

# Thrombolysis with Human Extrinsic (Tissue-Type) Plasminogen Activator in Rabbits with Experimental Jugular Vein Thrombosis

EFFECT OF MOLECULAR FORM AND DOSE OF ACTIVATOR, AGE OF THE THROMBUS, AND ROUTE OF ADMINISTRATION

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**ABSTRACT** A simple venous thrombosis model in rabbits was used for the quantitative evaluation of the thrombolytic effect of human extrinsic (tissue-type) plasminogen activator as compared with urokinase.

A thrombus was formed in an isolated segment of the jugular vein from a mixture of  $^{125}\text{I}$ -labeled fibrinogen, whole rabbit blood, and thrombin. In order to immobilize the thrombus during lysis, it was formed around a woolen thread introduced longitudinally in the lumen of the vein. Thrombotic extension of the clot was prevented by subcutaneous injection of heparin. The extent of thrombolysis was measured as the difference between the radioactivity introduced in the clot and that recovered in the vein segment at the end of the experiment. In control animals the extent of thrombolysis was  $5.6 \pm 1.4\%$  ( $n = 5$ ) after 6 h,  $14.5 \pm 1.7\%$  ( $n = 10$ ) after 30 h,  $16.0 \pm 1.5\%$  ( $n = 11$ ) after 78 h, and  $48.1 \pm 2.7\%$  ( $n = 10$ ) after 174 h (mean  $\pm$  SEM).

Extrinsic (tissue-type) plasminogen activator, highly purified from the culture fluid of a human melanoma cell line, was administered systemically or locally over a time period of 4 h and the percent thrombolysis measured 2 h after the end of the infusion. One- and two-chain extrinsic plasminogen activator had very similar thrombolytic potency. Systemic infusion resulted in a dose-dependent degree of thrombolysis. The activator-induced thrombolysis, after infusion of

100,000 IU ( $\approx 1$  mg protein), was  $\sim 75\%$  for fresh clots, 35% for 1-d-old clots, 30% for 3-d-old clots, and 50% for 7-d-old clots. The thrombolytic activity of urokinase was more than five times lower than that of extrinsic plasminogen activator: Infusion of 500,000 IU resulted in  $\sim 40\%$  lysis of fresh clots and 25% of 1-3-d-old clots, while 7-d-old clots appeared to have become resistant to urokinase. Local infusion resulted in a 5-10 times higher thrombolytic effect of both extrinsic plasminogen activator and urokinase.

Thrombolysis with extrinsic plasminogen activator was not associated with systemic activation of the fibrinolytic system as evidenced by unaltered plasma levels of fibrinogen, plasminogen, and  $\alpha_2$ -antiplasmin. Systemic infusion of urokinase resulted in significant thrombolysis only at doses that were associated with disseminated plasminogen activation. Local infusion of urokinase required a 5-10-fold higher dose than extrinsic plasminogen activator to obtain a similar degree of thrombolysis, which also occurred in the absence of systemic activation of the fibrinolytic system.

It is concluded that the extent of thrombolysis by extrinsic plasminogen activator is mainly determined by the dose of activator and its delivery in the vicinity of the thrombus and much less by the age of the thrombus or the molecular form of the activator. Extrinsic plasminogen activator appears to be superior to urokinase because of its higher (5-10-fold) specific thrombolytic activity and the absence of systemic activation of the fibrinolytic system, which results in defibrinogenation and a bleeding tendency.

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## INTRODUCTION

Extrinsic (tissue-type) plasminogen activator (EPA)<sup>1</sup> is a trypsinlike serine protease that may be isolated from the vessel wall (1), from tissues (2-4), or from the culture fluid of a human melanoma cell culture (5). It may be obtained either as a single-chain glycoprotein with 72,000  $M_r$ , or a two-chain disulfide-bonded proteolytic derivative (5, 6).

EPA, highly purified from the culture fluid of a human melanoma cell line (5), has been shown to specifically activate plasminogen in the presence of fibrin (7, 8) and to be a more specific and more potent thrombolytic agent than urokinase in artificial systems in vitro (9) and in experimental animal models in vivo (10, 11). The first experiments in humans have confirmed that infusion of EPA may result in thrombolysis without causing hemostatic breakdown and bleeding (12). All these findings suggest that EPA may provide a new thrombolytic agent with higher specificity and less side effects than those currently in use.

In view of eventual therapeutic application of this enzyme as a thrombolytic agent, a broader knowledge of the variables that determine the susceptibility of a thrombus to lysis is desirable. Therefore, we have developed a simple quantitative jugular vein thrombosis model in rabbits and have studied the influence of the dose and molecular form of EPA administered by local or systemic intravenous infusion on the lysis of fresh thrombi and on thrombi aged for 1, 3, or 7 d. The results provide quantitative evidence that thrombolysis with EPA occurs at lower doses than that with urokinase, and without affecting the hemostatic and fibrinolytic systems.

## METHODS

**Purified proteins.** One- and two-chain EPA were highly purified from the culture fluid of a human melanoma cell line by a method described elsewhere (5). The activity determined with plasminogen-enriched bovine fibrin films (5) was expressed in international units of urokinase. Equal units of EPA ( $M_r = 72,000$ ; 90,000 IU/mg sp act) and urokinase ( $M_r = 56,000$ ; 100,000 IU/mg sp act) correspond to approximately equal molar quantities.

