Both Circulating and Clot-Bound Plasminogen Activator Inhibitor–1 Inhibit Endogenous Fibrinolysis in the Rat

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The effects of both clot-bound and circulating plasminogen activator inhibitor–1 (PAI-1) on endogenous fibrinolysis were investigated in a rat model of pulmonary embolism. Iodine-125 fibrinogen–labeled blood–clot homogenates were delivered through the left jugular vein to the lung microvasculature, and the subsequent extent of the clot lysis was monitored by measuring the release of 125I-fibrin degradation products (FDPs) into the blood. Clots that had incorporated activated PAI-1 ex vivo were subsequently protected from dissolution in vivo in a dose-responsive manner (half-maximal concentration [IC\textsubscript{50}] = 4.3 µg/ml). PAI-1-containing clots also resisted lysis, as measured by the release of the specific FDP D-dimer. Plasma levels of plasminogen activator (PA) and PAI activity were unaltered by administration of PAI-1–containing clots, and the clot-protective effects of clot-bound PAI-1 were reversed by exogenous tissue-type plasminogen activator administration. Clot lysis was also inhibited in a dose-responsive manner (IC\textsubscript{50} = 58 µg/kg) by intravenous bolus delivery of activated PAI-1 to the circulation. The clot-protective effects of circulating PAI-1 were correlated with dose-dependent increases in plasma PAI-1 antigen and activity levels and decreases in plasma PA levels (IC\textsubscript{50} = 37 µg/ml). There was no evidence of any accumulation of circulating PAI-1 in the lungs. Latent PAI-1, whether delivered with or delivered after the clot homogenates, did not affect the clot-lytic process. Activated and latent PAI-1 was cleared from the circulation in a monophasic manner, with a half-life of approximately 32 and 7 minutes, respectively. The results indicate that both clot-bound and circulating PAI-1 are potent inhibitors of fibrinolysis in vivo. Clot-bound PAI-1 may inhibit PAs in the immediate vicinity of the clots, whereas circulating PAI-1 may act systemically by controlling overall levels of PAs present in the blood. (Arteriosclerosis and Thrombosis 1991;11:1276–1286)
smooth muscle cells\textsuperscript{24,25} synthesize and release PAI-1, especially after stimulation with inflammatory mediators and growth factors, including endotoxin, transforming growth factor-\textgreek{b}, tumor necrosis factor, and platelet-derived growth factor. Platelets store large amounts of PAI-1, which is released on platelet activation.\textsuperscript{6,7} PAI-1 also binds to fibrin\textsuperscript{26} and vitronectin,\textsuperscript{27} and the bound PAI-1 is capable of inhibiting t-PA and UK. Recent studies indicate that human thrombi\textsuperscript{28} as well as experimentally induced porcine thrombi\textsuperscript{29} contain greatly elevated levels of PAI-1 relative to the levels in the normal circulation. Thus, large amounts of platelet-derived PAI-1 or PAI-1 released from the vascular wall could accumulate at sites of vascular injury or forming thrombi by binding to fibrin or vitronectin and thereby generate an antifibrinolytic microenvironment.

Studies concerning the in vivo behavior and role of PAI-1 in the fibrinolytic system are limited. Knabb et al.\textsuperscript{30} demonstrated that high levels of circulating PAI-1 inhibited clot dissolution in a rabbit model, and we have recently demonstrated that whole-blood clot homogenates, which had incorporated PAI-1 ex vivo, were protected from lysis in a canine model of pulmonary embolism.\textsuperscript{31} Given the potential importance of PAI-1 to the generation of a prothrombotic state, we systematically examined the effects of both clot-bound and circulating PAI-1 on the fibrinolytic system in a rat model of pulmonary embolism.

Methods

Materials

Two-chain t-PA (2ct-PA; 920 IU/\mu g) and Immubind PAI-1 enzyme-linked immunosorbent assay (ELISA) kits were obtained from American Diagnostica, Greenwich, Conn. Human fibrinogen was purchased from Calbiochem, La Jolla, Calif., and predominantly single-chain t-PA (Activase) was obtained from Genentech, South San Francisco, Calif. Guanidine HCl was obtained from Schwarz/Mann, Cleveland, Ohio. Iodine-125--labeled Bolton-Hunter reagent and 125I--bovine serum albumin (BSA; 1 mCi/\mu g) were from NEN, Boston, Mass., and ICN Biomedicals, Costa Mesa, Calif., respectively. Fibrinogen was iodinated by the Iodogen method\textsuperscript{32} and possessed a specific radioactivity of 1 mCi/\mu g.

