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An Inhibitor of Activated Thrombin-Activatable Fibrinolysis Inhibitor Potentiates Tissue-Type Plasminogen Activator-Induced Thrombolysis in a Rabbit Jugular Vein Thrombolysis Model☆

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Abstract

When activated in vitro, thrombin-activatable fibrinolysis inhibitor (TAFI) slows clot lysis by cleaving the C-terminal lysine and arginine residues from partially degraded fibrin. An inhibitor of carboxypeptidase isolated from potato (CPI) reverses prolongation of clot lysis by inhibiting activated TAFI. We investigated in vivo effect of TAFI inhibition on tissue-type plasminogen activator (t-PA)-induced clot lysis using CPI in a rabbit jugular vein thrombolysis model. It was found necessary to further purify the CPI preparations from commercial sources by HPLC chromatography to remove endotoxin and anti-plasmin activity that would affect the endogenous fibrinolytic system. The effect of intravenous administration of the purified CPI with t-PA was determined by measuring thrombus weight at the end of 90 minutes in six groups of animals. In the control group receiving saline, the median thrombus weight was 116 mg. In the group that received CPI only (0.5 mg/kg bolus injection followed by 0.3 mg/kg/h infusion), the median thrombus weight was 121 mg. In the group that received t-PA at a dose of 10 μg/kg bolus followed by 67 μg/kg/h infusion, the median thrombus weight decreased to 86 mg. When CPI was coadministered with the same regimen of t-PA, the median value further decreased to 58 mg. When animals were given three times higher the dose of t-PA (30 μg/kg bolus followed by 200 μg/kg/h infusion) in the absence or presence of CPI, median thrombus weights were 56 mg and 0 mg, respectively. Our results demonstrate that systemic coadministration of the purified CPI improves clot lysis induced by t-PA.

Key Words: TAFI; Thrombolysis; Potato carboxypeptidase inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI) (EC 3.4.17.20), also known as plasma procarboxypeptidase B or procarboxypeptidase U, is a 60-kDa glycoprotein that

Abbreviations: TAFI, thrombin-activatable fibrinolysis inhibitor; CPI, carboxypeptidase inhibitor from potato; t-PA, tissue-type plasminogen activator; PAI-1, plasma plasminogen activating inhibitor-1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; FA-Ala-Arg, furylacryloyl-alanyl-arginine; PPACK, D-Phe-Pro-Arg chloromethylketone; HEPES, *N*-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]; PPP, platelet-poor plasma.

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circulates in plasma. It was originally described as a novel arginine carboxypeptidase generated in serum following coagulation [1,2]. The protein sequence was deduced from its cDNA sequence and indicated high homology to pancreatic carboxypeptidase B [3]. TAFI is activated in vitro by high concentrations of trypsin, thrombin, or plasmin to a zinc metalloprotease that hydrolyzes synthetic and natural peptides with C-terminal arginines and lysines [3–7]. The physiological activator of TAFI is likely a thrombin–thrombomodulin complex, since thrombomodulin enhances the catalytic efficiency of thrombin to activate TAFI by a factor of 1250, almost exclusively through its effect on k_{cat} [8–12].

Activated TAFI prolongs clot lysis time in vitro [5,8,10,11]. This effect is through the removal of C-terminal lysine and arginine residues that are newly exposed during partial degradation of fibrin clots by plasmin. This would reduce further binding of plasminogen as well as tissue-type plasminogen activator (t-PA) to these C-terminal basic residues in thrombus. The overall result is reduced plasmin production at the site of clots, which is dose-dependent with the half maximal effect obtained at activated TAFI concentration of around 1 nM. Since the concentration of circulating TAFI is over 50 nM, a sufficient amount of active enzyme can be generated in plasma following activation of a small percent of circulating TAFI to play a critical role in the regulation of endogenous fibrinolysis. In healthy individuals, a correlation between TAFI antigen level and in vitro clot lysis time has been observed [13]. The prolongation of clot lysis time is abolished when the activity of TAFI is inhibited with general inhibitors of carboxypeptidases such as guanidinoethylmercaptosuccinic acid and CPI.