Urokinase (Winkinase) was a gift from Dr. G. Murano (Bureau of Biologics, Food and Drug Administration, Bethesda, MD). Human fibrinogen was prepared according to Blombäck and Blombäck (13) and labeled with <sup>125</sup>I according to McFarlane (14). Human thrombin was purified as described by Fenton et al. (15).

**Experimental procedure.** New Zealand white rabbits with a body weight of  $2.4 \pm 0.3$  kg (mean  $\pm$  SD) were anes-

thesized by intramuscular injection of 0.5 ml Hypnorm (Duphar, Amsterdam, The Netherlands), containing 5 mg fluanisone and 0.1 mg fentanyl/kg body wt. Additional Hypnorm ( $\sim 0.3$  ml i.m. every 2 h) was administered to maintain anesthesia. Thyroidal uptake of radioiodide was blocked by administration of sodium iodide (0.5 ml i.v. of a 2% solution). A femoral vein catheter was introduced for blood sampling.

The surgical procedure to produce the artificial thrombus was as follows. An external jugular vein was exposed through a 5-cm paramedial incision in the neck. The vein was cleared over a distance of 4 cm up to the main bifurcation of the external jugular vein and the facial vein. Small side branches were ligated and the facial vein was cannulated with a 10-cm length of a Portex "pink gauge" cannula (Portex, Hythe, England). A woolen thread was then introduced in the lumen of the jugular vein over a distance of 4 cm with the use of an ordinary needle. When bleeding had ceased, the vein was clamped both proximally and distally to isolate a vein segment, which was then emptied of all blood by suction via the catheter. The volume of the segment was measured by injection of saline from a volumetric syringe until the vessel was fully distended. The anatomy of the veins and the essential steps of the surgical procedure are illustrated schematically in Fig. 1.

The procedure to form a thrombotic venous occlusion (referred to as a clot) was as follows. Approximately 10-20  $\mu$ l of <sup>125</sup>I-labeled human fibrinogen (containing  $\sim 500,000$  cpm) was aspirated in a 1.0-ml syringe followed by a volume of fresh rabbit blood corresponding to the measured volume of the isolated vein segment. The segment was then emptied by withdrawal of the saline through the side branch catheter, and 0.1 ml thrombin (100 NIH U/ml) was quickly injected, followed at once by the volume of blood containing the radioactive fibrinogen. Injection of air bubbles was avoided. Cotton swabs were then placed over the vessel to absorb

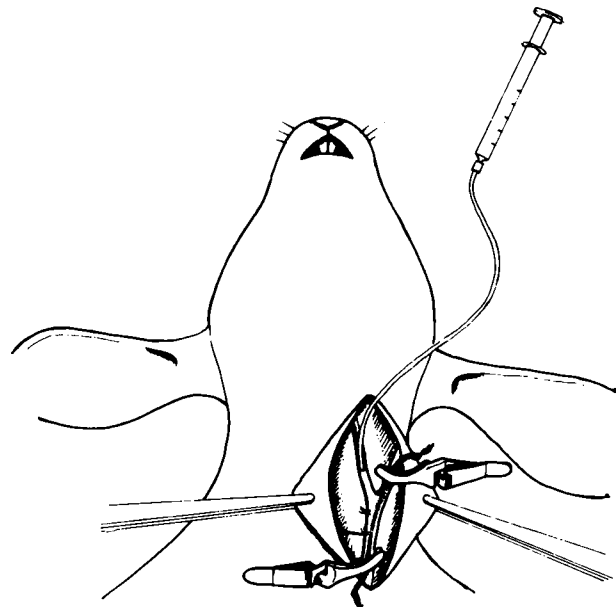


FIGURE 1 Schematic representation of the surgical procedure used to produce a jugular vein thrombosis in rabbits.

<sup>1</sup> Abbreviations used in this paper: AIL, activator-induced thrombolysis; EPA, extrinsic (tissue-type) plasminogen activator, highly purified from the culture fluid of a human melanoma cell line.

blood leaking from the vein segment. In all instances, the clot formed quickly and was allowed to age for 30 min before both vessel clamps were removed. A blood sample was drawn immediately after removal of the vessel clamps to measure the radioactivity in the blood. The cotton swabs were removed for radioisotope counting and the amount of radioactivity delivered to the clot was calculated by subtracting the swab losses, the radioactivity remaining in the syringe, and the total blood radioactivity (assuming a blood volume of 60 ml/kg body weight) from the original amount of radioactivity in the syringe.

In the acute experiments with fresh thrombi, the urine bladder was catheterized and a 21-g butterfly needle was introduced in the contralateral marginal ear vein for infusion of the thrombolytic agents, which was started immediately. The bladder was emptied every hour, the urine volume was measured, and an aliquot was taken for radioisotope counting. In the experiments with aged thrombi the catheters in the facial vein and in the femoral vein were removed, the vessels ligated, and the wounds closed. The animals were given an injection of 250,000 U i.m. procain benzylpenicillin/d and 500 units s.c. of heparin/kg body weight twice daily for 1, 3, or 7 d. The animals were then reanesthetized, and a catheter was introduced in the remaining femoral vein for blood sampling.