Plasminogen Activator Inhibitor--I

A nonglycosylated recombinant form of human PAI-1 (M\textsubscript{r}=43,000) was expressed intracellularly in Saccharomyces cerevisiae and purified to homogeneity.\textsuperscript{33} The purified PAI-1 was in a latent essentially inactive form as described by others\textsuperscript{10} and was activated by treatment with guanidine HCl.\textsuperscript{31,34} The guanidine HCl was subsequently removed by gel filtration in 0.05 M tris(hydroxymethyl)aminomethane HCl, 0.1 M NaCl, 0.01% Tween-80 (pH 7.4) (TNT buffer) at 4°C. The inhibitory activity of PAI-1 was determined by adding PAI-1 to a known amount of 2ct-PA and measuring the residual t-PA activity in a coupled amidolytic assay, which measures the conversion of plasminogen to plasmin by t-PA.\textsuperscript{28} One arbitrary unit (AU) is defined as the amount of PAI-1 required to inhibit one IU t-PA. Activated PAI-1 was approximately 35% active and possessed a specific activity of approximately 509 AU/\mu g. Latent PAI-1 had a specific activity of less than 5 AU/\mu g.

Measurement of Endogenous Fibrinolysis In Vivo

Male Sprague-Dawley rats, 270--400 g, were anesthetized with sodium pentobarbital (32 mg/kg body wt i.v.). The left jugular vein was cannulated for clot administration, and the right carotid artery was cannulated for blood withdrawal and a maintenance infusion of sodium pentobarbital (4 mg/hr). One milliliter blood was drawn and mixed with 1 \mu Ci 125I-fibrinogen and either 300 \mu l vehicle or PAI-1 (1.2--30 \mu g). Vehicle consisted of TNT buffer containing 120 mM guanidine HCl to control for the residual guanidine HCl remaining after PAI-1 activation. After the blood was allowed to clot for 1 hour at 37°C, the clot was homogenized to a fine suspension in a glass--glass homogenizer (Kon tes). The homogenate was centrifuged at 5,000g, washed three times in phosphate-buffered saline (PBS), and finally resuspended in 1 ml PBS before use. The extent of 125I-fibrinogen incorporation into the washed clot suspension ranged from 60% to 90%. At time zero, the homogenate (0.5 ml/kg body wt) was injected into the jugular vein of the anesthetized rats. Blood samples (0.2 ml) were drawn at intervals for determination of 125I-fibrin degradation products (FDPs). Total blood radioactivity was calculated with the assumption that the blood volume per weight ratio equaled 10%. In some cases the animals were killed by an intravenous injection of 1 ml KCl, and the lungs or other organs were obtained for counting. In other experiments t-PA (Activase) was administered 1 minute after clot delivery at 1 mg/kg body wt as a 10% loading dose, and the remainder was infused during the subsequent 60-minute period (0.026 ml/min). Care and handling of experimental animals conformed to the standards established by the Merck Sharp and Dohme Research Laboratories Institutional Animal Care and Use Committee, as recommended in National Institute of Health publication No. 86-23.

Plasminogen Activator Inhibitor--I Binding Studies

PAI-1 was radiolabeled with 125I--Bolton-Hunter reagent to a specific activity of approximately 4 \times 10^6 cpm/\mu g. After guanidine HCl activation, 125I--PAI-1 retains its inhibitory activity against t-PA by formation of 1:1 molar sodium dodecyl sulfate stable complexes (C.F. Reilly, unpublished observations). Binding studies were initiated by mixing duplicate samples of rat blood (1 ml) with activated or latent 125I--PAI-1 (2 ng) and increasing amounts of unlabeled activated PAI-1 (1.2--30 \mu g/ml). BSA binding was determined by adding 0.65 \mu Ci 125I-BSA to the rat blood instead of PAI-1. After a 1-hour incubation at 37°C, the clots were homogenized and washed as described above, and the radioactivity of each clot homogenate was...
determined in duplicate. To determine whether clot-bound PAI-1 was delivered to the lung, washed clot suspensions formed in the presence of $^{125}$I-PAI-1 and unlabeled PAI-1 were delivered via the left jugular vein to the rat lungs. The extent of PAI-1 delivered to the lungs was calculated from the amount of $^{125}$I-PAI-1 present in the lungs at 1 and 60 minutes after delivery.

**Determination of $\alpha$-D-Dimer Levels**

Rat whole-blood clots containing human fibrinogen were formed by adding 25 $\mu$l of a 20 mg/ml stock solution of human fibrinogen per milliliter blood (final concentration, 0.5 mg/ml) in the presence or absence of activated PAI-1 (30 $\mu$g/ml). The clots were homogenized and delivered to the lungs as described above. Blood samples were subsequently drawn into syringes containing 1/10 volume 3.8% sodium citrate and immediately centrifuged at 10,000 rpm for 2 minutes in an Eppendorf microfuge to obtain the plasma. The levels of human $\alpha$-d-dimer in the plasma samples were determined by a sandwich ELISA (Stago Diagnostica, Asnieres-sur-Seine, France).

