Aspirin Inhibits Vascular Plasminogen Activator Activity In Vivo

Studies Utilizing a New Assay to Quantify Plasminogen Activator Activity

Richard I. Levin, Peter C. Harpel, Deborah Weil, Tsun-San Chang, and Daniel B. Rifkin

Division of Cardiology, Department of Medicine, Department of Cell Biology, New York University School of Medicine, New York 10016; Division of Hematology-Oncology, Department of Medicine and the Specialized Center of Research in Thrombosis, The New York Hospital-Cornell Medical Center, New York 10021

Abstract. Vascular or tissue-type plasminogen activator (TPA) is a key enzyme in physiologic fibrinolysis. To study the role of prostaglandins in modulating the synthesis and release of TPA in vivo, we prospectively studied the effect of aspirin (650 mg/d × 2) on TPA activity in 13 human subjects before and after 10 min of forearm venous occlusion. TPA activity was quantified by a newly developed enzyme-linked immunosorbent assay that both measures and differentiates between TPA and urokinase (UK)-like plasminogen activator activity. This assay is based on the observation that the concentration of α_2-plasmin inhibitor-plasmin complexes in Reptilase-clotted plasma increases linearly in proportion to the amount of activator added. Resting TPA activity was higher in women than in men (0.56±0.59 vs. 0.15±0.11 U/ml, $P = 0.049$). Venous occlusion induced an eightfold rise in TPA activity in women (to 4.5 U/ml, $P = 0.006$) and a 15-fold rise in men (to 2.28 U/ml, $P = 0.004$), whereas UK activity was not detected. Aspirin inhibited the rise in TPA activity after venous occlusion by 69% in men ($P = 0.004$) and 70% in women ($P = 0.014$). In contrast, aspirin had no effect on pre- or post-occlusion hematocrits or Factor VIII-related antigen levels. There was no correlation between plasma salicylate level and percentage inhibition of TPA. Neither exogenous aspirin (0–1 μg/ml) nor salicylate (0–70 μg/ml) inhibited the generation of α_2-plasmin inhibitor-plasmin complexes by exogenous TPA or interfered with the assay system. We conclude that aspirin may have an antifibrinolytic effect in man that has not been previously described.

Introduction

The ability of the vascular endothelium to synthesize and release tissue or vascular plasminogen activator (TPA)[1] may be the major determinant of physiologic fibrinolysis (1). The factors that modulate TPA synthesis and release by the blood vessel wall in vivo, however, are largely unknown. Several studies suggest that prostacyclin may induce an increase in fibrinolysis, perhaps due to vascular plasminogen activator release (2–4). Further, Crutchely et al. (5, 6) have shown that arachidonate metabolites modulate the production of TPA by a variety of cells in tissue culture. Since aspirin inhibits prostaglandin synthetase (7, 8) and the production of prostacyclin by the blood vessel wall (9), we prospectively investigated the effect of aspirin on plasminogen activator activity in normal individuals before and after venous occlusion by using a new assay for plasminogen activator activity in vivo.

Methods for the assessment of TPA activity in blood have depended upon euglobulin fractionation of plasma, a method that is nonspecific and depends upon the variable precipitation of fibrinogen, plasminogen, and plasminogen activators (10, 11). To circumvent this problem, several new methods have been developed. These include lysine affinity chromatography

Address reprint requests to Dr. Levin, Division of Cardiology, Department of Medicine, New York University School of Medicine. Dr. Levin is the recipient of a National Institutes of Health Clinical Investigator Award (5 K08 HL00748). This work was done while Dr. Levin was a Visiting Investigator at Cornell University Medical College.

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1. *Abbreviations used in this paper:* ANOVA, analysis of variance; EACA-PBS-Tween, PBS containing Tween and epsilon amino caproic acid; ELISA, enzyme-linked, double antibody, immunosorbent assay; PBS-Tween, PBS containing Tween; TPA, tissue plasminogen activator; UK, urokinase; VIII:Rag, Factor VIII-related antigen.

to purify TPA from plasma (12) and an immunoradiometric assay for TPA that measures both active and inactive, inhibitor-bound plasminogen activator (13). We have recently developed an enzyme-linked, double antibody, immunosorbent assay (ELISA) that quantifies the complexes formed between plasmin and its plasma inhibitors, α_2-plasmin inhibitor and α_2-macroglobulin (14). Using this assay we have documented that α_2-plasmin inhibitor-plasmin complexes are formed in vivo. Elevated concentrations of these complexes have been identified in the blood of individuals with disseminated intravascular coagulation (14, 15) after UK infusion (14), and in patients with solid tumors without evidence for intravascular coagulation (16).

The sensitivity of this ELISA led us to believe that assay of the formation of plasmin inhibitor-plasmin complexes under appropriate conditions might provide a new approach for the quantification in plasma of functional TPA activity. In the present study we have determined that measurement of these complexes in clotted plasma provides a sensitive and quantitative measure of TPA activity. Our findings indicate that ingestion of 650 mg of aspirin on each of 2 d before venous occlusion results in a marked inhibition of TPA activity. Therefore aspirin may have an antifibrinolytic effect in man not previously appreciated.

Methods

Purification of TPA and of plasma proteins. Human melanoma cell TPA was prepared by minor modifications of previously described methods (17). The specific activity of the final product was 100,000 U/mg. The activator was stored at a concentration of 1,550 U/ml at −70°C. The fibrinolytic activity of the melanoma TPA was determined by the ^{125}I-fibrin plate assay as detailed previously (18) and standardized by comparison with urokinase (UK) (Abbokinase; gift of Abbott Laboratories, Chicago, IL). Antisera were raised in rabbits by using melanoma plasminogen activator that was purified by electrophoresis on a preparative 9% polyacrylamide gel under nonreducing conditions (19).