Recently, various groups have reported in vivo studies demonstrating that inhibition of activated TAFI enhances endogenous fibrinolysis as well as t-PA-induced thrombolysis in animal models [14,15]. In these experiments, inhibition of TAFI activity was achieved with CPI preparations obtained from commercial sources. It has been known that some preparations of CPI are contaminated with anti-plasmin activity and endotoxin, both of which can potentially affect the endogenous fibrinolytic system. Anti-plasmin activity would slow down clot lysis by blocking plasmin activity, whereas endotoxin would increase the plasma plasminogen activating inhibitor-1 (PAI-1) level and reduce t-PA activity [16–18], thereby masking the real effect of CPI on thrombolysis. Furthermore, an altered level of arginine carboxypeptidase activity has been seen in rats following an intravenous injection of endotoxin [19]. Therefore, in the current study we have further purified CPI preparations to remove the contaminants, and tested the hypothesis that inhibition of activated TAFI with purified CPI will enhance t-PA-induced thrombolysis in a rabbit jugular vein thrombolysis model.

1. Materials and Methods

1.1. Materials

Thromboplastin-HS with calcium, human plasmin, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), and D-Val-Leu-Lys-p-nitroanilide were purchased from Sigma (St. Louis, MO, USA). D-Phe-Pro-Arg chloromethylketone (PPACK) was from Calbiochem-Novabiochem Corporation (San Diego, CA, USA). Furylacryloyl-alanyl-arginine (FA-Ala-Arg) was from Bachem Biosciences Inc. (Bubendorf, Switzerland). CPI and pancreatic carboxypeptidase B (EC 3.4.17.2) were obtained from Sigma and Calbiochem-Novabiochem Corporation. C4 resin was from VYDAC (Hesperia, CA, USA). ^{125}I-labeled human fibrinogen was obtained from Amersham Life Science Inc. (Arlington Heights, IL, USA). Activase® (recombinant t-PA, 580,000 IU/mg) was purchased from Genentech Inc. (South San Francisco, CA, USA). Human thrombin (3000 NIH units/mg) was obtained from either Sigma or American Diagnostica (Greenwich, CT, USA). Limulus Amebocyte Lysate test kit was from Bio-Whittaker Inc. (Walkersville, MD, USA). Human thrombomodulin and TAFI were purified as described previously [20,21].

1.2. Purification of CPI

CPI preparations from two commercial sources were dissolved in Solvent A (0.1% trifluoroacetic acid in water) to give a final protein concentration of 5 mg/ml. The protein solution was loaded onto a reverse phase HPLC column (C4, 15–20 micron, 2.2×25 cm, equilibrated with Solvent A) at a flow

rate of 10 ml/min. The column was washed with Solvent A for 30 minutes followed by 20% Solvent B (0.1% trifluoroacetic acid in acetonitrile) for 25 minutes. CPI was eluted with a gradient of 20–40% Solvent B in 40 minutes, and absorbance at 280 nm was monitored. The column was further washed with 100% Solvent B to remove any other bound proteins. The protein peak corresponding to the purified CPI was lyophilized to remove solvents. The lyophilized samples were then resuspended in saline to determine carboxypeptidase inhibitory, endotoxin, and anti-plasmin activities. The level of endotoxin was determined using a standard Limulus Amebocyte Lysate test (BioWhittaker Inc.), and enzymatic activities were measured as described below. The purity of CPI was analyzed by HPLC, and the quantitation of CPI was based on amino acid analysis.

1.3. Measurement of CPI Inhibitory Activity

Inhibitory activity of CPI before and after HPLC chromatography was determined against activated TAFI using FA-Ala-Arg as a substrate. The quantitation of CPI before HPLC purification was done using Pierce BCA (Bicinchoninic Acid) protein assay, with purified CPI as a standard. TAFI was activated with 10 nM thrombin and 50 nM thrombomodulin for 10 minutes at room temperature in 20 mM *N*-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES) (pH 7.4) with 150 mM NaCl and 5 mM $CaCl_2$. The activation was stopped by addition of 1 μM PPACK. Activated enzyme (5 nM) was preincubated with various concentrations of CPI for 5 minutes prior to incubation with 0.4 mM FA-Ala-Arg in 20 mM Tris (pH 7.8) with 75 mM NaCl and 0.1% (w/v) CHAPS. The rate of hydrolysis of FA-Ala-Arg was determined by measuring absorbance at 336 nm every 20 seconds for 10 minutes.