**Bolus Administration of Plasminogen Activator Inhibitor-1**

$^{125}$I-fibrinogen-labeled rat clot homogenates, which had been prepared in the absence of PAI-1, were delivered to the rat lungs as described above. One minute later, activated or latent PAI-1 (20-300 $\mu$g/kg) in a volume of 300 $\mu$l was administered through the left jugular vein. Blood and plasma samples were taken at intervals to determine PAI-1 activity and antigen levels, PA activity levels, and $^{125}$I-FDP release.

**Determination of Plasminogen Activator Inhibitor-1 Antigen Levels in Rat Plasma**

Human PAI-1 levels in rat plasma were determined with a solid-phase immunological assay (Immubind PAI-1 ELISA kit), which measures both activated and latent PAI-1. Citrated plasma samples were tested undiluted and after a twofold to 20-fold dilution into TNT buffer. The absorbance values obtained were compared with standard curves constructed by adding known amounts of activated or latent PAI-1 to human PAI-1–depleted plasma at the appropriate dilution. The PAI-1 levels in the plasma samples were determined from the values that fell within the middle range of the standard curves, which were linear between 10 and 200 ng/ml. Similar results were obtained when the standard curves were generated in plasma pooled from normal rats.

**Other Procedures**

PAI-1 activity levels in citrated plasma were determined as described by Chmielewska and Wiman. The PA activity of freshly drawn acidified plasma was measured as described in the directions of the KabiVitrum COA-SET t-PA kit (KabiVitrum, Stockholm, Sweden). Results are presented as mean±SEM unless stated otherwise. Statistical significance ($p<0.05$) of differences among treatments was determined by a two-way analysis of variance of repeated measures and a one-sided Dunnett’s multiple-comparison test or Student’s $t$ test.

**Results**

**Plasminogen Activator Inhibitor-1 Binds to Rat Whole-Blood Clot Homogenates**

To establish whether PAI-1 binds to clots prepared from rat blood, increasing amounts of PAI-1 and trace amounts of $^{125}$I–PAI-1 were added to freshly drawn rat whole blood. After the blood was allowed to clot spontaneously for 1 hour, the clot was homogenized to a fine suspension and washed thoroughly to remove any unbound PAI-1, and the radioactivity of the final clot suspension was determined. After addition of activated or latent PAI-1 at 1.2, 6, and 30 $\mu$g/ml blood, 0.12±0.02, 0.67±0.8, and 3.55±0.24 $\mu$g/ml activated PAI-1 (four determinations) and 0.08±0.01, 0.28±0.2, and 1.57±0.24 $\mu$g/ml latent PAI-1, respectively (mean±range of two determinations), became tightly associated with the final clot homogenates. Thus, approximately 11% of the activated and 5.5% of the latent PAI-1 bound to the clot homogenates at each concentration examined. Because the association of PAI-1 with the clots could represent nonspecific trapping within the fibrin meshwork, $^{125}$I-BSA was added to rat blood, and the amount bound was determined. After clotting, 1.9±0.2% (three determinations) of the total BSA was found to be associated with the washed homogenates. Thus, most of the binding of activated and latent PAI-1 to the clots does not arise from nonspecific trapping within the clots.

**Delivery of Plasminogen Activator Inhibitor-1–Enriched Clots to the Rat Lung**

Homogenates containing activated $^{125}$I–PAI-1 were prepared as described above and delivered to rats through the left jugular vein. Clot suspensions delivered in this manner lodge in the microvasculature of the lung (see below). The animals were killed 1 or 60 minutes after clot delivery, and the amount of PAI-1 present in the lungs was established by measuring the radioactivity. Comparison of the amount of PAI-1 delivered to the rats relative to the amount of PAI-1 present in the lungs (Table 1) indicates that 74–82% of the PAI-1 was retained in the lung tissue 1 minute after administration. At 60 minutes, 59–67% of the PAI-1 was present in the lungs. These results indicate that most of the PAI-1, which was incorporated into the clots ex vivo, accumulates in the lungs. Furthermore, a considerable amount of PAI-1 remains in the lungs even after 60 minutes in vivo.

**Activated Plasminogen Activator Inhibitor-1–Enriched Clots Are Protected From Lysis In Vivo**

To establish whether clots containing PAI-1 were protected from dissolution in vivo, rat blood was
was low along the route of administration and in continued to increase in a linear manner for 60 minutes (Figure 1A). In contrast, the activated PAI-1-treated appeared in the blood within 10 minutes and continued to lodge in the microvasculature of the lung. Thus, the 125I-fibrin(ogen)-labeled clot suspensions were dispersed throughout both lungs and other tissues, including blood, heart, kidney, and liver. Therefore, the 125I-fibrin(ogen)-labeled clot suspensions were essentially identical in animals that received clots formed in the presence of vehicle or a high dose of unactivated latent PAI-1 (30 μg/ml) (Figure 1B).