α_2-Plasmin inhibitor was purified as previously detailed (20). Plasminogen was isolated from fresh plasma by lysine affinity chromatography (21) in the presence of soybean trypsin inhibitor (100 μg/ml plasma, Worthington Biochemical Corp., Freehold, NJ), which was followed by gel filtration chromatography (Bio-gel A-0.5m, Bio-Rad Laboratories, Richmond, CA). The concentration of each protein was determined by its extinction coefficient (α_2-plasmin inhibitor = 7.03 [22]; plasminogen = 17.0 [23]).

Plasmin was prepared by the activation of plasminogen with insolubilized UK as described previously (14). The activity of the plasmin was determined by active site titration (24). The plasmin was stored at −70°C in 50% glycerol, at a concentration of 2.0 mg/ml active enzyme.

Generation of inhibitor-plasmin complexes in plasma by the addition of plasminogen activators. Plasma was mixed with Tris (0.1 M)–HCl buffer, pH 7.4, containing disodium EDTA (0.01 M) and as indicated in the figure legends, varying concentrations of melanoma TPA or of UK diluted in Tris (0.1 M), pH 7.4, containing Tween 20 (0.05%) were added. The mixtures were incubated in 1.5-ml Eppendorf Tubes (Brinkmann Instruments, Inc., Westbury, NY) at 37°C with or without the addition of Reptilase (0.1 ml/ml plasma, reconstituted according to the manufacturer's instructions (Abbott Laboratories). Alternatively, the mixtures were clotted with human α-thrombin (0.02 ml containing 3 μg thrombin) or with thrombin and $CaCl_2$ (0.02 M final concentration). The final volume of the plasma mixture achieved a 1/2 dilution of the starting plasma. At varying intervals as indicated in the figures, the tubes were centrifuged 1 min (Eppendorf Micro Centrifuge, Brinkmann Instruments, Inc.). The supernatants were diluted 1/20 in phosphate-buffered saline (PBS) containing Tween 20 and epsilon amino caproic acid (EACA-PBS-Tween) as previously detailed (14). These samples were frozen at −20°C and assayed subsequently for α_2-plasmin inhibitor-plasmin complexes by the differential antibody ELISA. In this assay, 0.2 ml of the sample was applied to microtiter plates, the wells of which had been coated with the IgG fraction of rabbit anti-α_2-plasmin inhibitor antiserum. After incubation and washing, the immunoaffinity-purified $F(ab')_2$ fraction of rabbit anti-plasminogen IgG labeled with alkaline phosphatase was added. After incubation the substrate *p*-nitrophenyl phosphate was added and color development followed as detailed (14).

The stability of the melanoma plasminogen activator was studied by adding the activator to plasma and freezing portions at −70°C for 72 h. The thawed samples were diluted 1/20 in EACA-PBS-Tween, clotted with Reptilase, and incubated for 2 h at 37°C. The production of α_2-plasmin inhibitor-plasmin complexes was measured as described. The fresh plasma-TPA mixture was assayed immediately in a similar manner. In other studies, plasma containing added TPA was incubated in an ice bath or at 37°C for 2 h. The supernatant was assayed for the formation of α_2-plasmin inhibitor-plasmin complexes.

In these studies the curves of best fit were analyzed either by the four parametric logistic method of Rodbard and Hutt (25) or by regression analysis using a Hewlett-Packard model 9815A calculator with a model 9862A X-Y plotter (Hewlett-Packard Co., Palo Alto, CA).

Protocol for the human study. Informed consent was obtained from healthy human volunteers for participation in this study as approved by the Committee on Human Rights in Research. Subjects were randomly assigned to a first study period during which they ingested either aspirin or no drug. If assigned to the aspirin group, subjects ingested 650 mg of aspirin (Hudson Pharmaceutical Corp., West Caldwell, NJ), in the presence of one of us, both 18 and 2 h before blood collection. Aspirin was given to subjects after determining that tablets flocculated and broke up completely within 15 s in water brought to pH 3 by the addition of 0.1 N HCl. Subjects underwent venipuncture as described below and returned after 10 d during which no aspirin was ingested for the second part of the study.

One subject also participated in a randomized, double-blind, crossover trial during which he received either pulverized aspirin 650 mg (Hudson Pharmaceutical Corp.) or lactose powder in a No. 0 gelatin capsule (Lilly (Eli) International Corp., Indianapolis, IN).

Blood collection. Venous blood was drawn into plastic syringes from volunteers <1 min after the application of a venipuncture tourniquet and added to 3.8% sodium citrate (9:1, vol/vol) in plastic tubes. Blood was then collected from the opposite arm after venous occlusion of the forearm by a sphygmomanometer cuff inflated to 20 mmHg below systolic pressure for 10 min (12, 13, 26, 27). The plasma, obtained by centrifugation at 2,500 rpm for 10 min, was either used within 30 min of venipuncture in the plasmin inhibitor-plasmin complex generation assay (as described) or was frozen in plastic tubes at −70°C for subsequent analysis of salicylate and Factor VIII-related antigen (VIII:Rag) concentrations and for use in the generation of standard curves for each subject.

Effect of aspirin on the generation of inhibitor-plasmin complexes in plasma obtained before and after venous occlusion. Plasma or Reptilase-clotted plasma from the blood obtained before and after 10 min of

venous occlusion was incubated 2 h at 37°C as detailed above; and the formation of α_2-plasmin inhibitor-plasmin complexes was measured. Standard curves were prepared either from pooled normal plasma or from the donor's preocclusion plasma from each day of the test by the addition of varying concentrations of melanoma TPA as described above. In this manner, control samples were read against standard curves generated in control plasma from the same subject, and aspirin samples were read against standard curves generated in the subject's own aspirin-treated plasma. The spontaneous activity measured in the subject's pre- and postocclusion plasma samples was then converted to units of tissue activator by reference to the standard curve. Results were identical regardless of which plasma curve was used to determine TPA concentrations: pooled-plasma standard curves run on the day of the assay, subject's plasma standard curves as described, or composite standard curves generated by averaging the results of many standard analyses. In this paper, TPA concentrations reported are those determined by using the subject's own plasma for standard curve interpolation.