Intravenous infusion of EPA, urokinase, or solvent (0.3 M NaCl containing 0.01% Tween 80) was carried out by using a constant rate infusion pump. The infusions were given systemically through the contralateral marginal ear vein as a bolus injection of 2 ml (10% of the total volume) followed by an infusion of 18 ml over 4 h. Alternatively, the agents were given through a catheter introduced via the ipsilateral marginal ear vein into the immediate vicinity of the clot as a continuous infusion of 20 ml over 4 h. 6 h after the start of the infusion, the thrombosed segment of the jugular vein was removed after careful suturing of both ends and the remaining radioactive material was measured. The extent of thrombolysis was calculated as the difference between the radioactivity originally incorporated in the clot and the radioactivity in the vein segment, and expressed as percentage of the original radioactivity. The amount of radioactivity incorporated in the clot varied somewhat from one preparation of labeled fibrinogen to the other and with the age of the preparation. Thus, in the experiments with systemic infusions the isotope content of the clot was  $198,000 \pm 19,000$  cpm (mean  $\pm$  SEM) in the control groups ( $n = 22$ ),  $250,000 \pm 22,000$  cpm in the groups receiving 100,000 IU of EPA ( $n = 28$ ), and  $263,000 \pm 3,200$  cpm in the groups receiving 500,000 IU of urokinase ( $n = 14$ ). In the experiments with local infusions these values were  $335,000 \pm 34,000$  cpm for the controls ( $n = 14$ ),  $295,000 \pm 25,000$  cpm for the groups infused with 20,000 IU of EPA ( $n = 20$ ), and  $322,000 \pm 38,000$  cpm for the groups infused with 100,000 IU of urokinase ( $n = 13$ ). Activator-induced thrombolysis (AIL) was calculated as the difference between the means of the percentage thrombolysis in a given activator-infused group (A) and its control group infused with solvent (C), divided by the difference between the total radioactivity in the clot (100%) and the mean of the percentage thrombolysis in the control group; thus,  $AIL = A - C / 100 - C$ .

An isotope recovery balance was made by addition of the radioactivity in the recovered thrombus and in the blood at the end of the experiment (multiplied by three to correct for extravascular distribution) and was expressed as percentage of the original radioactivity in the clot. Urinary ra-

dioactivity was only measured in the experiments with fresh thrombi. It amounted to 20% in experiments with extensive clot lysis. In experiments with aged clots, quantitative urine collections were not performed. Thyroidal uptake of radioiodide or accumulation of labeled material in the lungs was found to be negligible.

2-ml blood samples were drawn into trisodium citrate (final concentration 0.011 M) and 1-ml samples into a mixture of 20 NIH U of thrombin and 250 U of aprotinin (Trasylol, Bayer, Leverkusen, West Germany) after removal of the vessel clamps and before the start of the infusion and at hourly intervals for 6 h. These plasma samples were used for measurements of radioactivity, fibrinogen (16),  $\alpha_2$ -antiplasmin (17), fibrinogen degradation products (18), and activated partial thromboplastin times (19). Plasminogen was measured with the chromogenic substrate S-2251 (20) after activation for 10 min at 37°C with 1,000 IU of urokinase/5  $\mu$ l plasma of which the plasmin inhibitors were first neutralized by acidification (21). EPA was measured with a two-site immunoradiometric assay (22).

The activated partial thromboplastin time before anticoagulation was  $29 \pm 0.5$  s (mean  $\pm$  SEM,  $n = 58$ ), before the start of the thrombolytic experiments (i.e., 14 h after the last injection of 500 IU s.c. of heparin/kg body weight) it was  $38 \pm 2.0$  s ( $n = 80$ ), and at the end of the experiments (i.e., 6 h after the heparin injection given at the beginning of the experiment) it was  $55 \pm 3.2$  s ( $n = 98$ ). These findings indicate that the aging of the clots and the thrombolytic experiments were performed under mild anticoagulation with heparin. This proved to be necessary to prevent extension of the clot and layering of the radioactive clot with cold fibrin, which would have interfered with the quantitation of the degree of thrombolysis by means of measurements of residual radioactivity in the clot.

A total of 185 experiments were performed. From these, 16 were excluded because of the following reasons: six rabbits died during the experiment probably due to an overdose of analgesics, in two rabbits part of the thrombolytic substance was infused paravenously, one animal developed a large hemorrhage in the neck, there were three technical failures due to rupture of the vein during surgery, one rabbit was eliminated because of a spinal fracture with paralysis, and three rabbits developed diarrhea and died before thrombolysis scheduled on the 7th d could be performed. 16 experiments, performed in non-anticoagulated rabbits with clots aged for 1 d, were omitted from the final analysis for reasons explained in the discussion. Thus, the present study is based on 153 completed and analyzed experiments.

*Analysis of the data.* The values reported in the tables and figures represent mean values  $\pm$  SEM;  $n$  represents the number of experiments. Statistical analysis of the data and determination of the level of significance was performed according to the  $t$  test (23).

