Complex Formation Between Urokinase and Plasma Protein C Inhibitor In Vitro and In Vivo

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Protein C inhibitor (PCI) and plasminogen activator inhibitors 3 (PAI-3; urinary urokinase inhibitor) are immunologically identical. The role of PCI for urokinase (uPA) inhibition in vivo was investigated. We therefore developed an enzyme-linked immunosorbent assay (ELISA) specific for uPA-PCI complexes: Rabbit anti-PCI IgG was immobilized on a microtiter plate and following incubation with uPA-PCI complex-containing samples, bound uPA-PCI complexes were quantified with a horseradish-peroxidase-linked monoclonal antibody (MoAb) to uPA. Using this assay, time, dose, and heparin-dependent complexes were detected when uPA was incubated with normal plasma or purified urinary PCI, whereas no complexes were measurable using PCI-immunodepleted plasma. Plasma samples containing 20 mmol/L benzamidine to prevent complex formation ex vivo from patients undergoing systemic urokinase therapy (1 x 10^6 IU/60 min intravenously [IV]) after myocardial infarction were also studied. uPA present in these plasma samples (up to 1,200 ng/mL) had only 43% to 70% of the specific activity of purified 2-chain uPA, suggesting that a major portion of uPA is complexed to inhibitors. In these plasma samples uPA-PCI complexes were present in a concentration corresponding to 21% to 25% of inactive uPA antigen. These data suggest that at high uPA concentrations, such as during uPA therapy, plasma PCI might contribute significantly to uPA inhibition in vivo.

The Balance between plasminogen activators and plasminogen activator inhibitors (PAl's) provides a major regulatory mechanism for the fibrinolytic capacity. In plasma, PAI-1 (endothelial-type plasminogen activator inhibitor) is the primary inhibitor of tissue plasminogen activator and urokinase.6-8 When these plasminogen activators are present at low concentrations. PAI-2 (placental-type PAI) is usually not found in plasma except during pregnancy. Other plasma serine protease inhibitors (eg, alpha-2 antiplasmin, antithrombin III, and alpha-2 macroglobulin) have also been described to interact with plasminogen activators.6,9-11 These inhibitors, however, react with tissue-type plasminogen activator (tPA) and urokinase with much lower rate constants than PAI-1.12,13 Although the plasma concentrations of these inhibitors are higher than that of PAI-1,13 they are thought to play a role for plasminogen activator inhibition only when the concentration of plasminogen activators exceeds the inhibitory capacity of PAI-1.13 Recently an additional, heparin-dependent inhibitor of urokinase has been demonstrated in plasma and urine14 which was designated PAI-3.15 We have shown that PAI-3 is immunologically identical to the heparin-dependent inhibitor of activated protein C (PC1),16 originally described by Marlar et al.17 and purified by Suzuki et al.18-20 PCI is a member of the serine protease inhibitor (serpin) superfamily of proteins.21 It inhibits activated protein C (APC), thrombin, factor Xa, factor XIa, plasma kallikrein, urokinase, and two-chain tissue plasminogen activator in reactions stimulated by heparin.21,22 The second order rate constants for the interaction of PCI with urokinase (1 x 10^7 M^-1 s^-1 without heparin, 4 x 10^7 to 9 x 10^8 M^-1 s^-1 with heparin) and two-chain tPA (<10^-10 M^-1 s^-1 without heparin, 1 x 10^-9 M^-1 s^-1 with heparin) are much lower than those for the interaction of these plasminogen activators with PAI-1 (>/=10^-7 M^-1 s^-1). However, the plasma concentration of PCI (/>=4 mg/mL) is two to three orders of magnitude higher than that of PAI-1.23 PCI might therefore be involved in the regulation of two major antithrombotic pathways, the protein C system and fibrinolysis. The interaction of PCI with APC in plasma has been studied by several groups,24-26 and APC-PCI complexes25 and decreased PCI levels24,25 indicating PCI consumption has been shown in plasma from patients with disseminated intravascular coagulation (DIC). The physiologic role of PCI/PAI-3 as plasminogen activator inhibitor, however, has not been defined previously, although complexes between this inhibitor and urokinase have been demonstrated in normal human urine.27

Therefore, we developed an enzyme-linked immunosorbent assay (ELISA) system for uPA-PCI complexes, which also allowed the measurement of uPA-PCI complexes formed in plasma. By means of this assay we demonstrate here that uPA-PCI complexes are, in fact, formed during thrombolytic therapy.