Since hemoconcentration occurred during the 10 min of venous occlusion, postocclusion TPA values were corrected by multiplying the calculated TPA concentration by a correction factor obtained by the formula: (1 − postocclusion hematocrit)/(1 − preocclusion hematocrit). Though this is a standard correction for venous occlusion studies, it is not clear whether it is a valid reflection of the partition of plasminogen activator between vascular and extravascular spaces (28, 29). Therefore, the data were also analyzed without the correction. Conclusions were identical and only corrected values are reported.

The relationship of melanoma TPA to the activator activity induced by venous occlusion was studied in the α_2-plasmin inhibitor-plasmin complex-forming assay. As detailed in Table I, postvenous occlusion plasma, or plasma to which TPA or UK was added was incubated 30 min with the IgG fraction of rabbit anti-melanoma TPA antisera or with the IgG fraction of serum from an unimmunized rabbit. The plasma was clotted with Reptilase; and a 0-time sample was obtained and incubated an additional 2 h at 37°C. The formation of complexes was determined by the ELISA assay. To test the activity of rabbit anti-melanoma TPA antisera against UK, the IgG fraction was preincubated with UK as indicated in Table I, before the addition of plasma that was then clotted with Reptilase and incubated 2 h at 37°C.

To study whether aspirin and salicylate influence the assay for TPA activity, aspirin (0–1 μg/ml) or salicylate (0–70 μg/ml) was added to plasma samples before the addition of TPA (0–7 U/ml) and subsequent clotting with Reptilase. Additions of salicylate or aspirin at the concentrations noted were also made after α_2-plasmin inhibitor-plasmin complexes had been generated by adding TPA (0–7 U/ml) to plasma that had been clotted with Reptilase. The ELISA was performed and the change in absorbance of samples containing identical amounts of TPA and various amounts of salicylate or aspirin was compared to determine whether salicylate or aspirin interfered with the performance of the ELISA.

Analysis of VIII:Rag. VIII:Rag was assayed by quantitative immunoelectrophoresis (30) using a monospecific antiserum (a gift of Dr. Connie Miller, Cornell University Medical College, New York).

Analysis of plasma salicylate. Salicylate concentrations in subjects' plasma were determined by minor modifications of the high performance liquid chromatographic method of Amick and Mason (31) by using toluic acid (Sigma Chemical Co., St. Louis, MO) as an internal standard. A standard curve was generated by analyzing plasma samples containing 0–75 μg/ml salicylate in triplicate. Regression analysis of salicylate observed vs. salicylate added yielded a straight line of the form $y = 0.94 x + 1$; $r = 0.997$. Concentrations of salicylate in subjects' plasma were determined in duplicate by reference to the standard curve.

Statistical analysis. The significance of differences noted in single comparisons was determined by the paired or unpaired t test while the significance of the influence of multiple factors and their interactions was analyzed by one-, two-, or three-way analysis of variance (ANOVA).

Results

Generation of α_2-plasmin inhibitor-plasmin complexes in plasma by melanoma cell TPA. Melanoma TPA (1 or 2 U/ml plasma) or buffer was added to plasma; and the mixtures were clotted with Reptilase and incubated at 37°C (Fig. 1). At varying intervals the tubes were centrifuged and the supernatant was diluted in EACA-PBS buffer and assayed for the formation of α_2-plasmin inhibitor-plasmin complexes by ELISA. The change in absorbance in the assays increased linearly with time and the rate of

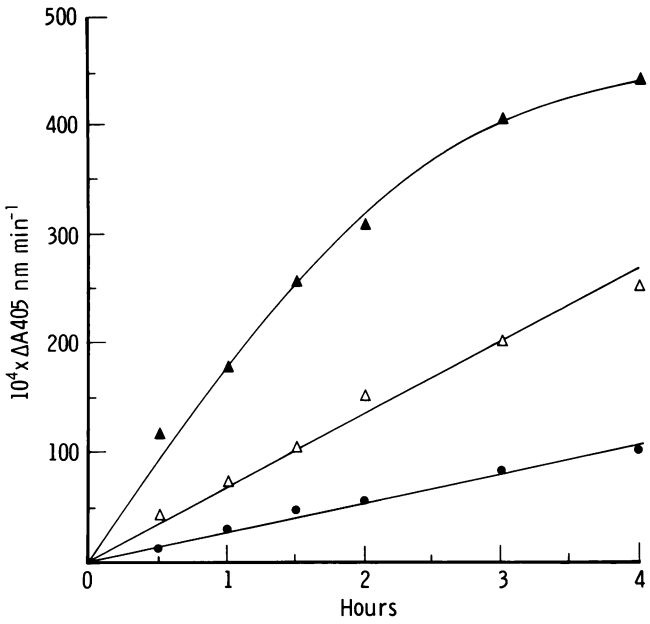

Figure 1. Time-dependent generation of α_2-plasmin inhibitor-plasmin complexes in Reptilase-clotted plasma by tissue plasminogen activator. Replicate samples of plasma (0.1 ml) were incubated in plastic tubes with Tris-EDTA buffer or with melanoma TPA (1 or 2 U/ml plasma) in a total volume of 0.19 ml. All samples were clotted with Reptilase (0.01 ml) and incubated at 37°C. A portion (0.07 ml) was removed from each tube at the times indicated on the abscissa and added to EACA-PBS-Tween (1.33 ml). These samples were assayed in triplicate for α_2-plasmin inhibitor-plasmin complexes by the immunosorbent assay. The activity generated in the plasma clotted in the absence of tissue plasminogen activator at each time was subtracted from the activity generated in the plasma samples containing the activator. The curves were analyzed by linear regression analysis: 2 TPA units (▲); for first four data points, $y = 159.9 x + 23.0$ ($r = 0.989$); 1 TPA unit, (△) $y = 67.5 x + 9.8$ ($r = 0.995$); spontaneous activity (●), $y = 28.2 x + 1.5$ ($r = 0.997$).

formation of these complexes was dependent upon the concentration of TPA added. Complex formation was observed in the Reptilase-clotted plasma in the absence of added TPA, indicating the presence of endogenous TPA activity in the plasma.