The amount of 125I-fibrin(ogen) remaining in the lungs was measured 60 minutes after delivery of either vehicle-treated clots or clots prepared in the presence of activated PAI-1 (30 μg/ml). Relative to the amount of 125I-fibrin(ogen) delivered (approximately 250,000 cpm/rat), significantly more radioactivity remained in the lungs of rats that had received the PAI-1–treated clots (vehicle, 51.4 ± 2.2%, n = 6; 30 μg/ml PAI-1, 77.8 ± 3.4%, n = 6; p < 0.0001 by Student’s t test).

The effect of PAI-1 on clot dissolution was also monitored by measuring the release of d-dimer from clots formed in the presence of vehicle or activated PAI-1 (30 μg/ml). D-Dimer is a specific FDP that is released from fibrin by plasmin. Antibodies are available that recognize human d-dimer; therefore, rat clot homogenates containing a small amount of human clots resisted lysis in a dose-responsive manner (p < 0.0001) (Figure 1A). At initial PAI-1 levels of 6 and 30 μg/ml, 125I-FDP levels were significantly less than in the vehicle-treated clots (p < 0.0001). The lowest level of PAI-1 employed (1.2 μg/ml) did not significantly affect blood levels of 125I-FDPs relative to controls. At 60 minutes, the 6 and 30 μg/ml levels of PAI-1 had reduced blood radioactivity by 60% and 85%, respectively, as judged by computing the areas under the curves in Figure 1A. The half-maximal effective dose (IC50) of PAI-1 at 60 minutes equaled 4.3 μg/ml. The inhibitory effects of PAI-1 on clot lysis were temporal, as 125I-FDP blood levels increased slowly with time, even at the highest dose of PAI-1 used (30 μg/ml, Figure 1A). Blood levels of 125I-FDPs were essentially identical in animals that received clots formed in the presence of vehicle or a high dose of unactivated latent PAI-1 (30 μg/ml) (Figure 1B).

The amount of 125I-fibrin(ogen) remaining in the lungs at 1 or 60 minutes after clot delivery was calculated from the total radioactivity present in the excised lungs.

**Table 1. Plasminogen Activator Inhibitor-1 Remaining in the Lungs at 1 and 60 Minutes After Clot Delivery**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Initial PAI-1 (μg/ml blood)</th>
<th>PAI-1 bound (ng/ml blood)</th>
<th>PAI-1 delivered (ng/kg)</th>
<th>PAI-1 remaining (ng/kg)</th>
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<td>76</td>
<td>56±3</td>
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<td>1,851±35</td>
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<td>60</td>
<td>6</td>
<td>937</td>
<td>469</td>
<td>315±24</td>
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<tr>
<td>30</td>
<td>3,697</td>
<td>1,848</td>
<td>1,231±90</td>
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</tr>
</tbody>
</table>

Activator iodine-125-labeled plasminogen activator inhibitor-1 (PAI-1) (6×10^5 cpm) was added to freshly drawn rat whole blood with the indicated amount of unlabeled activated PAI-1. One hour later clots were homogenized and washed, and the amount of PAI-1 that bound to the clot homogenates was determined as described in "Methods." Clot homogenates containing a known amount of PAI-1 were delivered (0.5 ml/kg) to the lungs (two rats at each dose). The amount of PAI-1 remaining (mean ± range) in the lungs at 1 or 60 minutes after clot delivery was calculated from the total radioactivity present in the excised lungs.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Line plots showing that clot-bound activated plasminogen activator inhibitor-1 (PAI-1) but not latent PAI-1 inhibits iodine-125-labeled fibrin degradation product (FDP) release from experimental pulmonary emboli. 125I-fibrin(ogen) containing clot homogenates, prepared in the presence of vehicle, activated PAI-1, or latent PAI-1, were delivered through the left jugular vein to the lungs at time zero. At indicated times (min) blood samples were withdrawn, and levels of 125I-FDPs were determined. Data are expressed as the percentage of administered radioactivity that was present in the total blood volume at each time point and assumes a blood volume to weight ratio of 10%. PAI-1 concentrations are per milliliter of blood before clot homogenization. Panel A: Activated PAI-1: ○, Vehicle, n = 8; ■, 1.2 μg/ml PAI-1, n = 7; ▲, 6 μg/ml PAI-1, n = 6; ●, 30 μg/ml PAI-1, n = 7. Panel B: Latent PAI-1: ○, Vehicle, n = 8; ▲, 30 μg/ml latent PAI-1, n = 7. Vehicle data in panel B were from the experiment described in panel A.
man fibrinogen and either vehicle or activated PAI-1 were prepared and delivered to the lungs. Figure 2 indicates that the plasma levels of D-dimer were significantly less in the PAI-1 treatment group than in the vehicle treatment group for 60 minutes after clot delivery (p<0.05).