The level of CPI in plasma samples was determined after dilution (1:3) with phosphate-buffered saline, and 1.2 nM pancreatic carboxypeptidase B (which is more stable than activated TAFI) was added. The remaining carboxypeptidase activity was measured with FA-Ala-Arg as a substrate and as described above for activated TAFI. A standard curve for CPI was constructed by spiking the plasma sample taken at time 0 with the same amount of pancreatic carboxypeptidase B and various concentrations of CPI in a range between 0.1 and 3.2 μg/ml.

1.4. Measurement of Anti-Plasmin Activity

Anti-plasmin activity in the CPI preparations was determined by preincubating plasmin (200 mU/ml) with various amounts of CPI for 5 minutes, and then the remaining plasmin activity was measured with a substrate, D-Val-Leu-Lys-p-nitroanilide (1.0 mM), in 50 mM Tris (pH 7.5) with 100 mM NaCl and 0.025% PEG6000. The rate of hydrolysis was monitored at 405 nm every 30 seconds for 10 minutes.

Anti-plasmin activity in plasma samples was measured by incubating diluted samples with excess amounts of plasmin for 5 minutes, and the remaining plasmin activity was assayed as above and compared to time 0 samples.

1.5. In Vitro Plasma Clot Lysis

Rabbit platelet-poor plasma (PPP) was obtained by centrifugation of citrated blood at 3000×*g* for 15 minutes at room temperature. In a 96-well microtitre plate, 30 μl of citrated plasma was mixed with 3 μl of 2 μM thrombomodulin and 17 μl of various concentrations of CPI and 60 μl of assay buffer (40 mM HEPES, pH 7.0, 150 mM NaCl and 0.01% Tween 80 v/v). The mixture was immediately added to another well containing 4 μl of 75 NIH units/ml thrombin, 2 μl of 1 M $CaCl_2$, and 4 μl of Activase® in a range between 0.9 and 2.1 μg/ml in separate aliquots. The total volume of the mixture was 120 μl. After mixing, the absorbance at 405 nm was measured at 37°C every minute using a SpectraMAX 250 Microplate Spectrophotometer (Molecular Device Corporation, Sunnyvale, CA, USA). Lysis time was defined as the time at which the absorbance is one-half of the difference between the plateau reached after clotting and the base-line value achieved at complete lysis.

1.6. Rabbit Jugular Vein Thrombolysis Model

A rabbit jugular vein thrombolysis model was set up as described originally by Collen et al. [22], with

some modifications. New Zealand white rabbits (~2.5 kg body weight) were anaesthetized with a mixture of 5–8% isofluorane in oxygen and maintained under 0.5–2.5% isofluorane in oxygen. The facial vein and both the right and left marginal ear vein were cannulated for delivery of citrated whole blood, intravenous infusion of test compounds, and collection of blood samples, respectively. A thrombotic occlusion was introduced by injecting a mixture of 300 μl autologous citrated whole blood (9:1 with 3.8% sodium citrate) and 80 μl thromboplastin with Ca^{2+} into a 2-cm isolated segment of the jugular vein via the cannula in the facial vein. After 10 minutes, a cotton thread was inserted through the thrombus to hold it in place. The thrombus was matured for 30 minutes prior to reestablishing blood flow and starting the infusion of test compounds. Blood samples were collected in 3.8% citrate (9:1, v/v) and PPACK (1 μM) prior to thrombus formation (denoted as time 0 sample), and 1, 10, 30, 60 and 90 minutes after initiation of compound administration to measure the level of CPI and α2-antiplasmin activity. At the end of 90 minutes, the thrombus was removed for wet weight measurement. All animals received care in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care International. The protocol was approved by the institutional Animal Care and Use Committee (Berlex Biosciences, Richmond, CA, USA).

Experimental animals were divided into six groups. Group 1 (n=14) received saline only. Group 2 (n=10) received CPI only (0.5 mg/kg bolus injection followed by 0.3 mg/kg/h infusion). Group 3 (n=12) received t-PA at a dose of 10 μg/kg bolus injection followed by 67 μg/kg/h infusion. Group 4 (n=12) received t-PA (same regimen as group 3) and CPI (same regimen as group 2). Group 5 (n=12) received a higher dose of t-PA of 30 μg/kg bolus injection followed by 200 μg/kg/hr infusion. And group 6 (n=7) received t-PA (same regimen as group 5) and CPI (same regimen as group 2).