Methods

Heparin sodium salt (about 140 U/mg; Fluka AG, Switzerland), S-2251 (Kabi, Sweden), horseradish peroxidase (Sigma, St. Louis), bovine serum albumin (BSA; Behringwerke, FRG), aprotinin (Bayer AG, FRG), and Protein A-Sepharose and CNBr-activated Sepharose-4B (Pharmacia, Sweden) were obtained as indicated. Urinary PCI was purified as described.28 Briefly, freshly voided human urine was collected on benzamidine (20 mmol/L final concentration) and incubated overnight with concanavalin A-(Con A) Sepharose (Pharmacia, Sweden) equilibrated in 0.05 mol/L Tris-HCl, 1.0 mol/L NaCl, 0.01% Tween 80, 20 mmol/L benzamidine, pH 7.4. The gel was washed with equilibration buffer and

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eluted with 0.6 mol/L α-methyl-D-mannoside in the same buffer. Eluted PCI-containing fractions were subjected without prior dialysis to monoclonal anti-uPA IgG (MPW5UK) Sepharose column, since none of the following purification steps removed contaminating plasminogen activator activity. Fallthrough fractions containing PCI activity but no plasminogen activator activity were dialyzed against 0.05 mol/L Tris-HCl, 0.2 mol/L NaCl, 0.01% Tween 80, pH = 7.4, and subjected to a heparin-Sepharose CL-6B column, equilibrated in the same buffer. The column was washed with equilibration buffer and eluted with a linear gradient between 0.2 and 0.8 mol/L NaCl in 0.05 mol/L Tris-HCl, 0.01% Tween 80, pH 7.4. PCI activity eluted at ~ 0.35 mol/L NaCl.

PCI-containing fractions were pooled, dialyzed against 20 mol/L Tris-HCl, 0.01% Tween 80, pH 7.8, and subjected to ion exchange chromatography on Q-Sepharose fast flow (Pharmacia, Sweden). Elution was performed with a linear gradient between 0 and 0.2 mol/L NaCl in the starting buffer and PCI eluted as the first protein from this column. All purification steps were performed at 4°C. Fractions were tested for PCI activity, as described. By this procedure PCI was purified to apparent homogeneity as judged from silver-stained sodium dodecyl sulfate (SDS) slab gels performed according to Lämmli. Urokinase was purchased from Serono (FRG). Its plasminogen activator activity was evaluated as described later by comparison with the international reference preparation obtained from the National Institute for Biological Standardization and Control, Holly Hill, UK and expressed as IU/mL. The urokinase preparation had a specific activity of 100,000 IU/mg. Polyclonal antibodies against plasma PCI were raised in rabbits as described previously, and the IgG fraction was purified by affinity chromatography on Protein A-Sepharose. PCI-immunodepleted plasma was prepared by treatment of pooled normal plasma with anti-PCI IgG coupled to CNBr-activated Sepharose-4B. Monoclonal antibody (MoAb) MPW5UK against uPA was obtained as described previously. Horseradish-peroxidase conjugation of goat anti-uPA IgG and MPW5UK was performed as described. Protein concentrations of PCI and urokinase were determined by the method of Lowry. The protein concentrations of the IgG preparations were calculated from A405 using an A405 of 14.

Blood from patients undergoing systemic urokinase therapy (1 × 10^6 IU/60 min IV) after myocardial infarction was collected on 20 mmol/L EDTA and 20 mmol/L benzamidine in intervals as indicated in the results section in Fig 1 before, during, and after urokinase infusion. The plasma obtained was stored in aliquots at -70°C until use. For control purposes plasma samples from one patient receiving systemic streptokinase (SK) therapy (1.5 × 10^6 U/60 min IV) were collected in the same way. All patients gave their informed consent to participate in the study, which was performed according to the Helsinki Agreement and which was approved by the local ethics committee. The normal plasma pool consisted of equal volumes of citrated plasma from 11 individual volunteers (six males, five females) and was stored in aliquots at -70°C until use.