The effect of clotting plasma with Reptilase on the generation of α_2-plasmin inhibitor-plasmin complexes by tissue plasminogen activator was compared with non-Reptilase-clotted plasma (Fig. 2). When concentrations of TPA from 0.25 to 4.0 U/ml plasma were added, a difference in the generation of α_2-plasmin inhibitor-plasmin complexes was observed between the clotted and unclotted samples (Fig. 2, left). Inhibitor-enzyme complexes were just detectable in the plasma incubated for 2 h at 37°C. However, when plasma was clotted with Reptilase, there was a 17-fold increase in the formation of complexes at these same concentrations of TPA. When higher concentrations of TPA were used (Fig. 2, right) the differences between enzyme-inhibitor complexes formed in clotted and unclotted plasma were less. The rate of formation of complexes in Reptilase-clotted plasma was about fourfold higher than in unclotted plasma.

The capacity of UK to induce formation of α_2-plasmin inhibitor-plasmin complexes was studied in Reptilase-clotted and unclotted plasma (Fig. 3). In contrast to the enhancing effect of fibrin on the formation of complexes as demonstrated for TPA (Fig. 2), no significant difference in the production of

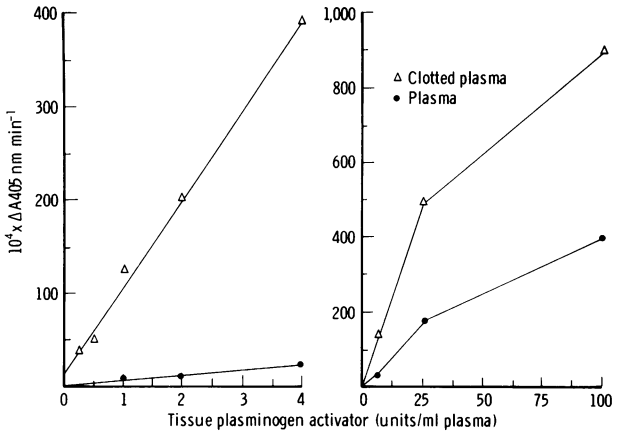

Figure 2. Comparison of the generation of α_2-plasmin inhibitor-plasmin complexes by varying concentrations of TPA in plasma or clotted plasma. Plasma (0.2 ml) was mixed with TPA at 4°C (*left:* 0.25, 0.5, 1, 2, and 4 U/ml plasma; *right:* 6.25, 25, 100 U/ml plasma) in Tris-EDTA-Tween buffer. Either buffer (•) or Reptilase (△) (0.02 ml) were added to bring the final volume of each mixture to 0.4 ml and the samples were incubated at 37°C. Portions were removed at 0 and 2 h from the mixtures containing the 0.25–4 U/ml, and at 0 and 30 min from those containing the higher concentrations and diluted 1/20 in EACA-PBS-Tween buffer. These samples were assayed for α_2-plasmin inhibitor-plasmin complexes. The change in absorbance of the sample obtained at 0 time was subtracted from that of the samples from later times. For the curve generated in Reptilase-clotted plasma (*left*): $y = 96.3\ x + 10.3$ ($r = 0.997$); and for that generated in plasma: $y = 5.5\ x - 0.06$ ($r = 0.999$).

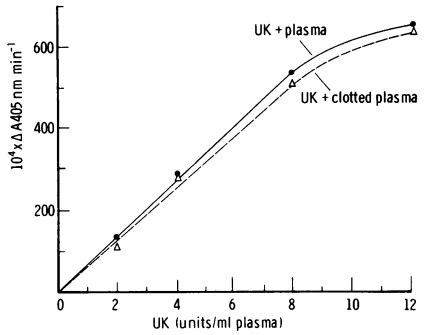

Figure 3. Generation of α_2-plasmin inhibitor-plasmin complexes by UK in plasma or clotted plasma. UK was added to plasma (0, 2, 4, 8, 12 U/ml plasma) and was incubated with (△ - - - △) or without (• —— •) the addition of Reptilase for 2 h at 37°C. The generation of α_2-plasmin inhibitor plasmin complexes was assayed as described in text.

complexes by UK was observed in plasma, regardless of whether the plasma was clotted with Reptilase.

Comparison of the effect of clotting plasma with Reptilase, thrombin, or thrombin with calcium on the generation of α_2-plasmin inhibitor-plasmin complexes by TPA. The rate of formation of α_2-plasmin inhibitor-plasmin complexes by TPA was influenced by the specific coagulant enzyme added to plasma (Fig. 4). Significantly less complex generation occurred when human thrombin rather than Reptilase was used to clot the plasma sample in the presence of varying concentrations of TPA. Approximately 1.7 times more TPA was required to yield the same change in absorbance in thrombin-clotted as in Reptilase-clotted plasma. To facilitate comparison of the effect of the different coagulant enzymes, the data in Fig. 4 were converted to nanomolar α_2-plasmin inhibitor-plasmin complex by using the standard curve in Fig. 2, left. Clotting plasma with thrombin resulted in a 33% decrease in the generation of complexes compared with the clotting of plasma with Reptilase (10.3 nM vs. 15.3 nM TPA U^{-1} h^{-1}). Furthermore, the addition to plasma of calcium chloride with thrombin resulted in an 82.3% decrease in the generation of α_2-plasmin inhibitor-plasmin complexes (2.7 nM U^{-1} h^{-1}).