1.7. Statistical Analysis

Statistical analysis was performed using the nonparametric Kruskal–Wallis one-way analysis of variance followed by the Mann–Whitney *U*-test.

2. Results

2.1. Purification of CPI

CPI obtained from either Sigma or Calbiochem-Novabiochem was further purified by HPLC chromatography. A typical absorbance profile at 280 nm exhibited one major peak with a number of minor peaks (Figure 1). Determination of the inhibitory activity against carboxypeptidase B showed that the major peak, which eluted at 27–36% acetonitrile, contained the inhibitory activity. The other peaks had no CPI activity. Fractions containing the major protein peak were pooled and lyophilized. Based on quantitation from amino acid analysis, the recovery of CPI was over 95%.

When the specific inhibitory activity of the purified CPI was compared to that of the original sample, there was no loss of activity (Figure 2). IC_{50} measured against activated TAFI was 0.35 μg/ml for CPI before and 0.44 μg/ml after HPLC chromatography. The level of endotoxin, however, was reduced over 4000-fold, from 2500 EU per mg to 0.6 EU per mg of CPI. Furthermore, HPLC chromatography of the CPI preparation also removed anti-plasmin activity (Figure 3). While the origin of anti-plasmin activity present in the CPI prepara-

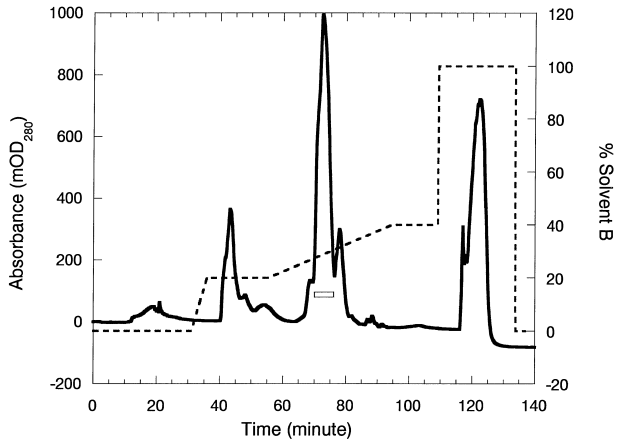

Fig. 1. HPLC chromatography of CPI. CPI preparations from either Sigma or Calbiochem-Novabiochem Corporation were dissolved in 0.1% trifluoroacetic acid in water and loaded onto an HPLC column. CPI was eluted with a gradient of 20–40% Solvent B (0.1% trifluoroacetic acid in acetonitrile). Absorbance at 280 nm is shown (solid line) and percent acetonitrile is given (dashed line). The dashed box indicates pooled fractions that contained the purified CPI.

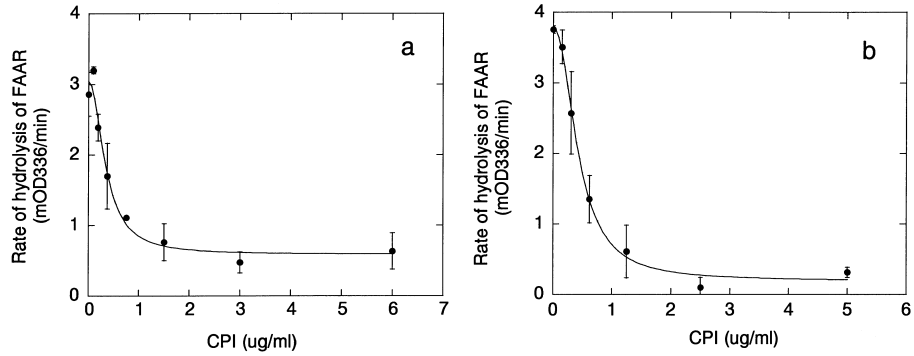

Fig. 2. IC_{50} determination for CPI before and after HPLC chromatography. Inhibitory activity of CPI was determined with activated TAFI as described in the Materials and Methods section. The rate of hydrolysis of FA-Ala-Arg (mOD at 336 nm/min) is plotted against CPI before (a) and after (b) HPLC chromatography. IC_{50} was calculated based on Michaels–Menten kinetics of competitive inhibition.

tions obtained from commercial sources is unknown, it is not inherent in CPI, as it can be purified away from CPI.