## RESULTS

The extent of thrombolysis and the isotope recovery balance are shown in Tables I and II. In the control groups infused with solvent (systemically and/or locally), the average degree of thrombolysis was  $5.6 \pm 1.4\%$  ( $n = 5$ ) after 6 h,  $14.5 \pm 1.7\%$  ( $n = 10$ ) after 30 h,  $16.0 \pm 1.5\%$  ( $n = 11$ ) after 78 h, and  $48.1 \pm 2.7\%$  ( $n = 10$ ) after 174 h. Extension of the clot was not observed in the experiments with fresh clots but occurred

TABLE I  
Thrombolysis and Isotope Recovery after Systemic Infusion

| Thrombus | Substance     | Dose    | n | Thrombolysis | P      | Isotope recovery |
|----------|---------------|---------|---|--------------|--------|------------------|
|          |               | IU      |   | %            |        | %                |
| Fresh    | Solvent       |         | 5 | 5.6±1.4      |        | 97.2±1.2         |
|          | One-chain EPA | 20,000  | 4 | 13.7±3.7     | <0.05  | 95.6±1.0         |
|          |               | 50,000  | 4 | 28.4±2.4     | <0.001 | 82.2±1.9         |
|          |               | 100,000 | 4 | 80.0±3.2     | <0.001 | 78.7±6.8         |
|          | Two-chain EPA | 20,000  | 4 | 11.5±0.7     | <0.02  | 97.3±0.8         |
|          |               | 50,000  | 4 | 38.7±5.6     | <0.001 | 77.3±2.1         |
|          |               | 100,000 | 4 | 71.3±5.5     | <0.001 | 69.2±7.0         |
|          | Urokinase     | 100,000 | 4 | 6.7±1.7      | NS     | 97.1±1.2         |
|          |               | 500,000 | 4 | 44.5±5.5     | <0.001 | 83.5±5.6         |
| 1-d      | Solvent       |         | 6 | 14.7±3.0     |        | 93.4±1.0         |
|          | One-chain EPA | 100,000 | 4 | 45.9±4.6     | <0.001 | 81.1±6.5         |
|          | Two-chain EPA | 100,000 | 4 | 44.2±5.3     | <0.001 | 81.6±2.6         |
|          | Urokinase     | 500,000 | 4 | 33.3±8.5     | <0.05  | 86.1±1.8         |
| 3-d      | Solvent       |         | 7 | 16.6±2.4     |        | 91.9±1.2         |
|          | One-chain EPA | 100,000 | 4 | 42.6±3.1     | <0.001 | 77.1±1.2         |
|          | Two-chain EPA | 100,000 | 4 | 38.0±2.6     | <0.001 | 80.7±3.3         |
|          | Urokinase     | 500,000 | 4 | 37.4±2.6     | <0.001 | 79.4±2.2         |
| 7-d      | Solvent       |         | 4 | 44.5±3.7     |        | 75.9±3.0         |
|          | Two-chain EPA | 100,000 | 4 | 73.9±1.5     | <0.001 | 58.2±5.1         |
|          | Urokinase     | 500,000 | 2 | 47.6±4.2     | NS     | 76.2±3.9         |

in two experiments with 78-h-old clots. Experience showed that this could be prevented by subcutaneous injection of heparin. Therefore, all experiments with aged clots reported in the present study were carried out under mild anticoagulation with heparin as described in Methods.

Systemic infusion of one- or two-chain EPA in animals with fresh clots resulted in a dose-dependent degree of lysis that was similar for both molecular forms of EPA (Table I). The extent of thrombolysis was ~13% with 20,000 IU of one- and two-chain EPA, 34% with 50,000 IU, and 76% with 100,000 IU. Extensive thrombolysis thus required the administration of 100,000 IU (~1 mg) of EPA. An equivalent molar amount of urokinase did not produce an increment above the spontaneous lysis, whereas a fivefold higher dose induced 45% thrombolysis.

Systemic infusion of 100,000 IU of one- or two-chain EPA in rabbits with clots aged for 1 or 3 d resulted in

44% thrombolysis, which was, however, less extensive than that obtained with the same dose in animals with fresh clots (76%). With 500,000 IU of urokinase, a similarly increased resistance to thrombolysis was observed in clots aged for 1 or 3 d (33% lysis) as compared with fresh clots (45% lysis). In clots aged for 7 d, the spontaneous degree of thrombolysis amounted to 45%. Infusion of 100,000 IU of EPA resulted in a significantly higher extent of thrombolysis (74%), whereas infusion of 500,000 IU of urokinase did not result in increased thrombolysis (48%).

The AIL determined as described in Methods, after systemic infusion of 100,000 IU of EPA, was 70% for fresh clots and 35, 30, and 50% for clots aged for 1, 3, or 7 d, respectively. With 500,000 IU of urokinase these values were 41, 22, 25, and 6% for fresh 1-, 3-, and 7-d-old clots, respectively.

