Plasminogen Activator in Normal Subjects After Exercise and Venous Occlusion: t-PA Circulates as Complexes With Cl-Inhibitor and PAI-1

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Exercise to exhaustion was associated with the appearance in plasma of plasminogen activator (PA) in several mol wt forms, as identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with zymography. A number of active bands, all immunologically identified as tissue-type PA (t-PA), were observed. The major form had an apparent mol wt of ~60,000 and is due to free t-PA. The other strong bands had apparent mol wts of ~110,000 and 180,000. The 110,000 band, also present in pre-exercise samples, represents t-PA complexed with its major inhibitor (PAI-1), and the 180,000 band is due to t-PA complexed with C1 inhibitor. The released forms of t-PA were cleared rapidly after cessation of exercise at exhaustion. Urokinase-type PA (u-PA) activity was also identified in pre- and postexercise samples at an apparent mol wt of ~50,000. This is consistent with its being free u-PA; no complexed forms of u-PA were observed. Qualitatively similar changes in plasma PA were observed after venous occlusion. Small quantities of plasmin were generated after strenuous exercise, as observed by detection of plasmin-α2-antiplasmin complex by two-dimensional immunoelectrophoresis in three of five subjects. This complex was cleared rapidly after cessation of exercise. Plasmin-α2-antiplasmin complex was not detected in any of the subjects after venous occlusion.

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FIBRINOLYTIC processes depend on the conversion of the plasma protein plasminogen to the protease plasmin by plasminogen activators. Two principal species of plasminogen activator (PA) have been identified, tissue-type PA (t-PA), identified in and purified from tissue extracts, and urokinase-type (u-PA), purified from urine. Both types of PA have been identified in human blood, but their relative importance and physiological roles remain uncertain.

For many years, strenuous short-term or sustained physical exercise and venous occlusion have been known to increase plasma fibrinolytic activity; this rise reflects increased levels of plasminogen activator, primarily t-PA, rather than formed plasmin. Numerous studies have confirmed these observations; most have used techniques such as the euglobulin lysis time (ELT) or fibrin plate lysis. Such methods, while primarily measuring overall PA activity, are materially influenced by other variables such as fibrinogen or plasminogen concentrations and by inhibitors of plasminogen activation or of plasmin itself.

We applied the technique of Granelli-Piperno and Reich to analysis of samples of whole plasma. This method uses sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate plasma proteins on the basis of molecular weight. Thereafter, PAs are identified zymographically by laying the washed gel on a detector gel that contains fibrin and plasminogen. PA activity is revealed after incubation by the development of visible bands of lysis in the opaque fibrin film in areas where PA has diffused into the detector gel and converted its plasminogen to plasmin with resultant local lysis of the fibrin. This technique separates PAs in plasma from one another, from plasminogen, and from the inhibitors of fibrinolysis. This allows their enumeration and characterization, in terms of their mol wt and immunological reactivity. This approach was used to study the response to strenuous standardized physical activity and to venous occlusion to investigate the contribution of the different species of PA.

MATERIALS AND METHODS

Blood samples were collected into 0.1 vol of 0.13 mol/L of sodium citrate and chilled on ice. Platelet-poor plasma was prepared by centrifugation at 1,000 g for 15 minutes at 4°C. All plasma samples were stored at -70°C.

ELT and fibrin plate lysis assays were performed as previously described. Fibrin plate assays were used to examine PA activity in both unfractionated plasma and in the euglobulin fraction. Plasmin-α2-antiplasmin complexes in plasma were detected by the two-dimensional immunoelectrophoresis technique described previously.

SDS-PAGE with zymography to detect PA was performed on plasma samples as previously described. Immunological identification of the PA was achieved by inclusion of specific immunoglobulin to either t-PA or u-PA in the detector gels. Antibodies to t-PA and u-PA were from Biopool (Hornefors, Sweden) and all other antisera were from Dako Immunochemicals, Copenhagen. Immunoblotting was performed as described by Burnette. Immunodepletion was performed on columns of immunoglobulin linked to Sepharose 4B, as described previously. C1-Inhibitor was kindly donated by Dr M. Smith, Department of Pathology, University of Dundee, Scotland.