**Combined assay for uPA activity and uPA antigen.** For quantitation of uPA activity and uPA antigen, a modification of an assay system described elsewhere was used. Briefly, microtiter plates (Microelsa, Greiner, Austria) were coated with a monoclonal anti-uPA antibody (MPW5UK; 10 μg/mL) in 0.01 mol/L Na carbonate buffer, pH 9.6. After blocking remaining binding sites with 1% BSA, uPA-containing samples supplemented with 20 mmol/L benzamidine, 20 mmol/L EDTA, 10 KIU/mL aprotinin were incubated with the immobilized antibodies for 90 minutes at 37°C. After washing the plates, bound uPA activity was quantified by using a mixture of plasminogen (0.5 μmol/L) and S-2251 (0.6 mol/mL) and comparing the amidolytic activity (A405) generated to a standard curve obtained with dilutions of purified urokinase (0.1 to 5 IU/mL), which had been previously calibrated with the international uPA standard. Whenever uPA antigen was determined from the same samples, the plates were washed, and bound uPA antigen was quantified using peroxidase-linked goat anti-uPA IgG (American Diagnostica Inc, USA; 7.5 μg/mL). Bound peroxidase activity was measured as described later for the

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**Fig 1.** uPA-PCI complexes in plasma samples from patients undergoing systemic urokinase therapy. Plasma samples from three patients receiving IV urokinase therapy (patients 1 to 3) and from one patient receiving streptokinase (SK) therapy (patient 4) were analyzed in the uPA-PCI complex ELISA. The final plasma concentration in the assay was 80%. The arrows indicate the start of the uPA (1 × 10^6 IU/80 min) or SK infusion (1.8 × 10^6 IU/80 min), respectively. Blood was collected before and in intervals after the start of the infusion into 20 mmol/L EDTA, 20 mmol/L benzamidine. Plasma samples obtained were made 10 KIU/mL in aprotinin. The uPA-PCI complex ELISA was performed as described in Materials andMethods. The relative uPA-PCI complex concentrations are given as A405 without correction for the respective plasma blank (see also y-intercept of the standard curve in Fig 6).
uPA-PCI complex ELISA. uPA antigen was determined using the same standard curve as for uPA activity and a specific activity of the purified uPA preparation of 100,000 IU/mg. When plasma samples from patients undergoing systemic urokinase therapy were tested in the uPA activity/antigen assay, they were supplemented with 10 KIU/mL aprotinin (final concentration) immediately after thawing. Each plasma sample was studied undiluted and after dilution (1:10, 1:20, and 1:40) in TBS, 1% BSA, 20 mmol/L benzamidine, 20 mmol/L EDTA, 10 KIU/mL aprotinin, pH 7.4. The buffer system used was TBS throughout the procedure, except for coating. Between each incubation step the plates were washed three times with TBS, 0.5% Tween 20, pH 7.4.

ELISA for uPA-PCI complexes. Microtiter plates (Microelisa) were coated with 100 µL rabbit anti-PCI IgG in 0.01 mol/L Na carbonate buffer, 0.02% Na azide, pH 9.6, overnight at 4°C. Remaining binding sites were blocked with 200 µL BSA (1%) in TBS, pH 7.4, for at least one hour at 37°C. Thereafter the plates were incubated with the samples for 90 minutes at 37°C. Samples were diluted as indicated in the results section either in TBS, 1% BSA, pH 7.4, or in plasma. Detection of bound uPA-PCI complexes was performed by incubation of the wells with 100 µL peroxidase-linked monoclonal anti-uPA IgG (MPW5UK, 5 µg/mL) for 90 minutes at 37°C. For quantitation of bound peroxidase, each well was incubated with 100 µL 0.1 mol/L Na2HPO4, 0.05 mol/L citric acid containing 1 mg/mL o-phenylenediaminidihydrochloride, and 0.03% H2O2. After ten minutes the reactions were stopped by addition of 100 µL 3N H2SO4 to each well, and A405 was determined using a Dynatech MR 600 Microplate reader. All A405 values above 1.0 were obtained after appropriate dilution of the respective sample and extrapolation of the photometer reading. Between each incubation step plates were washed three times with TBS, 0.5% Tween 20, pH 7.4.