The isotope recovery balance, calculated as described in Methods, ranged from 96 to 99% in the

TABLE II  
Thrombolysis and Isotope Recovery after Local Infusion

| Thrombus      | Substance     | Dose    | n | Thrombolysis | P        | Isotope recovery |
|---------------|---------------|---------|---|--------------|----------|------------------|
|               |               | IU      |   | %            |          | %                |
| 1-d           | Solvent       |         | 6 | 14.1±0.2     |          | 92.3±0.9         |
|               | One-chain EPA | 5,000   | 4 | 19.8±1.2     | <0.001   | 92.6±4.0         |
|               |               | 20,000  | 4 | 70.4±3.6     | <0.001   | 82.4±3.5         |
|               | Two-chain EPA | 5,000   | 4 | 14.5±0.6     | NS       | 92.0±2.0         |
|               |               | 20,000  | 4 | 66.1±7.3     | <0.01    | 76.8±3.8         |
|               | Urokinase     | 20,000  | 4 | 14.2±0.5     | NS       | 92.9±1.7         |
|               |               | 100,000 | 5 | 31.8±2.5     | <0.005   | 91.2±4.0         |
|               | 3-d           | Solvent |   | 4            | 14.8±1.3 |                  |
| One-chain EPA |               | 5,000   | 4 | 18.8±0.9     | <0.05    | 89.2±1.5         |
|               |               | 20,000  | 4 | 65.2±6.1     | <0.005   | 75.3±6.1         |
| Two-chain EPA |               | 5,000   | 4 | 16.9±1.7     | NS       | 90.1±1.0         |
|               |               | 20,000  | 4 | 54.0±4.8     | <0.001   | 88.5±2.9         |
| Urokinase     |               | 20,000  | 4 | 18.1±1.4     | NS       | 90.3±0.9         |
|               |               | 100,000 | 4 | 31.9±2.5     | <0.001   | 84.9±1.4         |
| 7-d           |               | Solvent |   | 4            | 55.5±5.0 |                  |
|               | Two-chain EPA | 20,000  | 4 | 77.5±2.7     | <0.01    | 59.5±6.2         |
|               | Urokinase     | 100,000 | 4 | 57.8±5.0     | NS       | 59.8±6.1         |

different groups of animals with fresh thrombi in which urine collections were performed. The radioactivity excreted in the urine amounted to 20% of the total, after infusion of 100,000 IU of EPA. The values of the isotope recoveries reported in Table I do not, however, include the urinary excretion because no urine collections were performed in animals with aged clots. If corrections could have been made for the urinary excretion of label in the experiments with aged clots, the isotope recovery balance would probably have been >90% in all groups. This indicates that the lysing thrombi were well kept in place by the woolen thread and that no significant loss by embolization occurred. This was confirmed by occasional measurements of radioactivity in the lungs.

Local infusion of one- or two-chain EPA in rabbits with clots aged for 1 or 3 d, resulted in a much higher degree of thrombolysis than that obtained by systemic infusion (Table II). Thus, 20,000 IU given locally produced 64% lysis in 1- or 3-d-old clots, whereas 100,000 IU systemically yielded 44% thrombolysis. Likewise, 100,000 IU of urokinase locally resulted in a degree of thrombolysis similar to that obtained with 500,000 IU systemically.

Local infusion of 5,000 IU of EPA or 20,000 IU of urokinase did not result in a significant increment of spontaneous thrombolysis, suggesting that these fibrinolytic substances have to be infused above a certain threshold rate. Local infusion of 20,000 IU of EPA still produced extensive thrombolysis of clots aged for 7 d, whereas 100,000 IU of urokinase did not, confirming that 7-d-old clots are resistant to urokinase but still susceptible to EPA.

The AIL obtained with local infusion of 20,000 IU EPA was 63, 53, and 49% for clots aged for 1, 3, or 7 d, respectively, as compared with 20, 20, and 5%, respectively, with 100,000 IU of urokinase. These results indicate that the amount of activator required for thrombolysis is 5 to 10 times lower when given in local infusion as compared with systemic infusion.

The isotope recovery balances in the animals with local infusion (Table II) were comparable with those in the groups in which a similar degree of thrombolysis was obtained by systemic infusion.

The changes in the blood radioactivity during thrombolysis are summarized in Fig. 2. After restoration of the blood flow, the radioactivity in the blood rose to ~5% of the injected radioactivity, probably