Subjects: Physical exercise. Healthy normal male subjects between the ages of 21 and 32 years were studied. Five subjects (VO2max 54, 58, 59, 60, and 62 mL/min/kg) were exercised to exhaustion on a bicycle ergometer at ~70% of their VO2max. Venous blood samples were withdrawn immediately prior to exercise, at intervals up to exhaustion, and for several hours postexhaustion.

Subjects: Venous occlusion. Healthy men and women aged 26 to 45 years were studied. After a 30-minute period of recumbent rest, a venous blood sample was obtained from one arm. A sphygmomanometer cuff was applied to the opposite arm, and the pressure was maintained at midway between systolic and diastolic (usually 90 to 100 mm Hg) for 15 minutes, after which further blood samples were obtained from both arms and the pressure in the cuff was released.

All subjects for both exercise and venous occlusion experiments gave informed consent in accordance with the Helsinki Agreement on Human Experimentation.
RESULTS

Zymography on plasma from normal resting subjects. SDS-PAGE with zymography on plasma samples from resting subjects produces two bands of plasminogen-dependent lysis, one of mol wt 110,000 and a faint one of 50,000, as well as a weak band of plasminogen-independent lysis of mol wt 95,000 (Fig 1). Inclusion of specific immunoglobulins to t-PA in the detector gel abolished the 110,000 band whereas immunoglobulin to u-PA abolished the faint 50,000 band. Neither had any effect on the plasminogen-independent activity (Fig 1).

Plasma from subjects after exercise or venous occlusion. Exercise or venous occlusion was associated with several changes in active bands of PA. A very broad band of PA of mol wt 55,000 to 65,000 appeared, sharply demarcated at its advancing border. The intensity of the band of 110,000 mol wt increased, and heterogeneity in this mol wt range appeared. A new band of activity of about 180,000 mol wt was observed.

Characterization of the PA activity appearing after exercise. The PA activity of plasma samples taken at exhaustion was examined using antibodies to t-PA and u-PA. Almost all the new bands of PA activity appearing in plasma after exercise were inhibited by antibody to t-PA (Fig 2). The exception was a crescent at the leading edge of the broad band of PA activity of ~60,000 mol wt that remained unaffected by this antiserum. This may be due to u-PA. Plasminogen-independent lysis did not appear to be affected by exercise (Fig 2). All bands identified in euglobulin fractions were inhibited by antiserum to t-PA (Fig 3). Thus, no u-PA-related activity appeared in euglobulin precipitates.

The t-PA in normal plasma is due to a complex of t-PA with its major inhibitor in the blood, PAI-1, and appears at a mol wt of ~110,000. This band was intensified on exercise. In addition, considerable heterogeneity of the t-PA-
related activity at \( \sim 110,000 \) mol wt was observed in postexercise plasma, but this remains unexplained. It was not due to plasminogen-independent activity, which does not appear to increase on exercise or venous occlusion (Fig 2).

The band of \( \sim 180,000 \) mol wt in postexercise plasma and euglobulin was due to a complex between t-PA and C1 inhibitor (Fig 4). Purified t-PA, incubated with excess C1 inhibitor, formed a complex that retained some PA activity (track b), as has been shown previously for t-PA–PAI-1.\(^{19,21}\) The complex of t-PA and C1 inhibitor had the same mobility with and without plasma and was indistinguishable from the 180,000 mol wt band seen in postexercise plasma. This band was removed from postexercise plasma by passage over a column of anti-C1-inhibitor immunoglobulin linked to Sepharose. On immunoblotting with antibodies to C1 inhibitor, postexercise plasma showed a very faint band of 180,000 mol wt in addition to a stronger band of \( \sim 120,000 \) mol wt, observed in both pre- and postexercise samples (data not shown).

