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Turnover of Human Extrinsic (Tissue-Type) Plasminogen Activator in Rabbits

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Key words

Extrinsic (tissue-type) plasminogen activator – Turnover in rabbits – Clearance mechanism – Inactivation

Summary

The turnover of highly purified human extrinsic plasminogen activator (EPA) (one- and two-chain form) was studied in rabbits. Following intravenous injection, EPA-activity declined rapidly. The disappearance rate of EPA from the plasma could adequately be described by a single exponential term with a t ½ of approximately 2 min for both the one-chain and two-chain forms of EPA.

The clearance and organ distribution of EPA was studied by using ^{125}I-labeled preparations. Following intravenous injection of ^{125}I-labeled EPA the radioactivity disappeared rapidly from the plasma also with a t ½ of approximately 2 min down to a level of 15 to 20 percent, followed by a small rise of blood radioactivity. Gel filtration of serial samples revealed that the secondary increase of the radioactivity was due to the reappearance of radioactive breakdown products in the blood. Measurement of the organ distribution of ^{125}I at different time intervals revealed that EPA was rapidly accumulated in the liver, followed by a release of degradation products in the blood.

Experimental hepatectomy markedly prolonged the half-life of EPA in the blood. Blocking the active site histidine of EPA had no effect on the half-life of EPA in blood nor on the gel filtration patterns of ^{125}I in serial plasma samples.

It is concluded that human EPA is rapidly removed from the blood of rabbits by clearance and degradation in the liver. Recognition by the liver does not require a functional active site in the enzyme. Neutralization in plasma by protease inhibitors does not represent a significant pathway of EPA inactivation in vivo.

Introduction

Plasminogen activation by extrinsic (vascular or tissue) plasminogen activators (EPA) represents a major pathway in the initiation of fibrinolysis in vivo. Some properties of EPA have been studied, such as their distribution in body tissues (1), release mechanisms (2) and biological significance (3).

Plasminogen activators have been highly purified from both human plasma (4) and human or animal tissues (5–7), but detailed biochemical and biological investigations have been hampered by the small amounts of purified material obtained. Recently, plasminogen activator has been purified in mg quantities from the culture fluid of a human melanoma cell line (8).

Two different forms of EPA have been purified (8, 9). One-chain EPA is presumably identical with the vascular or blood plasminogen activator, whereas the two-chain form represents a proteolytic breakdown product of the single-chain activator (8, 9). The availability of sufficient amounts of EPA has enabled us to initiate a series of studies on its biochemical (8–10), biological (11, 12) and thrombolytic (13, 14) properties. In the present study we report data on the turnover of this activator in rabbits.

Materials and Methods

One-chain and two-chain extrinsic plasminogen activator (EPA) was highly purified from the culture fluid of a human melanoma cell line by a method described elsewhere (8). The activity was expressed in international units (IU) of urokinase by comparison of its fibrinolytic activity on plasminogen-enriched bovine fibrin films, as previously described (8). The activator solutions were kept frozen in 0.15 M NaCl, containing 10 µl Tween 80 per liter. Both one-chain and two-chain EPA were labeled with ^{125}I according to McFarlane (15). Free ^{125}I was then removed by affinity chromatography on zinc chelate-sepharose. Further purification of the ^{125}I-labeled EPA was achieved by gel filtration on Ultrogel AcA 34, whereby radioactive aggregates and breakdown products were removed and only the main radioactive peak with a M_r of ~ 70,000 was recovered. The ^{125}I-labeled EPA preparations contained approximately 1 atom of iodine per molecule, had a specific activity of 100 IU/µg and appeared to be unaltered as evidenced by their specific activity, electrophoretic mobility, size distribution and binding to fibrin.

Irreversible blocking of the active site histidine of ^{125}I-EPA was achieved by incubation (10 min room temperature) with 10^{-4} M (final concentration) of D-Phe-Pro-Arg-CH_2Cl (16), a synthetic peptide chloromethylketone, which inhibits two-chain EPA with a rate constant of 5,000 $M^{-1}s^{-1}$ and one-chain EPA with a rate constant of 300 $M^{-1}s^{-1}$ (unpublished observations).

Gel filtration was performed at 4°C, on 2.5 × 40 cm Ultrogel AcA 34 (LKB, Bromma, Sweden) columns, equilibrated with 1 M NaCl, 0.1 M Tris, 0.01 M Na-Citrate, pH 7.4, containing 10 µl Tween 80 per liter. 3 ml fractions were collected at a flow rate of 25 ml/hr. Plasma samples of 1 ml were applied to the columns.