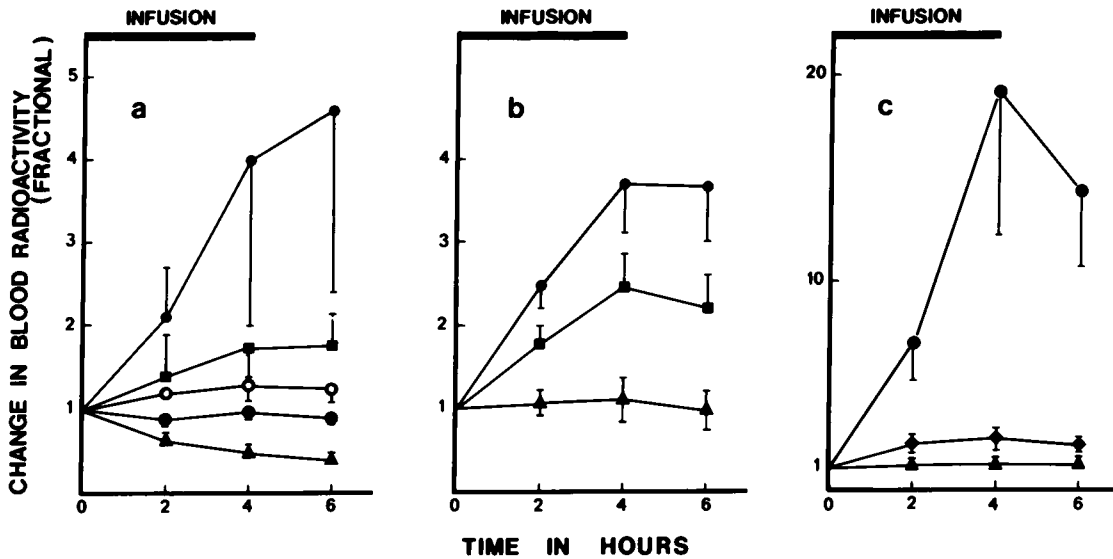


FIGURE 2 Evolution of the blood radioactivity during infusion of EPA or urokinase in rabbits with jugular vein thrombosis. a. Dose-response in rabbits with fresh thrombi.  $\blacktriangle$ — $\blacktriangle$ , solvent ( $n = 5$ );  $\odot$ — $\odot$ , 20,000 IU ( $n = 8$ );  $\circ$ — $\circ$ , 50,000 IU ( $n = 8$ ); and  $\bullet$ — $\bullet$ , 100,000 IU ( $n = 8$ ) of EPA;  $\blacksquare$ — $\blacksquare$ , 500,000 IU of urokinase ( $n = 4$ ). b. Dose-response in rabbits infused systemically with solvent ( $\blacktriangle$ — $\blacktriangle$ ,  $n = 10$ ), 100,000 IU EPA ( $\bullet$ — $\bullet$ ,  $n = 28$ ), and 500,000 IU urokinase ( $\blacksquare$ — $\blacksquare$ ,  $n = 14$ ) (all groups combined). c. Dose-response in rabbits infused locally with solvent ( $\blacktriangle$ — $\blacktriangle$ ,  $n = 12$ ), 20,000 IU EPA ( $\odot$ — $\odot$ ,  $n = 20$ ), and 100,000 IU urokinase ( $\blacklozenge$ — $\blacklozenge$ ,  $n = 12$ ) (all groups combined). The data represent means and the vertical bars SEM.

due to washing out of nonclotted labeled fibrinogen and to a lesser extent of free radioactivity. In the experiments with fresh thrombi (Fig. 2 a), the blood radioactivity thereafter slowly declined in the control group and in the low-dose urokinase group (not shown) to about 50% of the initial value after 6 h. Systemic infusion of 20,000 IU of EPA resulted in a delayed decrease of the blood radioactivity. With 50,000 IU of EPA, the blood radioactivity rose by 25%, with 500,000 IU of urokinase by 75%, and with 100,000 IU of EPA by 400%. In all experiments with systemic infusion taken together the blood radioactivity did not change significantly in the control groups, but rose 2.5-fold after infusion of 500,000 IU of urokinase and 3.5-fold after infusion of 100,000 IU of EPA (Fig. 2 b). Local infusion of 100,000 IU of urokinase produced a 2.5-fold increase of the blood radioactivity, whereas 20,000 IU of EPA produced a nearly 20-fold increase (Fig. 2 c). Local infusion of 5,000 IU of EPA or 20,000 IU of urokinase did not produce a rise in the blood radioactivity. These findings confirm the higher specific thrombolytic activity of EPA as compared with urokinase.

The changes in the concentration of EPA in the blood are summarized in Fig. 3. Infusion of 5,000 IU (55  $\mu$ g) of EPA produced a steady-state EPA level in

the blood during the entire course of the infusion of  $0.79 \pm 0.13$  IU or  $8.7 \pm 1.4$  ng ( $n = 32$ )/ml (mean  $\pm$  SEM of all measurements 2 and 4 h after the start of the infusion). With 20,000 IU (220  $\mu$ g) the concentration rose to  $3.4 \pm 0.31$  IU or  $37 \pm 3.4$  ng/ml ( $n = 40$ ), and with 100,000 IU (1.1 mg) to  $14.5 \pm 2.0$  IU or  $160 \pm 22$

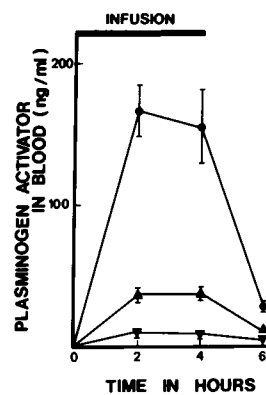


FIGURE 3 Concentration of EPA in plasma during its infusion.  $\blacktriangledown$ — $\blacktriangledown$ , all groups infused locally with 5,000 IU ( $n = 16$ );  $\blacktriangle$ — $\blacktriangle$ , all groups infused locally with 20,000 IU ( $n = 20$ );  $\bullet$ — $\bullet$ , all groups infused systemically with 100,000 IU ( $n = 20$ ). The data represent means and the vertical bars SEM.