2.2. In Vitro Effect of Purified CPI on Clot Lysis

In vitro effects of the purified CPI on clot lysis were determined in rabbit plasma using a microtitre-plate clot lysis assay. Addition of thrombin and thrombomodulin to rabbit PPP caused 2- to 2.5-fold prolongation of clot lysis time induced by t-PA. This was likely due to the activation of rabbit TAFI, since purified CPI reversed this prolongation in a dose-dependent manner (Figure 4). IC_{50} of CPI was determined to be about 0.2 μg/ml in rabbit plasma at a t-PA concentration of 0.067 μg/ml. Inhibition

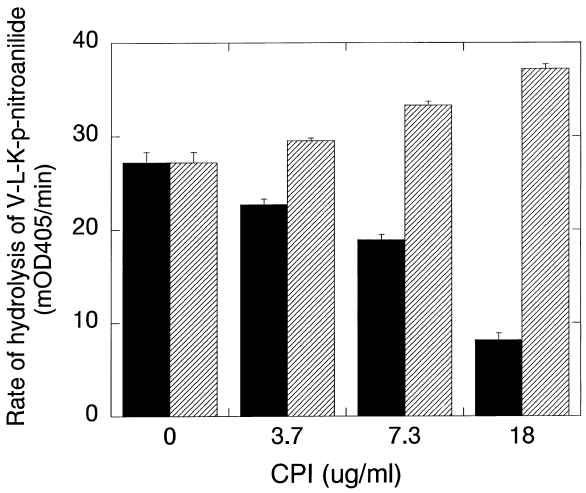

Fig. 3. Anti-plasmin activity in CPI preparations. Anti-plasmin activity was determined by preincubating plasmin with CPI before (solid bar) and after (crosshatched bar) HPLC chromatography, followed by measuring the remaining plasmin activity with 1.0 mM D-Val-Leu-Lys-p-nitroanilide. The rate of hydrolysis of the substrate (mOD at 405 nm/min) is plotted against various concentrations of CPI (n=3 for each concentration).

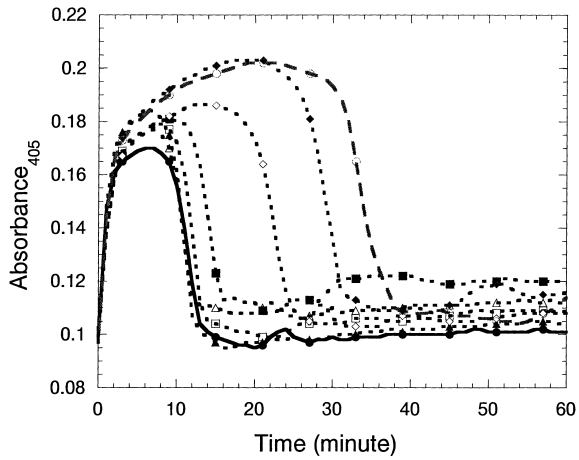

Fig. 4. CPI dose-response in plasma clot lysis. The lysis profile was obtained as described in the Materials and Methods section; in the absence of thrombomodulin and CPI (solid line with closed circle); in the presence of thrombomodulin and absence of CPI (dashed line with open circle); and in the presence of thrombomodulin and various concentrations of the purified CPI (dotted lines). The concentrations of CPI tested were 3.4 μg/ml (open triangle), 1.7 μg/ml (closed triangle), 0.85 μg/ml (open square), 0.43 μg/ml (closed square), 0.21 μg/ml (open diamond), and 0.11 μg/ml (closed diamond).

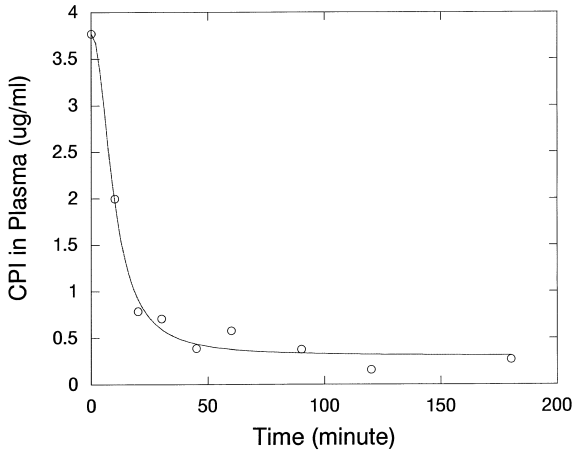

Fig. 5. Plasma clearance of CPI in rabbit. The purified CPI (1 mg/kg body weight) was administered by intravenous bolus injection into two anaesthetized rabbits. Citrated plasma samples were collected at times 0, 1, 10, 20, 30, 45, 60, 90, 120, and 180 minutes, and the plasma concentration of CPI was measured as described in Materials and Methods section. The plasma clearance profiles were similar in both animals.

of TAFI activity with the purified CPI, therefore, enhanced t-PA-induced clot lysis in vitro.