Turnover of Unlabeled One-Chain and Two-Chain EPA

New Zealand White rabbits with a bodyweight of about 3 kg were anesthesized with "Hypnorm®" (0.5 ml, i. m.) and pentobarbital (0.3 ml, i. v.). A catheter was introduced in the superficial femoral vein for blood sampling (Portex-white, Portex, Hythe, U. K.). Unlabeled one-chain and two-chain EPA (20,000 IU in 3 ml) were injected in one rabbit each. At fixed time intervals up to 30 min, 4 ml blood samples were collected in trisodium-citrate (final concentration 0.01 M), immediately transferred to an ice bath and centrifuged at 0°C. The euglobulins of the plasma were precipitated at pH 5.8 and dissolved in the same volume of gelatin-buffer (0.05 M Na-barbital-0.1 M barbituric acid pH 7.75 containing 2.5 mg calf skin gelatin per ml). The euglobulin fibrinolytic activity was measured on plasminogen-enriched bovine fibrin films as described elsewhere (8).

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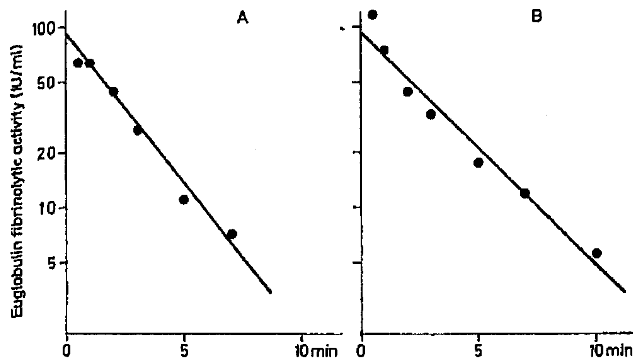

Fig. 1 Turnover of unlabeled EPA in rabbits. The disappearance rate of the euglobulin fibrinolytic activity from plasma was measured following intravenous injection of 20,000 IU EPA. A: two-chain EPA, B: one-chain EPA

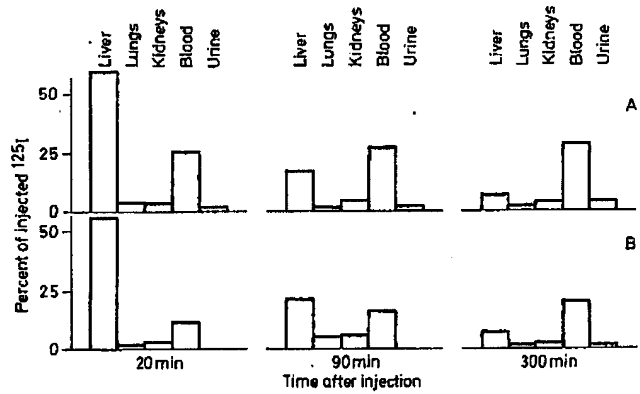

Fig. 4 Organ distribution of radioactivity after injection of ^{125}I-labeled EPA. A: unblocked two-chain EPA, B: unblocked one-chain EPA. The animals were sacrificed 20 min, 90 min or 300 min after injection.

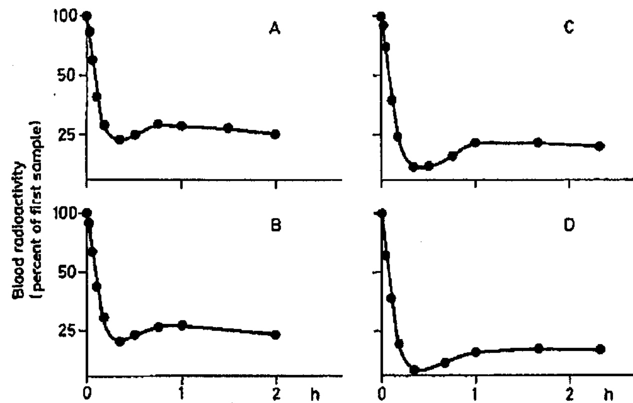

Fig. 2 Turnover of ^{125}I-labeled EPA in rabbits. The blood radioactivity after injection of ^{125}I-labeled EPA was measured and expressed in percent of the radioactivity in the first sample. A. two-chain EPA (n = 6), B. two-chain EPA, active site blocked (n = 3), C. one-chain EPA (n = 3), D. one-chain EPA, active site blocked (n = 1). The data represent mean values. The S. E. M. were less than 5 percent of the mean values.