Stability of TPA in plasma. TPA (4 U) was preincubated with plasma for varying periods either at 0° or 37°C before clotting with Reptilase, and the generation of α_2-plasmin inhibitor-plasmin complexes was followed. The activity of TPA remained relatively stable at both temperatures for the first hour, then declined in a linear fashion. The rate of decay of TPA was significantly greater at 37° than at 0°C. After 2 h of incubation at 37°C only 34% of the starting activity remained, while at 0°C the activity declined to 52.5% of the starting activity. TPA incubated for 2 h with buffer, either at 37° or 0°C, and then tested for α_2-plasmin inhibitor-plasmin-forming activity in the Reptilase-clotted plasma, demonstrated no loss of activity.

TPA was also labile in plasma when frozen at −70°C. Freezing caused a 30% loss of the endogenous plasminogen activator

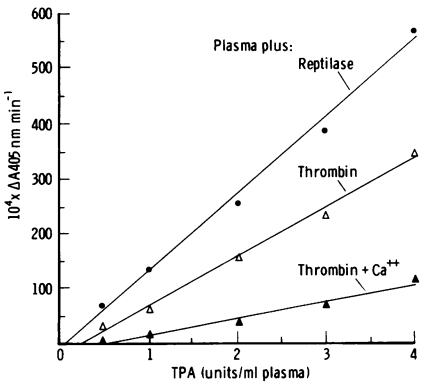

Figure 4. Effect of Reptilase, thrombin or thrombin with Ca^{++} on the tissue plasminogen activator-induced generation of α_2-plasmin inhibitor-plasmin complexes in plasma. Normal plasma (0.2 ml) was incubated with 0, 0.5, 1, 2, 3, or 4 U TPA/ml plasma diluted in Tris-Tween buffer. The mixtures were clotted with thrombin (0.02 ml containing 3 μg thrombin) (△) or Reptilase (0.02 ml; ●) and made to a total volume of 0.4 ml with Tris (0.1 M, pH 7.4)-EDTA (0.01 M)-Tween buffer. Alternatively, the mixtures were clotted with thrombin and $CaCl_2$ (0.02 M final concentration; ▲). After a 2-h incubation at 37°C, 0.05 ml of the supernate was added to 0.95 ml EACA-PBS-Tween. These samples were assayed for α_2-plasmin inhibitor-plasmin complexes by the immunosorbent assay. The change in absorbance of the sample without added plasminogen activator was subtracted from those samples with the activator. Curves were fitted by regression analysis. For normal plasma clotted with: Reptilase, $y = 139.5\ x - 8.3$, $r = 0.99$; thrombin, $y = 86.7\ x - 14.3$, $r = 0.99$; and thrombin plus Ca^{++}, $y = 28.9\ x - 11.4$, $r = 0.98$.

activity in normal resting plasma. When TPA was added to plasma that was subsequently frozen, a somewhat greater loss of plasminogen activator activity was observed.

Effect of rabbit anti-human melanoma TPA on the generation of α_2-plasmin inhibitor-plasmin complexes in TPA-treated plasma, in postvenous occlusion plasma and urokinase-treated plasma. Plasma to which two different concentrations of TPA were added, or plasma obtained after 10 min of venous occlusion was incubated 30 min either with buffer, the IgG fraction of rabbit anti-human melanoma TPA, or normal rabbit IgG before clotting with Reptilase and complex formation was measured. The antibody directed against TPA almost completely inhibited the complex-forming activity of TPA treated-plasma and of postvenous occlusion plasma while normal rabbit IgG had no effect (Table I). In contrast, the rabbit anti-melanoma TPA antibody did not inhibit UK.

Effect of venous occlusion on plasminogen activator activity in vivo. Seven men and six women were enrolled in this study. The characteristics of the subjects are listed in Table II. While the ages of the men and women were similar ($P = 0.271$), women had lower hematocrits ($P = 0.007$). The "resting level" of TPA, defined as the amount of plasminogen activator activity present in venous blood obtained after <1 min of venous occlusion induced by the application of a venipuncture tourniquet,

Table I. Effect of Rabbit Anti-TPA Antibody on Plasminogen Activator Activity in Plasma Incubated with TPA, UK, or in Plasma Obtained after Venous Occlusion

Incubation mixture*	Units‡	% Activity
	TPA	
TPA (4 U)-plasma	2.0±0.02	100
TPA + RaTPA	0.09±0.006	4.5
TPA + IgG	1.75±0.013	87.5
TPA (2 U)-plasma	0.78±0.01	100
TPA + RaTPA	0.02±0.07	2.6
TPA + IgG	0.72±0.012	92.3
Stasis plasma	0.94±0.02	100
Stasis + RaTPA	0.02±0.01	2.1
Stasis + IgG	0.89±0.01	94.7
	UK	
UK-plasma	4.0±0.32	100
UK + RaTPA	3.6±0.25	90
UK + IgG	3.5±0.06	87.5