Plasma contains another basic carboxypeptidase, carboxypeptidase N, which also hydrolyzes a small-molecule substrate such as FA-Ala-Arg. In contrast to TAFI, carboxypeptidase N is constitutively active and does not require activation. However, we, as well as others, have shown that carboxypeptidase N is not inhibited by CPI [23]. In the absence of exogenous thrombomodulin, there was no pronounced effect of CPI on clot lysis time (data not shown), indicating that there is no significant activation of TAFI in normal plasma.

2.3. In Vivo Effect of Systemic Administration of the Purified CPI on t-PA-Induced Thrombolysis

In vivo effects of the purified CPI on clot lysis were investigated in a rabbit jugular vein thrombolysis model by measuring the wet weight of the thrombus after a 90-minute infusion of test compounds. To determine the dosing regimen of CPI, the plasma clearance rate was measured following a bolus injection of 1 mg/kg of the purified CPI in two rabbits (Figure 5). The half-life of CPI in plasma was estimated to be around 10 minutes in both rabbits. Based on the plasma half-life of CPI, the following dosing regimen of CPI was decided: a bolus injection (0.5 mg/kg), followed by an infusion (0.3 mg/kg/hr) for 90 minutes.

In preliminary studies, a thrombus was formed in the presence of 2 μCi ^{125}I-labeled human fibrinogen, and the extent of thrombolysis determined based on the radioactivity in the thrombus was compared with the thrombus weight. We established a good correlation between the thrombus weight and the percentage of radioactivity remaining in the thrombus, as shown in Figure 6. Therefore, the study was carried out without the use of radioactivity, and the thrombus weight was used as an endpoint in the subsequent experiments.

The results were analyzed using the Mann–Whitney *U*-test and are shown in Figure 7. The median values of thrombus weight obtained for saline group (n=14), t-PA at a medium dose (n=12), and t-PA at a higher dose (n=12) were 116 mg, 86 mg, and 56 mg, respectively, demonstrating that the extent of clot lysis was dependent on the concentration of t-PA. When CPI was coadministered with the medium dose of t-PA (n=12), the thrombus weight was reduced significantly from 86 to 58 mg (p<0.007). Since CPI was given as an intravenous bolus injection, followed by an infusion, the actual plasma level of CPI determined was found to be greater than or equal to 0.7 μg/ml in most of the animals that received CPI. When CPI was used with the higher dose of t-PA (n=7), the median value also decreased from 56 to 0 mg. It is noteworthy that the extent of clot lysis obtained with the medium dose of t-PA administered together with CPI was similar to that achieved with a threefold higher dose of t-PA given alone. Thus, the systemic administration of CPI improved the potency of t-PA by threefold. In this model, CPI alone (n=10) did not enhance endogenous fibrinolysis, as the median value of the thrombus weight was 121 mg compared to 116 mg in saline group.

Systemic activation of plasminogen by t-PA was determined by measuring the plasma level of α2-anti-plasmin activity in animals. It has previously been shown that the C-terminal lysine residues of α2-antiplasmin are important for its interaction with plasmin, and pretreatment of α2-anti-plasmin with pancreatic carboxypeptidase B leads to a loss of its ability to rapidly inhibit plasmin [24]. Inhibition of TAFI activity with CPI may protect α2-anti-