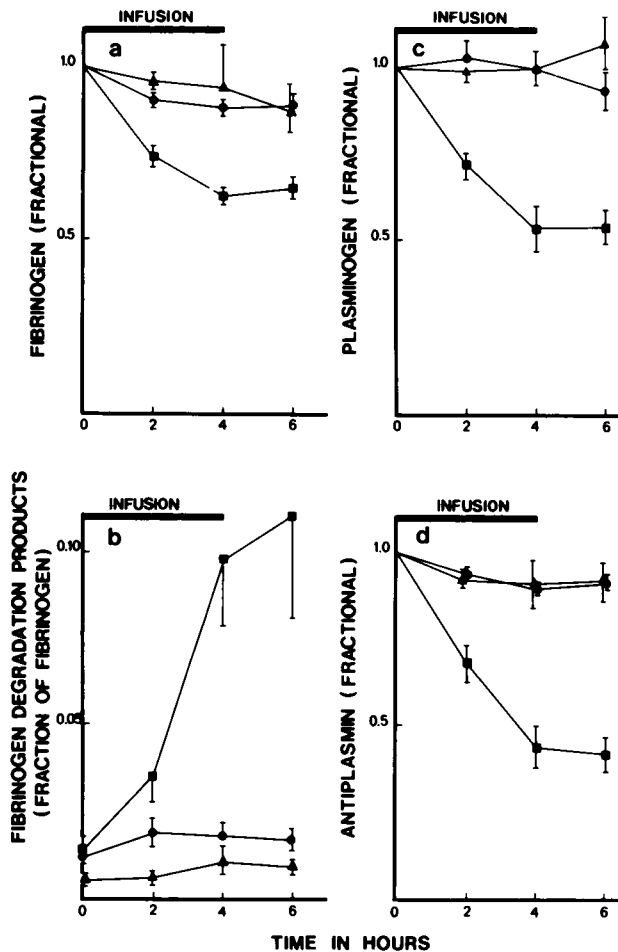


FIGURE 4 Evolution of relevant hemostatic parameters during infusion of EPA or urokinase in rabbits with jugular vein thrombosis. ▲—▲, all groups infused with solvent ( $n = 9$ ); ●—●, all groups infused with 100,000 IU EPA ( $n = 28$ ); ■—■, all groups infused with 500,000 IU urokinase ( $n = 14$ ). The data represent means and the vertical bars represent the SEM.

ng/ml ( $n = 56$ ). These findings indicate that the concentration of EPA in the blood is proportional to the rate of its infusion and that a constant steady-state concentration is obtained with a constant infusion rate.

Fig. 4 presents the evolution of the most relevant hemostatic parameters in blood during and 2 h after the infusion. The fibrinogen level remained essentially unchanged in the control groups and the groups infused with 100,000 IU of EPA (Fig. 4 a) and only very small amounts of fibrinogen degradation products were generated in these groups (Fig. 4 b). Infusion of 500,000 IU of urokinase, however, was associated with partial defibrinogenation evidenced by a decrease of the plasma fibrinogen level to 65% and the generation

of fibrinogen degradation products up to a concentration equivalent to 10% of the plasma fibrinogen. Infusion of EPA or urokinase at the concentrations used in the other experimental groups did not produce measurable changes in the plasma levels of fibrinogen or fibrinogen degradation products. In line with these observations, infusion of 500,000 IU of urokinase provoked extensive systemic activation of the fibrinolytic system with a drop of the plasminogen level to ~55% of the preinfusion value (Fig. 4 c) and of  $\alpha_2$ -antiplasmin to ~40% (Fig. 4 d). No evidence for systemic activation of the fibrinolytic system was observed in the other experimental groups.

## DISCUSSION

The present study was undertaken to further investigate the thrombolytic properties of EPA *in vivo* and to evaluate some of the factors that determine the sensitivity of a thrombus to lysis. We have previously studied the thrombolytic effect of EPA in rabbits with an experimental pulmonary embolus (10) and in dogs with experimental femoral vein thrombosis (11). In the former model, only very limited thrombolysis was obtained. In retrospect, this appeared to be due mostly to very limited accessibility to the clot and not to poor reactivity of the rabbits' fibrinolytic system (24). The femoral vein thrombosis model in dogs yielded higher degrees of thrombolysis but this model is too expensive in terms of experimental animals, surgical equipment, and amounts of thrombolytic agents required to apply it on a larger scale. In order to circumvent some of these problems, we have developed a jugular vein thrombosis model in rabbits that can be studied in a normal laboratory environment and in which the extent of thrombolysis can be accurately quantitated. Our model is similar to a vena cava inferior thrombosis model recently described (25) but it has the advantage that the surgical intervention is minimal, does not require opening of the peritoneal cavity, and therefore is well suited to study the lysis of clots aged *in vivo*. The rabbit jugular vein thrombosis model is simple; the failure rate is <10%. The model is also quantitative and very reproducible as evidenced by the small SEM values. One limitation, however, is that thrombolysis of aged clots *in vivo* can be quantitated only if additional deposition of unlabeled fibrin on the clot surface is prevented by heparinization. Indeed, systemic infusion of 100,000 IU of EPA in anticoagulated rabbits with clots aged for 24 h yields 45.0% lysis, whereas the apparent lysis was only 31% if anticoagulation was omitted. We have selected to use a standard infusion time of 4 h, not only to be able to complete the experiments within a working day but also because prolonged anesthesia without adequate monitoring and

correction of vital parameters (oxygenation, blood, pH, etc.) probably would be associated with a variety of complications that might confound the results. This short infusion time further limits the extrapolation of results obtained in an experimental "thrombosis" model to patients with clinical thrombosis in whom prolonged infusion for a few days is feasible.