* Plasma (0.2 ml) was preincubated in duplicate with either 0.8 or 0.4 U TPA (TPA-plasma) with and without 80 μg of the IgG fraction of rabbit anti-melanoma plasminogen activator (RaTPA) (TPA-plasma + RaTPA) or with normal rabbit IgG (TPA-plasma + IgG) diluted in Tris-EDTA buffer for 30 min at 25°C in a total volume of 0.38 ml. Reptilase (0.02 ml) was added and the mixture incubated for 2 h at 37°C. Samples were taken after Reptilase addition at 0 and 2 h and diluted 1/20 in EACA-PBS-Tween buffer for assay for α_2-plasmin inhibitor-plasmin complexes. Alternatively, plasma was obtained from blood collected after 10 min of venous occlusion of the forearm (stasis plasma) and incubated with buffer or with 80 μg of rabbit anti-melanoma plasminogen activator or with normal rabbit IgG and processed as detailed above. To test for the effect of the anti-melanoma antibody against UK, 80 μg of rabbit anti-melanoma plasminogen activator IgG or normal rabbit IgG were preincubated with UK (0.8 U) for 30 min at 25°C. Plasma (0.2 ml) was added to the mixture that was then clotted with Reptilase (0.02 ml) in a total volume of 0.4 ml and incubated 2 h at 37°C. Samples were obtained as described above for ELISA.

‡ The change in absorbance in the ELISA for α_2-plasmin inhibitor-plasmin complexes was converted to TPA units by using a standard curve. A similar curve was constructed for converting the ELISA values to UK units.

was significantly greater in women (0.56 U/ml) than in men (0.15 U/ml, $P = 0.049$). These results are similar to those of previous studies, which have found fibrinolytic activity to be greater in premenopausal women than in men (32), and are similar to preocclusion values recently demonstrated using other assays (12, 13). No plasminogen activator activity was measurable in unclotted plasma samples, thereby eliminating a contribution of UK-like activity in resting plasma. While both the hematocrit and TPA concentrations were different in men and

Table II. Comparison of Male and Female Subject Characteristics

	n	Age*	Hematocrit‡	TPA§	VIII:Rag‖
Men	7	35.3±8.0	44.1±1.5	0.15±0.11	116±93
Women	6	32.7±7.0	40.7±2.6	0.56±0.59	128±34
Total	13	34.1±7.3	42.5±2.6	0.34±0.44	122±70
P		0.271¶	0.007	0.049	0.392

* Figures show the mean±SD.
‡ Blood values listed were obtained from the preocclusion venous sample in the control state.
§ TPA activity is expressed in units per milliliter of plasma.
‖ VIII:Rag concentrations are expressed as percent of the control, pooled plasma = 100%.
¶ Differences between men and women were analyzed by the unpaired *t* test.

women, there was no relationship between the hematocrit and the resting level of TPA by linear regression analysis (data not shown).

The stimulus of venous occlusion induced a 15-fold rise in TPA concentrations in men (0.15±0.11 to 2.28±1.49, mean±SD, $P = 0.004$) and an eightfold rise in women (0.56±0.59 to 4.5±2.66, mean±SD; $P = 0.006$) (Fig. 5). Whereas the percentage increase was greater in men, the absolute increase of TPA induced by venous occlusion was greater in women (3.94±2.46) than in men (2.13±1.38). While venous occlusion induced a marked rise in TPA activity, no increase in UK or fibrin-independent plasminogen activator activity was detected (data not shown).

Effect of aspirin on the release of vascular plasminogen activator activity. Fig. 6 demonstrates the effect of aspirin on the increase in TPA activity induced by venous occlusion in the 13 subjects. Aspirin did not affect the preocclusion activity of TPA. The control value for the 13 subjects was 0.34±0.44 U/ml and the value after aspirin ingestion was 0.32±0.12 U/ml (mean±SD, $P = 0.34$). However, aspirin markedly inhibited the rise in TPA activity after venous occlusion. In the control state TPA activity increased by 2.14±1.43 U/ml plasma in men and 3.94±2.46 U/ml plasma in women after venous occlusion, while after aspirin ingestion TPA activity increased only by 0.67±0.85 U/ml plasma in men (mean±SD, $P = 0.004$) and 1.17±0.54 U/ml plasma in women (mean±SD, $P = 0.014$). While gender influenced both the resting activity of TPA and the response of TPA activity to venous occlusion as shown above, gender did not influence the inhibition of TPA activity by aspirin. Aspirin inhibited the increase in TPA activity after venous occlusion by 69% in men and by 70% in women (effect of gender on inhibition of TPA activity by aspirin: $P = 0.45$). While all subjects showed inhibition of TPA release by aspirin, there was marked individual variation as shown in Fig. 6, with percentage inhibition compared with the control state ranging from 15 to 100%. The inhibition was not due to an effect of aspirin on the resting hematocrit or the extent of hemoconcentration during venous occlusion because there were no differences between the control and aspirin period resting hematocrits (42.5±2.6 vs. 42.5±2.7) or the increased hematocrits after hemoconcentration due to venous occlusion (50.98±4.4 vs. 50.85±3.74, mean±SD; effect of aspirin on hematocrit values: $P = 0.81$, two-way ANOVA).

To determine whether the inhibition of TPA activity after venous occlusion by aspirin was consistent, a single subject was tested on three occasions during a 1-yr period. On two occasions the subject was randomly assigned to either no drug or aspirin and returned 10 d later for the opposite arm of the study. On the third occasion, the subject was randomly assigned first to placebo (lactose) or aspirin and the experiment was conducted in a double-blind, placebo-controlled, crossover manner. Aspirin inhibited the rise in TPA from the pre- to postocclusion state on each of the three test periods, including the double-blind

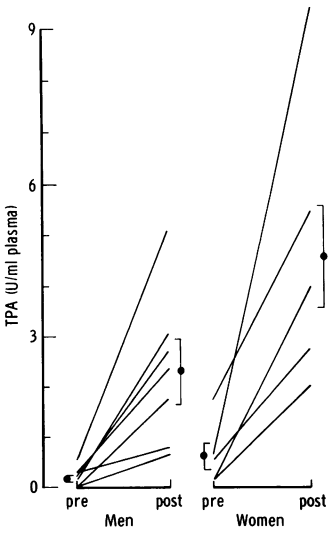

Figure 5. Effect of venous occlusion on TPA activity. The individual responses of the seven men (*left*) and six women (*right*) as well as the mean±SEM (with the postocclusion values corrected for the change in hematocrit as described in Methods) are shown. Two-way ANOVA showed that both venous occlusion ($P < 0.00001$) and gender ($P = 0.02$) significantly affected the TPA activity.