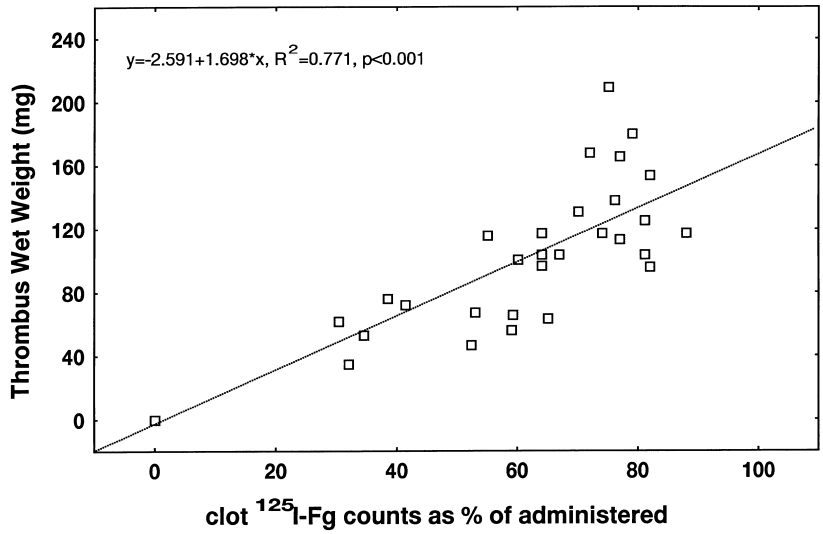

Fig. 6. Correlation between thrombus wet weight and clot count. ^{125}I-labeled thrombus was formed in the jugular vein by injecting a mixture of 300 μl citrated autologous whole blood, 80 μl thromboplastin with Ca^{2+}, and 2 μCi ^{125}I-labeled human fibrinogen (^{125}I-Fg). The amount of radioactivity administered was calculated by subtracting the radioactivity remaining in the syringe and the catheter, and the radioactivity found in the blood 1 minute after initiation of compound administration (assuming a blood volume of 60 ml/kg body weight), from the original amount of radioactivity in the syringe. At the end of 90 minutes, the thrombus was removed from the vessel for wet weight and radioactivity count. Clot ^{125}I-Fg counts were expressed as percentage of counts administered.

plasmin, thus leading to a reduced consumption of circulating α2-anti-plasmin. However, no significant decrease in plasma α2-anti-plasmin activity was observed in any of the groups (data not shown). This is probably due to the fact that the concentrations of t-PA used here were too low to cause major systemic activation of plasminogen.

3. Discussion

The fibrinolytic system is critical in removing clots from circulation and maintaining patency [25–29]. The first step in fibrinolysis is generation of a limited amount of plasmin from plasminogen by t-PA. Once initiated, fibrinolysis accelerates by a mechanism that utilizes newly exposed C-terminal basic residues in fibrin chains resulting from the partial degradation of fibrin. These C-terminal basic residues are sites that bind more plasminogen and plasminogen activator, leading to amplification of plasmin production [30,31]. When TAFI is activated during fibrinolysis in vitro, it catalyzes the removal of these C-terminal basic residues, significantly limiting plasmin production on the clot surface [10]. The net result of TAFI activation is an inhibition of clot lysis, providing a natural regulator of fibrinolysis.

It has been demonstrated that in vitro clots formed from plasma deficient in coagulation factors VIII, IX, X, or XI were lysed by t-PA prematurely [32–34]. When these plasma samples were supplemented with the missing factor, which results in a generation of a large amount of thrombin, or with an addition of thrombomodulin, which enhances activation of TAFI by thrombin, premature lysis of the clots was prevented. Thrombin also activates factor XIII, plasma transglutaminase, to XIIIa, which catalyzed intramolecular cross-linking of fibrin chains as well as intermolecular cross-linking of α2-anti-plasmin to fibrin, thereby rendering clots more resistant to lysis [35,36]. However, reduced activation of TAFI, and not factor XIIIa, was the main cause of premature lysis, since pretreatment of clots with antibody to TAFI reversed the protection of clots from premature lysis by the missing factor or thrombomodulin. Furthermore, thrombomodulin inhibits activation of factor XIII by thrombin [37]. These data indicate that activation of TAFI facilitates the stabilization of clots from premature lysis.

