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 inhibitor 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 10^4 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.

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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.29 Briefly, freshly voided human urine was collected on benzamidine (20 mmol/L; final concentration) and incubated overnight with cancavalin 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 to 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 mmol/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 Laemmli. 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, Holtz 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 A_{490} using an A_{1%} 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 IU/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, 0.02% Na azide, 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 (0.66 mol/L) and comparing the amidolytic activity (A_{405}) 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 (1.5 × 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 and Methods. The relative uPA-PCI complex concentrations are given as A_{490} 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 NaH2PO4, 0.05 mol/L citric acid containing 1 mg/mL o-phenylenediamine dichloride, 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 time > 20 minutes 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 Arel. 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 Arel 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 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 A490 values measured for these samples were in the same range as the A490 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. A490 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 A490 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
The plasma concentration of PCI, on the other hand, is two
orders of magnitude higher than that of PAL-1,8,13
or PAL-3), described by Stump et al,14 are immunologically identical.16 PCI inhibits
activated protein C and urokinase with similar rate con-
stants,26,28 and in vitro-activated protein C and urokinase
compete for this PCI in plasma and urine.10 The reaction rate
between urokinase and PCI is, however, much lower than the
reaction rates between urokinase and PAI-1 and PAI-2,
respectively, even in the presence of stimulating heparin.13,23
The plasma concentration of PCI, on the other hand, is two
to three orders of magnitude higher than that of PAI-1,1,13 the
primary urokinase inhibitor in plasma. Therefore the
present study was performed, since PCI might play a role for
uPA inhibition in vivo in plasma, especially when high
urokinase concentrations are reached such as during throm-
bolitic therapy.

Using polyclonal anti-plasma PCI-IgG and a monoclonal
anti-uPA antibody, an ELISA was developed by us that was
specific for uPA-PCI complexes. In this assay system com-
plex formation between PCI and urokinase was demon-
strated in vitro in purified and complex systems. PCI in
plasma formed time-, dose-, and heparin-dependent com-
plexes with urokinase. The urokinase concentrations used in
these in vitro experiments (30 to 1,000 IU/mL) exceeded the
inhibitory capacity of PAI-11 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.11,13
Murano et al11 studied heparin-dependent inhibition of uro-
kinase at high concentrations in plasma and in antithrombin
III-depleted plasma. They suggested that heparin cofactors
other than antithrombin III might contribute to uPA inhibi-
tion 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 sys-
temic 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 corre-
sponded to approximately one fifth to one fourth of the
inactive uPA antigen concentration. Assuming a PCI plasma
inhibitory capacity of by far; however, they were in
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