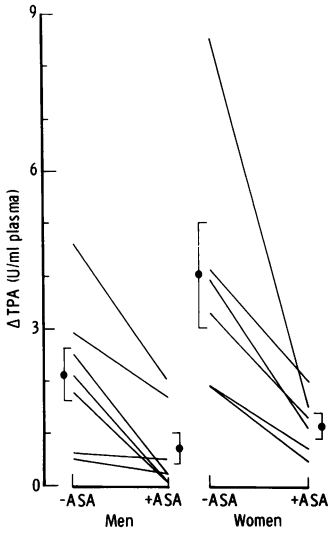

Figure 6. Effect of aspirin (ASA) on the rise in TPA activity induced by venous occlusion. The individual responses of the seven men (*left*) and six women (*right*) as well as the mean±SEM (with the postocclusion values corrected for the change in hematocrit as described in Methods) are shown. Aspirin inhibited the rise in TPA concentrations after venous occlusion by 69% in men ($P = 0.004$) and 70% in women ($P = 0.014$).

cycle, by 96, 78, and 64%, respectively. Similar results have been noted in two additional subjects. Thus, the inhibition of TPA release by aspirin appears to be a consistent response of an individual subject.

Plasma salicylate concentration and TPA activity. To determine whether the marked variability documented in the suppression of TPA activity by aspirin was due to variation in the absorption and metabolism of aspirin, salicylate levels were measured in all preocclusion plasma samples from both control and aspirin test periods. Salicylate levels in all control plasma samples were below the level of detection of the method (<1 μg/ml). Plasma salicylate concentrations after aspirin ingestion were significantly higher in women (55.4±6.4 μg/ml, mean±SD) than in men (34.5±6.8 μg/ml, mean±SD, $P = 0.0001$), as has been noted previously (33, 34). However, there was no significant relationship between percentage inhibition of TPA activity and salicylate concentration for the group as a whole ($r = 0.3$, $P = 0.16$) or for either men or women analyzed separately.

Effect of aspirin or salicylate on the assay for TPA activity. The addition of aspirin (0–1 μg/ml) or salicylate (0–70 μg/ml) to the assay system for TPA activity either before or after the addition of exogenous TPA to plasma did not affect the assay ($P > 0.4$; two-way ANOVA comparing standard curves; data not shown).

Effect of aspirin on the rise in VIII:Rag induced by venous occlusion. Venous occlusion induced a significant rise in VIII:Rag concentrations in all 13 subjects both in the control state (28.5%) and after aspirin ingestion (29.4%) but aspirin had no significant effect on VIII:Rag levels (effect of occlusion: $P = 0.0001$; effect of aspirin: $P = 0.795$, two-way ANOVA). Thus, while aspirin caused a marked inhibition of TPA activity after venous occlusion it caused no inhibition of VIII:Rag release.

Discussion

In this study we have quantified TPA activity in blood by measuring the generation of α_2-plasmin inhibitor-plasmin complexes that form in clotted plasma. The assay has made it possible to measure resting levels of blood plasminogen activator activity in normal individuals; to quantify and further characterize the increased levels of plasminogen activator activity induced by venous occlusion of the forearm; and to demonstrate that aspirin markedly inhibits this increased activity.

Fibrin catalyzes the activation of plasminogen by TPA in purified systems (35–38). The present studies show that fibrin also accelerates the TPA-induced generation of α_2-plasmin inhibitor-plasmin complexes in plasma. At plasma concentrations of TPA from 0.25 to 4.0 U/ml, there is an increase in α_2-plasmin inhibitor-plasmin complexes in both clotted and unclotted plasma that is proportional to the amount of activator added. However, clotting with Reptilase results in approximately a 17-fold enhancement in the rate of formation of complexes. At higher concentrations of TPA, 25–100 U/ml plasma, the stimulatory effect of fibrin on complex generation was reduced to fourfold. In contrast to these observations with TPA, clotting plasma with Reptilase failed to enhance the generation of α_2-plasmin inhibitor-plasmin complexes by UK, a finding that confirms and extends to plasma previous results utilizing purified systems (35–39).

A comparison of the effect of Reptilase, isolated from the venom of *Bothrops atrox,* that cleaves fibrinopeptide A from fibrinogen, and thrombin, which cleaves both fibrinopeptides A and B from fibrinogen, indicates that significantly more complexes were formed when plasma treated with TPA was clotted with Reptilase than with thrombin. These results, that are as yet unexplained, extend the observations of Ranby et al. (40) who have observed in purified systems that the activation of plasminogen by TPA is fivefold greater in the presence of Reptilase-clotted fibrinogen than in the presence of thrombin-clotted fibrinogen.

The addition of calcium chloride to the solution containing thrombin produced a decrease in the α_2-plasmin inhibitor-plasmin complexes generated by TPA in plasma as compared with thrombin alone. These results may be explained by the findings of Aoki and co-workers (41–43) who have demonstrated that α_2-plasmin inhibitor is covalently linked to fibrin by fibrin-stabilizing factor after activation by thrombin and calcium. They have postulated that this cross-linking of the inhibitor produces fibrin clots resistant to plasmin-induced fibrinolysis by increasing the local concentration of α_2-plasmin inhibitor as well as by masking plasminogen binding sites on fibrin. These mechanisms may explain the apparent inhibitory effect of calcium on the generation of plasmin inhibitor-plasmin complexes observed in the present study.