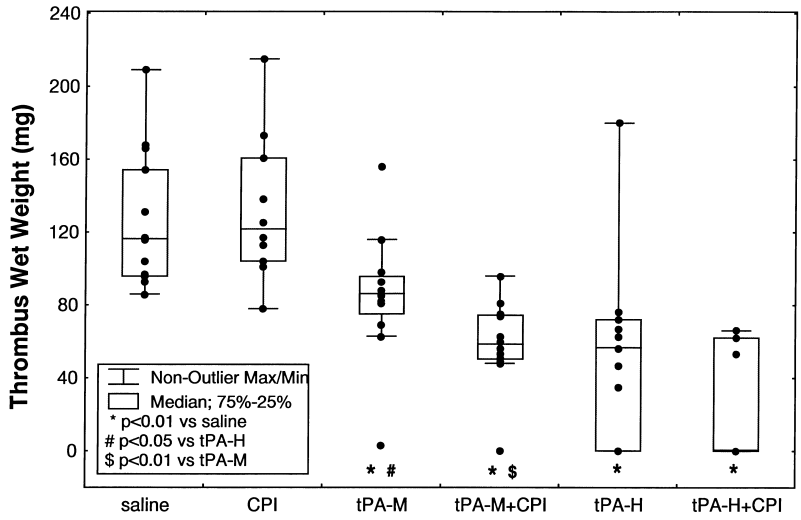

Fig. 7. Effect of the purified CPI on thrombolysis in rabbit. A thrombus was formed in the jugular vein as described in Materials and Methods section. The effect of intravenous coadministration of CPI (0.5 mg/kg bolus injection followed by 0.3 mg/kg/h infusion) with t-PA was investigated by measuring thrombus weight at the end of 90 minutes infusion. Doses of t-PA used were as follows: M, 10 μg/kg bolus injection followed by 67 μg/kg/h infusion; H, 30 μg/kg bolus injection followed by 200 μg/kg/h infusion. Statistical analysis was performed using the nonparametric Kruskal–Wallis one-way analysis of variance followed by the Mann–Whitney *U*-test. Each dot represents individual animals. The median value is indicated by the middle horizontal line in each case, except for tPA-H plus CPI, where it falls on 0. Non-outlier (individual values that lie within twice the size of the box away from the median) maximum and minimum values are also displayed.

In the current study, we extended in vitro observations on the effect of activated TAFI to investigate whether thrombolysis could be enhanced in vivo by inhibiting TAFI activity. It has recently been reported that CPI enhanced t-PA-induced clot lysis of thrombus formed in an arterio-venous shunt and in an abdominal aorta [15,38].[1] However, in these studies, CPI from commercial sources was used without further purification. We detected a large amount of endotoxin in CPI preparations, which is not surprising, since CPI was isolated from potato tuber. Endotoxin can trigger an altered endogenous fibrinolytic state within 2 hours of its administration in a number of different species [16–18]. For example, intravenous infusion of 20 μg/kg/h of endotoxin (about a fivefold higher dose than that found in CPI preparations before purification) into rabbits for 6 hours resulted not only in an increased plasma level of PAI-1 but also in the death of some animals. In rats, endotoxin administration was associated with an altered plasma level of carboxypeptidase B activity [19]. Therefore, we purified CPI preparations to ensure that endotoxin would not influence endogenous fibrinolysis in our studies. The HPLC chromatography also removed anti-plasmin activity from CPI preparations obtained from two different commercial sources. Without purification, the amount of anti-plasmin activity that would have been injected might potentially result in a 30% reduction in plasmin activity (assuming 60 ml of blood per kg body weight, then a bolus injection of 500 μg/kg of CPI solution would be diluted to ~8 μg/ml). The level of anti-plasmin activity would decrease as it was cleared, but its half-life is unknown. Thus, purification of CPI was warranted to ensure that our results were interpretable.

In our model, a thrombus was formed in situ in contact with endothelium. In this setting, physiological interaction with thrombomodulin and other vessel wall components can take place. Our results demonstrated that the systemic coadministration of purified CPI significantly increased the thrombolytic potency of t-PA. This data confirms our hypothesis that TAFI is activated in vivo and plays a regulatory role in fibrinolysis. We did not observe

[1] It should be noted that reference 38 appeared as a full paper in an October 15, 1999, issue of Blood after submission of the present manuscript.

any effect of CPI on endogenous fibrinolysis in the absence of t-PA administration, as had been shown previously [14]. Although these studies were carried out in the same jugular vein thrombolysis model, Minnema et al. [14] used a higher concentration of CPI (20 μg/ml) and CPI was incorporated into the clots at the time of clot formation. Such differences might account for the failure to observe an acceleration of endogenous fibrinolysis in the current study. In conclusion, an inhibitor of TAFI activity enhances the thrombolytic efficacy of t-PA and may potentially be beneficial as an adjunct to thrombolytic therapy.

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