The present results confirm and extend the conclusions of our two earlier studies that EPA is a thrombolytic agent with a specific activity, which is an order of magnitude greater than that of urokinase. In addition the present study shows again and in more detail that EPA can induce extensive thrombolysis without disseminated activation and consumption of the fibrinolytic system.

Some additional findings may be important for the development of therapeutic regimens with EPA in man. Firstly, it appears that the one- and the two-chain forms of activator have very similar thrombolytic properties. This finding is in agreement with their similar kinetic properties (8) but at variance with the borderline-significant difference found in the dog femoral vein thrombosis model (11). Secondly, it appears that in vivo aging of a clot has relatively little influence on its susceptibility to lysis by EPA in contrast to that with urokinase infusion. A third important conclusion of the present study is that the efficacy of the thrombolytic agents (both EPA and urokinase) is much higher when they are delivered into the immediate vicinity of the thrombus. Local infusion of 20,000 IU of EPA resulted in a higher degree of thrombolysis than systemic infusion of 100,000 IU. For urokinase the higher efficacy of local vs. systemic infusion was also evident although somewhat less marked.

The effect of increasing doses of EPA and urokinase was investigated in rabbits with fresh clots. Infusion of increasing amounts of EPA yielded increasing degrees of thrombolysis, but the dose response appeared not to be linear both for EPA and for urokinase.

The steady-state concentration of EPA in the blood was found to be directly proportional to the rate of infusion and was maintained at a constant level throughout the infusion period (measured half-way and at the end). We have previously shown that the  $t_{1/2}$  of EPA in rabbits is 2–3 min corresponding to a catabolic rate of 35 or 23% of the plasma pool/min. Infusion of 5,000 IU of EPA over 4 h corresponds to an infusion rate of  $\sim 20$  IU/min or assuming a plasma volume of 40 ml/kg (body weight 2.5 kg),  $\sim 0.2$  IU/ml plasma per min. Thus, a plasma level of 0.79 IU/ml is within the range expected from the  $t_{1/2}$  of EPA in rabbits and the infusion rate. Likewise, infusion of 20,000 IU over 4 h, or 80 IU/min, or 0.8 IU/ml plasma per min is in agreement with a steady-state plasma level of 3.4 IU/ml and a clearance rate of 25–35%/

min. Infusion of 100,000 IU over 4 h or 4 IU/ml plasma per min counterbalances a clearance of 3.5–5 IU/ml resulting in a plasma level of  $\sim 15$  IU/ml.

These considerations can probably be extrapolated to patients in the following way. The  $t_{1/2}$  of EPA in man has been estimated to be 10–15 min, corresponding to a clearance rate of 7% of the plasma pool per min. For a body weight of 70 kg and a plasma volume of 35 ml/kg this corresponds to a clearance of 175 ml plasma/min. Thus, to maintain a steady-state EPA level of 1 IU/ml, an infusion rate of 250,000 IU/24 h should be maintained. This is in reasonable agreement with actual levels of EPA measured in a few patients after infusion of EPA (unpublished observations).

The levels of EPA in blood required to obtain significant thrombolysis in 4 h are, however,  $>1$  IU/ml. This level is much higher than the normal basic level of  $\sim 0.2$  IU/ml in man and comparable with the peak level, which is transiently obtained following exhaustive physical exercise or venous occlusion (22). This may explain why the physiological dissolution of a thrombus in vivo in man, which fortunately occurs spontaneously in the majority of cases, is a time-consuming process. From the present experiments we cannot extrapolate to which extent a moderate increase of the EPA level in blood for prolonged periods of time may affect thrombus dissolution in man. In two selected cases systemic infusion of 5–7.5 mg (450,000–675,000 IU) EPA over 24 h resulted in thrombolysis (12) but in a few patients with extensive deep vein thrombosis over very long segments such a regimen did not result in clearance of the veins (unpublished observations). In the framework of our present results this is not too surprising because in the latter patients neither a high EPA level nor a prolonged period of a moderate increase of this level was obtained. It follows that the treatment of extensive deep vein thrombosis will probably require in the order of centigram quantities of EPA by systemic infusion and probably 5–10 times less when local administration is feasible. These expectations are in agreement with the clinical observation that significant thrombolysis in deep vein thrombosis with urokinase is only obtained with very high doses infused in one day or with prolonged infusion for several days.

Finally, it appears that rheological parameters and particularly the accessibility of EPA for a clot or its delivery in high concentration into the vicinity of a thrombus are probably major determinants for the thrombolytic process. This is exemplified not only by the different thrombolytic efficacy of systemic vs. local infusion of the thrombolytic substances but also by the higher resistance of clots produced in vitro and injected into the lungs (10) as compared with jugular



vein thrombi. It follows that supportive measures that direct the flow of EPA towards a clot or that avoid its dilution into collateral circulation may be determining factors for successful thrombolysis.

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