or the resuspended ammonium sulfate precipitate extract was sufficient to permit the detection of guanidine-activatable PAI-1. Because active and latent human PAI-1 migrate quite differently on molecular sieving columns,10,11 we extended these observations to include the active and latent forms of the porcine molecule. Therefore, our data suggest that the differences in the migration of the two forms of PAI-1 during gel filtration accounts for the reported selective purification of active PAI-1 from porcine platelets. Finally, in the absence of sequence data for porcine PAI-1, the similar elution profile on a series of standard chromatographic columns does not indicate a structural basis for differences between the activity of porcine and human platelet PAI-1.

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