Serial studies on individuals exercised to exhaustion. Serial studies in the subjects exercised on the bicycle ergometer allowed observation of the time scale of the development of these new bands of PA activity and correlation of their development with changes in the crude studies of plasma PA activity by the fibrin plate and euglobulin lysis time techniques.

Figure 5 (top) shows that the broad band of PA activity of \( \sim 60,000 \) mol wt started to appear in whole plasma at between 30 and 60 minutes, the time when PA activity became detectable in whole plasma applied to fibrin plates. It was fully developed at 60 minutes. This band remained detectable in the 95-minute specimen taken 15 minutes after complete exhaustion and cessation of all exercise, but had completely disappeared 4 hours after cessation of exercise. The t-PA–C1 inhibitor complex (180,000 mol wt) was also fully developed at 60 minutes, was weaker at 95 minutes—15 minutes after exercise ceased—and had disappeared completely 4 hours after cessation of exercise.

The activities of the euglobulin fractions of these plasmas

![Figure 4](image)

**Fig 4.** Zymography of (A) tissue plasminogen activator (t-PA) (5 mlU); (B) t-PA (5 mlU) plus C1 inhibitor (12 \( \mu \)g), preincubated for 30 minutes at 37°C; (C) t-PA plus C1 inhibitor as in (B) plus normal plasma; (D) postexercise plasma; and (E) postexercise plasma after passage over antibodies to C1 inhibitor–Sepharose.

are shown in Fig 5 (bottom). A series of changes occurred similar to that observed in plasma. The 110,000-band of t-PA–PAI-1 complex is clearly present in the euglobulin fraction; no activity was seen in the supernatant after euglobulin preparation. All the euglobulin PA was identified as t-PA (Fig 3). An additional band just above the 110,000 mol wt band was apparent in the 60- and 80-minute samples. The nature of this is unexplained, but it may be a complex of t-PA with another plasma inhibitor. No convincing evidence for a complex with \( \alpha_2 \)-antiplasmin or \( \alpha_1 \)-antitrypsin came from immunoblotting with antisera to these inhibitors.

**Generation of plasmin during exercise.** Intravascular generation of plasmin was examined using two-dimensional immunoelectrophoresis against antiserum to \( \alpha_2 \)-antiplasmin. Figure 6 shows that at the point of exhaustion a small but definite second peak was detected. Similar peaks were detected in three of the five subjects studied. This slow-moving peak represents plasmin–\( \alpha_2 \)-antiplasmin complex.\(^{22}\) The complex was almost completely cleared 15 minutes after exercise ceased.

**Patterns of PA after venous occlusion.** Blood samples obtained with and without venous occlusion were analyzed by the same methods. The observed changes in fibrinolytic parameters (data not shown) were indistinguishable from...
Those caused by strenuous exercise, except that no plasmin-α2-antiplasmin complex was observed in any of the subjects studied.

**DISCUSSION**

This study examined the relative roles played by t-PA and u-PAs in the well-known increase in fibrinolytic activity following physical exercise and venous occlusion. The technique of SDS-PAGE followed by zymography allowed demonstration that this response is primarily due to t-PA-related material, which appears in several different mol wt forms, all neutralized by antiserum to purified t-PA.

The appearance in plasma samples of the broad band of t-PA-related material of ~60,000 mol wt in the exercise experiment coincided in time with the appearance of detectable PA activity in whole plasma when applied to fibrin plates. This band probably reflects free t-PA in the plasma, even though it migrates as a broader band of faster mobility than purified t-PA in the absence of plasma. Albumin considerably broadens the t-PA band and increases its mobility. In experiments not illustrated here, we showed that the addition of purified t-PA to normal plasma results in its migration as a broad band of ~60,000 mol wt, indistinguishable from that seen in postexercise plasma samples in this study.