Complex formation between uPA and PCI in vitro was studied in the following way: PCI-containing samples (ie, dilutions of plasma or purified urinary PCI, both in TBS, 1% BSA, pH 7.4) were incubated at 37°C with urokinase in the absence or presence of heparin directly on anti-PCI IgG-coated wells. This procedure was chosen for all in vitro studies after having ensured that similar amounts of uPA-PCI complexes were measured when PCI and uPA were preincubated either in the absence or in the presence of immobilized anti-PCI IgG. The reactions between uPA and PCI were stopped after different incubation times (0 to 60 minutes) by adding 10 µL benzamidine (20 mmol/L final concentration) to the preincubation mixture (final volume 100 µL). Whenever data for incubation times > 0 are given, 20 mmol/L benzamidine was added to the PCI-containing sample prior to the addition of uPA. Benzamidine, a reversible, competitive inhibitor of trypsinlike enzymes, has been shown previously to prevent interactions of urokinase with protease inhibitors in plasma. After the addition of benzamidine the samples were incubated for 90 minutes with the anti-PCI IgG-coated plate at 37°C.

Plasma samples from patients undergoing IV urokinase therapy after myocardial infarction were also studied. They were tested at a final plasma concentration of 80% and contained 20 mmol/L EDTA and 20 mmol/L benzamidine from the blood sampling on. Each plasma sample was supplemented with 10 KIU/mL aprotinin (final concentration) immediately after thawing, since these samples were also tested in the uPA activity/antigen assay, which routinely uses 10 KIU/mL aprotinin. Unless otherwise indicated, the relative uPA-PCI complex concentration present in plasma samples was given as A405. The following procedure was chosen whenever uPA-PCI complexes were quantified: Different concentrations of purified urinary PCI (0, 1.25, 2.5, and 5 µg/mL) were incubated with different concentrations of purified uPA (25, 50, and 100 IU/mL) in the presence of heparin (30 µg/mL). After 60 minutes at 37°C, 20 mmol/L benzamidine, 10 KIU/mL aprotinin, and 20 mmol/L EDTA (final concentrations) were added to each incubation mixture. An aliquot of each sample was diluted 1:40 in TBS, 20 mmol/L benzamidine, 20 mmol/L EDTA, 10 KIU/mL aprotinin, pH 7.4, and analyzed in the uPA activity assay. A second aliquot of each incubation mixture was analyzed in the uPA-PCI complex ELISA after dilution in normal plasma containing 20 mmol/L benzamidine, 20 mmol/L EDTA, 10 KIU/mL aprotinin to yield the same final plasma concentration as in the unknown plasma sample. From the uPA activity assay IU/mL uPA inhibited by PCI was calculated for each uPA concentration used as the difference in plasminogen activator activity after incubation with buffer and after incubation with PCI. Standard curves were obtained by plotting uPA inhibited by PCI as obtained from the uPA activity assay—v A405 obtained for the same sample in the uPA-PCI complex ELISA.