The plasma levels of PA and PAI-1 activity did not change after delivery of the vehicle- or PAI-1-treated clots. PA levels ranged from 3.9-4.3 IU/ml and 3.5-4.4 IU/ml before and 30 and 60 minutes after delivery of vehicle-treated (n=2) and PAI-1-treated (n=2) clots, respectively. PAI-1 activity levels before and 30 and 60 minutes after clot delivery ranged from 9.2 to 12.6 AU/ml and from 12.0 to 15.4 AU/ml in the same vehicle- and PAI-1-treated rats, respectively.

Exogenous Tissue-Type Plasminogen Activator Reverses the Clot-Protective Effects of Plasminogen Activator Inhibitor–1

If clot lysis in the above model is initiated by endogenous PA activation of the fibrinolytic system and if PAI-1 suppresses clot dissolution by directly inhibiting this process, high levels of exogenously added PA should accelerate the clot-lytic process and reverse the effects of PAI-1. Figure 3 indicates that exogenous t-PA accelerates 125I-FDP release relative to non-t-PA-treated controls. Moreover, clots formed in the presence of a high concentration of activated PAI-1 (30 µg/ml) lysed as rapidly in the presence of exogenous t-PA as the vehicle-treated clots.

Activated Plasminogen Activator Inhibitor–1 in the Circulation Inhibits Clot Dissolution In Vivo

The above experiments addressed the role of PAI-1, which was prebound to the clots, on the subsequent fate of the clots. We also determined whether PAI-1 systemically administered to the circulation inhibits clot lysis. Activated PAI-1 was delivered by bolus intravenous injection 1 minute after 125I-fibrinogen-labeled clots were directed to the lungs. Under these conditions PAI-1 significantly reduced 125I-FDP blood levels in a dose-dependent manner (p<0.0001) (Figure 4A). Relative to vehicle, PAI-1 at 20, 100, and 300 µg/kg reduced blood radioactivity levels by 25%, 62%, and 74%, respectively, as judged by computation of the area under the curve at the 60-minute time point. The IC50 of activated PAI-1 was 58 µg/kg. Activation of PAI-1 was critical for its clot-protective effects because blood levels of 125I-FDPs were nearly identical at all times after administration of vehicle or latent PAI-1 (300 µg/kg) (Figure 4B).

Activated Plasminogen Activator Inhibitor–1 in the Circulation Inhibits the Systemic Fibrinolytic System

To elucidate the mechanism underlying the clot-protective effects of activated PAI-1 shown in Figure 4A, plasma levels of PAI-1 antigen, PAI-1 activity,
and PA activity were determined after bolus administration of activated PAI-1 to rats bearing lung clots. PAI-1 antigen levels increased immediately after bolus administration of 20, 100, and 300 μg/kg activated PAI-1 (Figure 5). The initial levels of PAI-1 were similar to the values predicted from the administered dose, assuming a 10% blood volume to weight ratio and a uniform distribution in the circulation. PAI-1 antigen cleared from the circulation in a monophasic manner, with a half-life \( t_{1/2} \) of 27.6 ± 0.9 minutes (mean of 20, 100, and 300 μg/kg values).

PAI-1 activity in plasma increased 10-, 43-, and 114-fold immediately after delivery of activated PAI-1 at 20, 100, and 300 μg/kg, respectively, relative to the basal levels present in rat plasma (13.3 ± 0.7 AU/ml, \( n=6 \)) (Figure 6). The initial levels of PAI-1 activity at the three different doses were close to those predicted from the antigen levels measured in Figure 5. PAI-1 activity subsequently cleared from the circulation in a monophasic manner, with a \( t_{1/2} \) of 35.3 ± 2.4 minutes (mean of 20, 100, and 300 μg/kg values). Essentially similar pharmacokinetic behavior has been observed in rabbits, where activated PAI-1 activity and antigen cleared from the circulation through a predominant β phase, with a \( t_{1/2} \) of approximately 30 minutes.34

Within 10 minutes of activated PAI-1 bolus administration, decreases in the levels of PA activity in the circulation were apparent (Figure 7). The reductions in PA levels were sustained for 60 minutes and were dependent on the initial dose of PAI-1. Administration of PAI-1 at 20 and 100 μg/kg resulted in a 35.7 ± 0.9% and a 71 ± 2.5% inhibition of PA activity in plasma, respectively (mean inhibition at 10, 30, and 60 minutes). Administration of PAI-1 at the highest dose (300 μg/kg) did not result in further PA inhibition (69.7 ± 5.3%) relative to the 100 μg/kg dose. Thus, one of the components responsible for PA activity in rat plasma may resist inhibition by PAI-1. The IC50 of activated PAI-1 equalled 37 μg/kg.