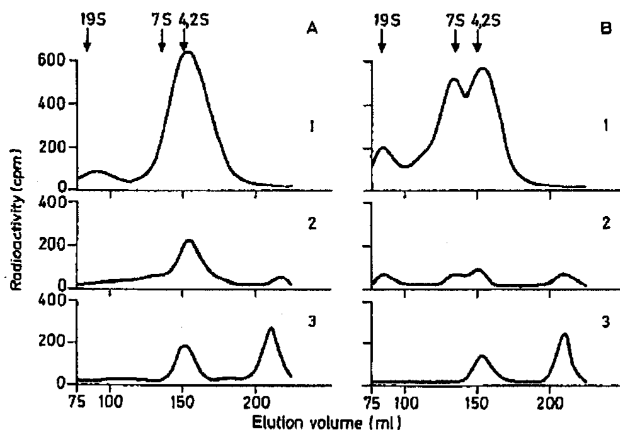

Fig. 3 Gel filtration patterns of ^{125}I in plasma samples following injection of ^{125}I-labeled EPA. A: two-chain EPA; B: one-chain EPA, 1:0.5 min, 2:20 min and 3:120 min after injection of EPA. 19 S, 7 S and 4.2 S: elution position of macroglobulins, IgG and albumin, respectively.

Turnover of ^{125}I-Labeled EPA

^{125}I-labeled EPA was injected in 13 rabbits. The turnover of the following ^{125}I-labeled materials was studied: one-chain EPA (3 rabbits), one-chain EPA with the active site blocked (1 rabbit), two-chain EPA (6 rabbits) and blocked two-chain EPA (3 rabbits). In order to prevent thyroid uptake of ^{125}I, 1 ml of a 1 percent solution of NaI was given intravenously 30 min before injection. Approximately 2μCi ^{125}I-EPA (500 ng protein) was administered by a single bolus injection via a marginal ear vein. At fixed time intervals, 4 ml blood samples were collected in citrate. 20 μl D-Phe-Pro-Arg-CH_2Cl 10^{-2} M was immediately added to the blood samples in order to prevent reaction with plasmatic inhibitors in vitro. After radioisotope counting, the blood samples were centrifuged and the plasma was stored at −20°C for further analysis. At 20 min, 90 min or 300 min after ^{125}I-EPA injection the animals were sacrificed and the ^{125}I content of liver, lungs, kidneys and urine was determined.

One-chain ^{125}I-EPA was injected in two rabbits which were functionally hepatectomized by ligating the hepatic artery and the portal vein. The disappearance rate of ^{125}I from the blood was followed for 45 min after which the animals were sacrificed and the ^{125}I content of different organs measured.

Results

Turnover of Unlabeled One-Chain and Two-Chain EPA

Fig. 1 illustrates the disappearance rate of the fibrinolytic activity in the euglobulin fraction of rabbit plasma following injection of 20,000 IU EPA (one-chain and two-chain). The euglobulin fibrinolytic activity increased to approximately 100 IU/ml after the injection of EPA. Assuming a plasma volume of 40 ml/kg this represents a recovery of approximately 60 percent of the injected EPA activity. The euglobulin fibrinolytic activity declined rapidly, with a half-life of about 3 min for one-chain EPA and of 2 min for two-chain EPA.

Turnover of ^{125}I-Labeled EPA

Fig. 2 shows the plasma disappearance curves of ^{125}I after injection of ^{125}I-labeled EPA. Very similar curves were obtained for the one-chain EPA (3 experiments) and the two-chain EPA (6 experiments) preparations as well as for preparations with blocked or unblocked active center. The blood radioactivity initially declined very rapidly with a t $^1/_2$ of about 2.5 min. Subsequently a slight rise in blood radioactivity was followed by a very slow decline over a few hrs.

Gel Filtration of Plasma Samples

Fig. 3 shows typical elution patterns on Ultrogel AcA 34 of plasma samples obtained at different time intervals following injection of ^{125}I-EPA.

Thirty seconds after injection of ^{125}I-labeled two-chain EPA (Fig. 3A1) the radioactivity elutes as a small peak (5–10 percent of total) in the void volume and a main peak in the 4.2 S region, corresponding to unchanged EPA with a M_r of ~ 70,000. The elution patterns of later samples (Fig. 3A2, 3A3) are characterized by disappearance of the small peak at the void volume, by a progressive decrease of the activity eluting in the 4.2 S region and by the appearance of a new peak in the total volume of the column, corresponding to radioactive breakdown products.

The radioactive component corresponding to intact EPA initially disappeared with a t $^1/_2$ between 2 and 3 min both for unblocked and blocked ^{125}I-labeled two-chain EPA. A small amount of radioactive material (less than 5 percent of the initial radioactivity) however was still found in the position of ^{125}I-EPA after 120 min. This component may represent a labeled contaminant with a much longer t $^1/_2$ than EPA.