RESULTS

Figure 2 shows the results obtained in the uPA-PCI complex ELISA when purified urinary PCI was incubated with urokinase. Urokinase (0, 10, 50, or 100 IU/mL) was incubated with purified PCI (3 µg/mL) in the absence (○ △) or presence (● ▲) of heparin (30 µg/mL final concentration) at 37°C. The reactions were stopped either after 0 (○ ●) or after 20 minutes (△ ▲) by the addition of 20 mmol/L benzamidine (final concentration). Thereafter the samples were analyzed in the uPA-PCI complex ELISA using rabbit anti-PCI IgG as first antibody and a peroxidase-linked MoAb to uPA for antigen detection as described in Materials and Methods.
with purified uPA in the absence and presence of heparin. The amount of complex formed after 20-minute incubation increased with increasing urokinase concentrations, and higher complex concentrations were measured when PCI and uPA were incubated in the presence of heparin. Addition of 20 mmol/L benzamidine to PCI prior to incubation with uPA prevented complex formation. Time dependence of uPA-PCI complex formation in plasma is shown in Fig 3. As can be seen from the figure, the amount of complex formed increased with increasing incubation time. Also in plasma, higher complex concentrations were measured in the presence than in the absence of heparin. In the presence of heparin, half maximal complex formation was observed after five to ten minutes, maximal complex formation after ~30 minutes. The amount of uPA-PCI complex formed increased also with increasing plasma concentration, as seen in Fig 4A. In the presence of heparin, the highest uPA-PCI complex concentrations were determined for plasma concentrations between 3.5% and 7%. No complexes were seen when 20 mmol/L benzamidine was added to the plasma samples before the addition of uPA. When PCI-immunodepleted plasma was incubated with uPA either for 0 or for 20 minutes, the A405 measured at all plasma concentrations used never exceeded the buffer blank. Dependence of uPA-PCI complex formation on the urokinase concentration in diluted plasma is shown in Fig 4B. Addition of 20 mmol/L benzamidine to the diluted plasma before the addition of urokinase prevented complex formation completely at low uPA concentrations and to >85% at the highest uPA concentration used (1,000 U/mL final concentration). Complex formation between uPA and plasma PCI increased also with increasing heparin concentrations; using 4.4% plasma and 20-minute incubation time, maximal uPA-PCI complex concentrations were measured at heparin concentrations >3 µg/mL (Fig 5). No decrease in the stimulatory effect of heparin...
was observed by increasing the heparin concentrations up to 120 μg/mL.

As seen from the data shown in Figs 2 to 5, the ELISA is specific for uPA-PCI complexes. To determine whether or not PCI forms complexes with uPA in plasma not only in vitro but also in vivo, we studied plasma samples from patients undergoing uPA therapy in the uPA-PCI complex ELISA. These samples were used at a final plasma concentration of 80%. They contained 20 mmol/L benzamidine from the blood sampling on and were supplemented with 10 KIU/mL aprotinin. It has been shown previously using a combined uPA-activity/antigen assay that in concentrated plasma, 20 mmol/L benzamidine completely blocks ex vivo interactions between urokinase and plasma inhibitors up to uPA concentrations as measured during thrombolytic therapy. In Fig 1, results obtained in the uPA-PCI complex ELISA for plasma samples from three patients (patients 1 to 3) receiving urokinase therapy are shown. It can be seen from the figure that peaks of uPA-PCI complexes occurred in all patients after onset of the uPA infusion. For control purposes plasma samples from one patient receiving streptokinase therapy (patient 4) were also tested. At all time points A_490 values measured for these samples were in the same range as the A_490 values measured for the other patients’ plasma samples before onset of the therapy.

For quantification of uPA-PCI complexes, different concentrations of purified urokinase were incubated either with buffer or with different concentration of purified PCI for 60 minutes. An aliquot of each sample was analyzed in the uPA-PCI complex ELISA and another aliquot in the uPA activity assay. From the uPA activity assay, uPA inhibited by PCI was determined for each uPA and each PCI concentration as the difference in plasminogen activator activity between uPA incubated with buffer and the same concentration of uPA incubated with PCI. For analysis in the uPA-PCI complex ELISA, standards were diluted in plasma (final concentration 80%) containing 20 mmol/L EDTA, 20 mmol/L benzamidine, and 10 KIU/mL aprotinin to employ the same assay conditions for standards and patients’ samples. A standard curve was obtained by plotting uPA inhibited by PCI vs A_490 measured for the same sample in the uPA-PCI complex ELISA (Fig 6).