The presence of clot homogenates in the lungs did not influence the pharmacokinetic behavior of activated PAI-1. Nearly identical clearance profiles were measured for PAI-1 antigen (Figure 8A), PAI-1 activity (Figure 8B), and inhibition of plasma PA
Activated PAI-1 was delivered by bolus intravenous administration to rats bearing clots. At indicated times (min) plasma samples were obtained, and the levels of PAI-1 activity were determined. ○, 20 μg/kg PAI-1; ●, 100 μg/kg PAI-1; ▲, 300 μg/kg PAI-1. Results are mean±range from two experiments. Basal levels of PAI-1 activity present in normal rat plasma equaled 13.3±1.6 AU/ml (n=6). AU, arbitrary unit.

The pharmacokinetic behavior and effect on plasma PA activity of a high dose of latent PAI-1 were also evaluated in rats bearing clots. After bolus delivery of latent PAI-1 at 300 μg/kg, PAI-1 antigen levels increased dramatically and subsequently cleared from the circulation in a monophasic manner, with a t1/2 of approximately 6.8 minutes (Figure 9A). In contrast, PAI-1 activity levels increased slightly (approximately twofold) after delivery and subsequently cleared from the circulation with a t1/2 of approximately 39 minutes (Figure 9B). The minor increase in PAI-1 activity relative to the increase in antigen and the similarity between the clearance of latent PAI-1 activity (t1/2=39 minutes) and activated PAI-1 activity (t1/2=35 minutes) suggest that the activity increase was due to the small amount of active PAI-1 inherently present in latent PAI-1 preparations. The delivery of latent PAI-1 did not significantly alter the levels of PA activity in rat plasma (Figure 9C). This finding does not appear to be due to the more rapid clearance of latent PAI-1 (t1/2=7 minutes) from the circulation, as the average latent PAI-1 antigen levels were similar throughout the first 30 minutes to those of activated PAI-1 at 100 μg/kg, a dose that inhibited PA activity by 71%. Results similar to those presented in Figure 9 were also obtained after delivery of latent PAI-1 (300 μg/kg) to rats without clots in the lungs (data not shown).

Activated Plasminogen Activator Inhibitor–1 in the Circulation Does Not Bind to the Clot Homogenates In Vivo

To determine whether activated PAI-1 delivered to the circulation binds to clots already present in the lungs, [125I]–PAI-1 (2 ng) was added to unlabeled PAI-1 (100 μg/kg), and the mixture was administered to the systemic circulation 1 minute after clot homogenates were directed to the lungs. PAI-1 was also administered to control animals without clots. Sixty minutes later, the amount of radioactivity in the lungs was measured. Relative to the total radioactivity administered, there was slightly more radioactivity present in the lungs of the rats that bore clots (0.81±0.12%, n=5) than in the lungs without clots (0.60±0.03%, n=5). However, the values were not significantly different (p=0.07, one-sided Student's t test).

Discussion

The effects of both clot-bound and circulating PAI-1 on fibrinolysis have been evaluated in a rat model of pulmonary embolism. The results indicate that elevated levels of PAI-1 in the circulation can...
directly suppress the systemic fibrinolytic process. These findings provide direct experimental evidence supporting the idea that the net fibrinolytic potential of the circulation is regulated by the balance between PAI-1 and the PAs present. Moreover, the data confirm and extend our previous results that clot-bound PAI-1 inhibits clot dissolution in vivo and fully support the notion that the localization of PAI-1 within a thrombus provides an important thrombus-stabilizing effect.

We first established that PAI-1 binds to blood clots and subsequently protects those same clots from lysis in vivo, as has been previously demonstrated in a canine model. When increasing amounts of activated PAI-1 were added to rat whole blood, the concentration of PAI-1 that bound to the final clot suspension rose proportionally. Activation of PAI-1 apparently enhances its binding capability because greater than twofold more activated PAI-1 than latent PAI-1 bound to the clots at every concentration examined. PAI-1 may bind to the clots through an association with the fibrin component because activated PAI-1 and to a lesser extent latent PAI-1 bind to fibrin in vitro. The ability of PAI-1 to bind to the clots does not appear to be directly related to its ability to inhibit t-PA, however, as more latent PAI-1 bound to the clots (5.5%) than would be expected from its t-PA-inhibitory activity (<0.5% active). Wagner et al reached the same conclusion after studying PAI-1 binding to fibrin. As suggested by the extent of PAI-1 found in the lung tissue after delivery of the PAI-1–containing clots, the inhibitor is delivered with the clots to the lungs and remains tightly bound to the clots even after 60 minutes in vivo. Thus, the effects of clot-bound PAI-1 on clot
lysis are due to the PAI-1 that was delivered with the clots to the lungs.