Studies of the stability of TPA added to plasma or of TPA induced by venous occlusion using our assay demonstrated a lability of activity at both 37° and −70°C, therefore making it necessary to perform the generation assay on freshly collected plasma. These findings are consistent with those previously reported that estimate the half-life of the activator to be 90–105 min in plasma incubation mixtures (44).

The Reptilase-clotted plasma obtained from 13 subjects in the resting state generated α_2-plasmin inhibitor-plasmin complexes equivalent to the addition of 0.34±0.44 (mean±SD) TPA units to plasma. No such generation was observed in unclotted plasma, indicating the absence of detectable UK activity. These findings are consistent with two recent reports using different methods, which have documented similar plasma concentrations of plasminogen activator activity (12, 13).

Enhanced blood fibrinolytic activity has been observed after venous occlusion of the forearm, presumably due to the release of plasminogen activator from the vascular endothelium (26, 45, 46). In the present study, the generation of α_2-plasmin inhibitor-plasmin complexes in Reptilase-clotted, postocclusion plasma was increased 15-fold in men and eightfold in women as compared with the preocclusion plasma. This activity appeared to be due to TPA since no generation of complexes was observed in unclotted plasma. These observations are similar

to those of Comp et al. (12) and Rijken et al. (13) who also documented increases in TPA after 10 min of venous occlusion.

Preincubation of plasma with the IgG fraction of rabbit anti-human melanoma TPA before the addition of exogenous TPA abolished complex formation. This antisera almost completely inhibited the complex-forming activity of plasma obtained after venous occlusion but did not significantly affect the activity induced by UK. These findings confirm the validity of the assay system in measuring TPA and are consistent with previous data, indicating that plasminogen activator from human vessel wall and that present in blood after exercise, venous occlusion or 1-desamino-8-D-arginine vasopressin infusion is TPA (13, 17, 47, 48).

Aspirin ingestion produced a marked inhibition of TPA activity induced by venous occlusion. This was due solely to an inhibition of plasminogen activator activity and not to some other effect of aspirin on the test system since we demonstrated that neither aspirin nor salicylate at concentrations present in vivo in the patient's own plasma inhibited either the generation of α_2-plasmin inhibitor-plasmin complexes by exogenous TPA or interfered with the assay method. We interpret these observations to indicate that aspirin inhibits TPA before its presence in the circulation, and suggest that aspirin is most likely acting by inhibiting release of TPA from the vessel wall. Aspirin did not suppress all phenomena due to venous stasis because neither the increase in VIII:Rag nor the hemoconcentration induced by venous occlusion was affected by aspirin. All individuals tested showed inhibition of TPA activity by aspirin, though there was marked variation in the degree of inhibition ranging from 15 to 100%. This variability, in turn, was not related to the salicylate level, therefore raising the possibility that the inhibitory effect of aspirin on TPA activity may be related to the acetylating capacity of aspirin rather than to the steady-state concentration of salicylate. Men and women were affected equally by aspirin and this suppressive effect was consistent in the several individuals tested over 9–12 mo.

The mechanism by which aspirin inhibits plasminogen activator activity is not defined but there are several biochemical pathways that may be involved. Venous occlusion induces a rise in prostacyclin (a vasodilatory, antiaggregatory prostaglandin) (49) as well as in plasminogen activator and VIII:Rag (50), substances that are produced by vascular endothelial cells (51–53). Aspirin inhibits the production of prostacyclin by the endothelium by acetylating endothelial cyclooxygenase (54). We can speculate that the rise in plasminogen activator after venous occlusion is mediated by prostacyclin and that inhibition of plasminogen activator release is due to inhibition of prostacyclin synthesis. Alternatively, Comp and his co-workers (55, 56) have demonstrated that activated Protein C (C_a), a vitamin K-dependent inactivator of Factors V and VIII, also induces fibrinolysis by causing a rise in plasminogen activator. They have suggested that this is an incompletely understood phenomenon, requiring blood and enhanced by cellular elements. Thus, acetylation of one or several of these factors or Protein C itself might result in an inhibition of plasminogen activator release.

Because aspirin prevents platelet aggregation by inhibiting cyclooxygenase and resultant production of thromboxane A_2 (7, 8, 54), it has been used in attempts to alter the natural history of atherosclerotic (57–61) and thromboembolic disease (62, 63). Earlier studies that used relatively large doses of aspirin (≥325 mg/d) demonstrated a statistically significant benefit from aspirin only in men and for the prevention of stroke or venous thrombosis but not for myocardial infarction (57–63). However, a recent study (64) has demonstrated a benefit of lower dose aspirin (325 mg/d) in patients with unstable angina.

The beneficial effects of aspirin have been ascribed to inhibition of platelet function and this area has been studied extensively. The effect of aspirin on fibrinolysis has been less well defined. Menon (65) and Moroz (66) both demonstrated an increase in whole blood fibrinolytic activity after ingestion of 1.8 g of aspirin, but Moroz showed that this was due entirely to the fibrinolytic action of cellular elements. In contrast, Ghezzo et al. (67) showed a decrease in fibrinolysis induced by both cellular and plasma factors after ingestion of 1 g of aspirin/d for 4 d. Studies in animals have shown both an antithrombotic (68, 69) and a "paradoxical" thrombotic effect of aspirin (69–71). The present studies have demonstrated a marked effect of aspirin on vascular plasminogen activator activity in vivo after stimulation by venous occlusion in a small group of normal subjects. Caution should be used, however, in extrapolating these observations to the microenvironment of thrombus formation where fibrinolysis is dependent upon multiple complex interactions on which the effect of aspirin is unknown.

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