For one of the patients (patient 3) shown in Fig 1, the time course of the plasma concentrations of uPA antigen, inactive uPA antigen, and uPA in complex with PCI is shown in Fig 7. The concentration of inactive uPA antigen was calculated as the difference between uPA antigen concentration and uPA activity using a specific activity of 100,000 IU/mg for purified uPA. uPA in complex with PCI was calculated from the A_490 value measured for the respective plasma sample in the uPA-PCI complex ELISA using the standard curve shown in Fig 6 and a specific activity of 100,000 IU/mg purified uPA. In the patients’ plasma, obtained 30 and 90 minutes after the start of the uPA infusion, 30% and 57%, respectively, uPA antigen was inactive. uPA-PCI complexes

Fig 5. Heparin concentration dependence of uPA-PCI complex formation in plasma. Urokinase (100 IU/mL) was incubated with 4.4% plasma diluted in TBS, 1% BSA, pH 7.4, in the presence of different concentrations of heparin (0 to 15 μg/mL) for 0 (○) or 20 minutes (●) at 37°C. The complexes formed were measured as described in Materials and Methods.

Fig 6. Quantification of uPA-PCI complexes. Purified urinary PCI (0 to 5 μg/mL) was incubated with purified urokinase (25 to 100 IU/mL) for 60 minutes in the presence of heparin (30 μg/mL final concentration). Each incubation mixture was analyzed in parallel in the uPA-PCI complex ELISA and in the uPA activity assay as described in Materials and Methods. “uPA inhibited” was calculated as the difference in plasminogen activator activity between a certain uPA concentration incubated with buffer and incubated with PCI, respectively. “uPA-inhibited” was then plotted vs A_490 measured for the same sample in the uPA-PCI complex ELISA after dilution of each sample in plasma (80% final concentration) containing 20 mmol/L benzamidine and 10 KIU/mL aprotinin. The standard curve obtained was used to quantify uPA-PCI complexes measured in plasma.
The plasma concentration of PCI, on the other hand, is two orders of magnitude higher than that of PAL-I and PAL-2, respectively, in vitro. However, the reaction rate between urokinase and PCI is much lower than the reaction rates between urokinase and PAI-1 and PAI-2, respectively, even in the presence of stimulating heparin. The plasma concentration of PCI in plasma formed time-, dose-, and heparin-dependent complexes with urokinase. The urokinase concentrations used in these in vitro experiments (30 to 1,000 IU/mL) exceeded the inhibitory capacity of PAI-1 by far; however, they were in the same range as the uPA-plasma concentrations measured during uPA therapy (up to 1,200 ng/mL).

Interaction of urokinase with serine protease inhibitors in plasma has been studied by several authors, and it has been shown that besides PAI-1, mainly alpha-2 antiplasmin and antithrombin III play a role for uPA inhibition in plasma. Murano et al studied heparin-dependent inhibition of urokinase at high concentrations in plasma and in antithrombin III-depleted plasma. They suggested that heparin cofactors other than antithrombin III might contribute to uPA inhibition in plasma, since antithrombin III-depleted plasma still exhibited some residual heparin-dependent uPA inhibitory capacity. As seen from the data presented in this report, the interaction of uPA with PCI might explain this previous observation.

The data here demonstrate that complexes between plasma PCI and urokinase are also formed in vivo, as shown by studying plasma samples from patients undergoing systemic urokinase therapy. In all three patients studied, a considerable amount of uPA-PCI complexes was measurable during urokinase therapy. These complexes were quantified using a standard curve obtained by reacting purified uPA with purified PCI and measuring in parallel experiments uPA inhibition and uPA-PCI complexes in these standard samples. We calculated for one of the patients studied that 30% to 57% of the total uPA antigen (up to 1,200 ng/mL) present in plasma during the first 90 minutes after the start of uPA infusion was inactive. uPA-PCI complexes corresponded to approximately one fifth to one fourth of the inactive uPA antigen concentration. Assuming a PCI plasma concentration of 4 μg/mL, 2% to 5% of total plasma PCI would therefore participate in complex formation with uPA. These data suggest that PCI acts in fact as plasminogen activator inhibitor not only in vitro but also in vivo. PCI might therefore play a role for the in vivo regulation of uPA activity, at least when uPA is present in plasma in high concentrations, such as during thrombolytic therapy.

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