Activated PAI-1, delivered prebound to the clot homogenates, subsequently protected those clots from the endogenous fibrinolytic system of the rat lung in a dose-responsive manner. Blood levels of $^{125}\text{I}-\text{FDPs}$ were significantly lower in rats with PAI-1–treated clots than in rats that had received vehicle–treated clots. Moreover, the levels of $^{125}\text{I}$–fibrin(ogen) remaining in the lungs with PAI-1–treated clots were significantly higher than in controls. This latter observation eliminates the possibility that the reductions in blood radioactivity resulted from alterations in the rate of FDP clearance from the circulation.

Additional experiments indicated that the release of the defined FDP, d-dimer, from rats bearing PAI-1–treated clots was significantly reduced. Together, these data indicate that the observed reductions in circulating $^{125}\text{I}$–FDPs are related directly to PAI-1 inhibition of the rate of clot dissolution in the lung microvasculature. Because the delivery of exogenous t-PA to the rats accelerated the rate of clot lysis and completely reversed the clot-protective effects of the clot-bound PAI-1, PAI-1 most likely inhibited clot dissolution by directly inactivating the endogenous PAs responsible for initiation of the fibrinolytic process. In contrast to activated PAI-1, clot-bound latent PAI-1 did not affect clot dissolution. Although this may be related to the relatively weaker binding of latent PAI-1 to the clots, other data (see below) indicate that fibrinolysis was also unimpaired in the presence of latent PAI-1 in the circulation.

Clot lysis in the absence of PAI-1 or its inhibition in the presence of clot-bound PAI-1 occurred without changing the normal levels of PAI or PA activity in the general circulation. Thus, the clot-protective effects of PAI-1 can be ascribed to the PAI-1 that was delivered with the clots and not to the increases in plasma PAI-1 that can occur after surgery or as an acute-phase type of reaction. Because the introduction of clot-bound PAI-1 did not lead to a generalized inhibition of PAs in the systemic circulation, clot-bound PAI-1 probably inhibits PAs locally in the microenvironment of the clot homogenates that were lodged in the lung tissue.

PAI-1 circulates in human plasma at levels of approximately 10 ng/ml. Thus, the initial levels of PAI-1 added to the rat blood ex vivo and required for clot protection (IC$_{50}$ = 4.3 μg/ml, Figure 1A) appear to be well above what is considered the normal physiological levels of PAI-1. This discrepancy could arise from the use of human PAI-1 to inhibit the rat fibrinolytic system or from the presence of reversible PA inhibitors in plasma that interfere with PAI-1–PA binding.

In addition, the PAI-1 employed in these studies is only partially (35%) active after guanidine HCl activation. Because only 11% of the added PAI-1 actually bound to the final blood clot homogenates, the concentration of PAI-1 required to protect clots in vivo may be 473 ng/ml blood clot. The normal complement of platelets in 1 ml blood can release more than 200 ng PAI-1 on activation.

Next we asked if circulating PAI-1 inhibited endogenous fibrinolysis in this model. Bolus intravenous administration of activated PAI-1 led to a dose-dependent inhibition of clot lysis, as measured by suppression of the $^{125}\text{I}$–FDP levels in blood. Knabb et al. demonstrated that continuous intravenous infusion of PAI-1 also suppresses clot lysis in a rabbit model. In our experience, inhibition of clot dissolution was correlated with dose-dependent increases in the plasma levels of PAI-1 activity and antigen after PAI-1 administration. The increases in PAI-1 antigen and activity levels were accompanied by dose-dependent decreases in the levels of PA activity in plasma and probably reflect direct inactivation of PAs by PAI-1 in the circulation. The relatively large amounts of circulating PAI-1 required for inhibition of plasma PA activity as well as suppression of clot lysis may be due to the same factors noted above for clot-bound PAI-1. Importantly, comparison of the effects of activated PAI-1 on clot lysis and plasma PA activity (Table 2) yields similar inhibition values at each dose. These results are reflected by the respective IC$_{50}$ values for inhibition of clot lysis (58 μg/kg) and inhibition of plasma PA activity (37 μg/kg). These data suggest that the increased stability of clots in the presence of circulating PAI-1 stems from PAI-1–mediated inhibition of PAs in the circulation and the resultant diminution in fibrinolytic potential. This view is supported by the finding that only minimal amounts of circulating PAI-1 could have been associated with the clots in vivo. Identical PAI-1 pharmacokinetic profiles were observed in the presence and absence of clots in the lungs; moreover, little if any of a radiolabeled PAI-1 preparation delivered to the circulation was associated specifically with the clots present in the lungs. The lack of binding relative to that observed when blood is allowed to clot ex vivo may arise from the cross-linked nature of the clots, which would physically limit the access of PAI-1. We infer from these data that the effects of circulating PAI-1 on fibrino-