The t-PA-related band of ~180,000 mol wt was shown to be due to a complex with C1 inhibitor. A complex was formed in vitro with purified proteins, when C1 inhibitor was present in excess, and the complex retained some PA activity on zymography. Its mobility was unaffected by the presence of plasma proteins and its apparent mol wt was indistinguishable from the 180,000 mol wt band observed after exercise to exhaustion. The band was removed from postexercise plasma by passage over a column of antibodies to C1 inhibitor.

The 110,000 mol wt band in normal samples is a complex of t-PA with its major circulating inhibitor, a platelet release product of ~48,000 mol wt, which is also produced by endothelial cells and is known as PAI-1. This band of complex is intensified during exercise, an increase that is most clearly observed on zymography of euglobulin fractions (Fig 5). A similar increase in this PA band in the euglobulin fraction after venous occlusion was reported. The t-PA-PAI-1 complex clearly is precipitated in the euglobulin fraction and was not detected in the supernatant fraction. In contrast, free PAI-1 is distributed about equally between the euglobulin and soluble fractions (N.A. Booth and B. Bennett, unpublished observations). The nature of the weaker band of slightly slower mobility than the t-PA-PAI-1 complex, observed in this study, is uncertain.

Evidence has been presented by others for the presence of complexes of t-PA with α2-antiplasmin, C1 inhibitor and α1-antitrypsin in plasma. In this study, no complexed forms of PA with α2-antiplasmin or with α1-antitrypsin could be demonstrated by immunoblotting; in each case, only a single band of the free inhibitor could be seen. Immunoblotting of normal and postexercise plasma with antiserum to C1 inhibitor showed only a very faint band of 180,000 mol wt in the postexercise sample, in addition to the band of ~120,000 mol wt seen in both samples. Thus, immunoblotting is a less sensitive technique than zymography for the detection of traces of this complex.

In contrast to t-PA, no striking changes in plasma u-PA activity were observed after exercise. The 50,000 mol wt band of u-PA seen in resting plasma was also present after exercise, possibly slightly intensified. The interpretation is complicated by the fact that u-PA and free t-PA in the postexercise plasma are poorly separated in this electrophoretic system, in which the presence of a high concentration of albumin causes the t-PA to spread so that it migrates almost as rapidly as u-PA. Thus, only in the presence of antibodies to one of these PAs can the two types be distinguished (Fig 2). Because the techniques used in this study are not quantitative, no definite conclusion on changes in plasma u-PA concentration after exercise can be reached from these data. If there is a change, however, it is minor and all the detectable u-PA is free, uncomplexed to inhibitor. u-PA in patients with alcoholic cirrhosis is also detectable only in free form in plasma, even though in vitro studies show the PA inhibitor in plasma to be capable of forming complexes with u-PA as well as t-PA. This is probably because zymography detects only two-chain u-PA, and most u-PA in plasma is in the single-chain form. Single-chain u-PA is stable in plasma and is not susceptible to inhibition by PAI-1.

Despite the considerable increase in total PA activity in the blood after exercise and venous occlusion and the pres-
ence of free t-PA, it is evident from these studies that very little plasmin was generated in most subjects during the observations. The method using two-dimensional immunoelectrophoresis against antiserum to α₂-antiplasmin is sensitive enough to detect quantities of plasmin associated with clinical hemorrhage but remains a relatively crude instrument in a study such as this. It did detect the generation of significant amounts of plasmin-α₂-antiplasmin complex in three men exercised to exhaustion. Similarly, Collen and co-workers noted a small increase in this complex, assayed by latex agglutination, in three of four subjects after repeated exhausting exercise. In another study, in which complex was assayed by a radioimmunoassay, less marked increases in complex were noted after exercise than after venous occlusion, in contrast to our results. All these studies suggest that in some physiological as well as pathological situations sufficient plasminogen activator may be released to overcome inhibition of plasminogen activation and to result in generation of small quantities of plasmin in the circulating blood.

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REFERENCES


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