### Table 2. Circulating Plasminogen Activator Inhibitor-1 Inhibition of Clot Lysis and Plasma Plasminogen Activator Activity

<table>
<thead>
<tr>
<th>PAI-1 (μg/kg)</th>
<th>Clot lysis (%)</th>
<th>PA activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>100</td>
<td>62</td>
<td>71</td>
</tr>
<tr>
<td>300</td>
<td>74</td>
<td>70</td>
</tr>
</tbody>
</table>

Activated plasminogen activator inhibitor-1 (PAI-1) was delivered to rats bearing clots in the lungs by bolus intravenous administration at the doses indicated. The effect on clot lysis was obtained from the experiment described in Figure 4A by computing the area under the curve (AUC) at 60 minutes and setting the percent inhibition = 1 – (AUC for PAI-1/AUC for vehicle). The effect of circulating PAI-1 on plasminogen activator (PA) activity was calculated from data presented in Figure 7. Percent inhibition = 1 – (average PA activity at 10, 30, and 60 minutes after PAI-1 delivery/PA activity before PAI-1 delivery).
lysis are not due to a localized accumulation of PAI-1 in the vicinity of the clot homogenates. The introduction of latent PAI-1 to the circulation failed to alter the clot dissolution process, despite the fact that initial antigen levels were reached that equaled those of an equivalent dose of activated PAI-1. The inability of latent PAI-1 to stabilize the clots is most likely related to the minimal changes in plasma PAI-1 and PA activity that followed its administration. These data support the idea that the clot-protective effects of activated PAI-1 are directly related to its ability to inhibit PA initiation of the fibrinolytic pathway. These results also eliminate the possibility that a significant portion of the latent PAI-1 became activated in vivo. Because latent PAI-1 is normally present in plasma and is elaborated from activated platelets, the determination of its in vivo role, if any, remains elusive.

Our results predict that PAI-1 regulates the fibrinolytic system through localized as well as systemic mechanisms. PAI-1 released locally from activated platelets or perhaps the vascular wall itself could accrue at sites of thrombus formation, presumably by binding to the fibrin component. In this regard, human arterial thrombi obtained at autopsy and experimentally induced porcine thrombi contain 155–213-fold more PAI-1 than does a comparable volume of plasma. PAI-1 concentrated in this manner could directly inactivate any PAs present and thus suppress local fibrinolytic events. The thrombi would remain protected until the finite levels of PAI-1 present were overwhelmed by PAs continuously recruited from the circulation. As the effects of PAI-1 would be entirely localized at the thrombus interface, it may represent a mechanism that stabilizes thrombi without changing the overall systemic fibrinolytic pathways. In addition, localization of PAI-1 to a thrombus would prevent its rapid clearance from the circulation. This model also predicts that excessive PAI-1 accumulation at localized sites, due to prolonged platelet activation or PAI-1 release from an activated vascular wall, is potentially prothrombotic. Moreover, clinical detection of an associated prothrombotic state could be impaired because the plasma levels of PAI-1 and PA activity remained unchanged after the introduction of PAI-1–enriched clots to the lungs.

PAI-1 also appears to regulate the systemic fibrinolytic pathway. Under normal circumstances, excessive activation or suppression of the fibrinolytic system may be prevented by the approximately equivalent balance existing between the levels of PAs and PAI-1 present in the circulation. As our data indicate, however, increases in plasma PAI-1 levels would lead directly to reductions in the levels of circulating PAs. Fewer PAs would be available for recruitment to the site of a thrombus, and thus, the overall fibrinolytic system would be inhibited. These latter observations may be particularly relevant to human studies in which concomitant increases in PAI-1 levels and reductions in circulating PA activity have been associated with myocardial infarction.

Acknowledgments
We wish to thank Tim Schoefield for the statistical analyses, Barbara Francis for preparing the iodinated fibrinogen, and Paul Friedman for his support.

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**KEY WORDS** • plasminogen activator inhibitor-1 • fibrin • thrombosis • fibrinolysis • plasminogen activators • tissue-type plasminogen activator
Both circulating and clot-bound plasminogen activator inhibitor-1 inhibit endogenous fibrinolysis in the rat.

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doi: 10.1161/01.ATV.11.5.1276

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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