Thirty seconds after injection of ^{125}I-labeled one-chain EPA (Fig. 3B1) the radioactivity elutes as a minor peak at the void volume and two main peaks, one in the 7 S region and one in the 4.2 S region. Identical patterns were obtained after injection of blocked or unblocked ^{125}I-EPA. These results suggest either dimerisation of one-chain EPA, or binding to another plasma protein with a M_r of ~ 70,000. Since the active site of EPA seems not to be required for the generation of the component with an apparent M_r of 150,000, complex formation with an inhibitor of EPA in plasma cannot explain this phenomenon. In later samples (Fig. 3B2, 3B3) the radioactivity eluting in the void volume, the 7 S region and the 4.2 S region decreases rapidly. Concomitantly, a new peak probably representing small breakdown products appears at the total volume of the column.

The t $^1/_2$ of the component eluting in the 4.2 S region was between 2 and 3 min both for unblocked and blocked ^{125}I-EPA. Again a small amount of radioactive material, eluting in the position of ^{125}I-EPA persisted after 120 min.

The evolution of the blood radioactivity after injection of ^{125}I-labeled one-chain EPA was followed in two hepatectomized rabbits. No significant disappearance of plasma radioactivity occurred within 45 min. Gel chromatography on Ultrogel AcA 34 revealed the lack of ^{125}I-EPA fragmentation, as no additional radioactive peak at the total volume was formed (not shown).

Organ Distribution of ^{125}I Following Injection of ^{125}I-Labeled EPA

The organ distribution of radioactivity in rabbits sacrificed 20, 90 or 300 min after injection of ^{125}I-EPA is represented in Fig. 4. The results were very similar for one-chain and two-chain EPA, whether unblocked or blocked (not shown). Twenty min after injection, about 60 percent of the injected radioactivity was found in the liver, after 90 min roughly 20 percent and after 300 min about 6 percent. Little radioactivity (2 to 3 percent) was found in the kidneys, urine and lungs. The blood radioactivity reached a plateau at about 25 percent of the injected amount.

Discussion

With the recent development of a purification method for human extrinsic plasminogen activator yielding milligram amounts of highly purified material (8) it has become possible to investigate its thrombolytic properties (11, 13, 14). Since EPA appears to induce specific thrombolysis in animals with experimental thrombosis, we have initiated a series of studies aiming at the development of EPA into a new thrombolytic drug. In order to devise possible therapeutic schemes we have in the present study investigated the turnover of EPA and its mechanisms of clearance from the blood.

Our findings indicate that the t $^1/_2$ of unlabeled purified EPA in plasma is 2–3 min in rabbits. This is in agreement with earlier reports (17, 18) that the t $^1/_2$ of activator released into the blood in humans is 10–15 min, considering that the turnover rates of plasma proteins in different species are inversely proportional to the bodyweight. We found no difference in t $^1/_2$ between one-chain and two-chain EPA suggesting that the partial proteolytic degradation does not significantly alter the turnover properties of EPA.

The t $^1/_2$ of ^{125}I-labeled one-chain and two-chain EPA was similar to that of unlabeled EPA and no difference was observed between EPA with a free or an irreversibly blocked active site. This observation indicates that a functional active site is not important for the life span of EPA in the blood and that the neutralization of the activator in the blood stream does not occur by enzymatic inhibition. These findings also suggest that reversible blocking of the active site of EPA with active site titrants such as p-anisic acid (19) will not prolong its biological half-life.

The use of ^{125}I-labeled EPA also allowed to study its mechanisms of clearance from the blood. After injection of ^{125}I-EPA a rapid accumulation of tracer was found in the liver (about 60 percent of the injected amount after 20 min) followed by a progressive appearance of small degradation products in the blood. The rate of uptake of EPA by the liver was similar for one-chain and two-chain activator, either with blocked or unblocked active site. This indicates that the liver contains receptor(s) for EPA but that these receptor(s) do not interact with the active site of the enzyme.

The predominant role of the liver in the clearance of EPA from the blood was confirmed by our observation that its half-life became very long in rabbits with a functional hepatectomy.

Gel filtration of serial plasma samples in a 1 M NaCl containing buffer also revealed that the two-chain EPA circulates as an uncomplexed molecule with an apparent M_r of 70,000 whereas the single-chain EPA, whether blocked or unblocked elutes in two peaks, one with $M_r \simeq 70,000$ and one with $M_r \simeq 150,000$. Whereas the former peak probably represents monomeric EPA, the latter may represent either dimerized EPA or a complex with a carrier protein of similar molecular weight. Since a functional active site is not required for formation of the M_r 150,000 component, complex formation with an inhibitor cannot explain this phenomenon.

In conclusion our study indicates that EPA has a short half-life in the blood (2–3 min in rabbits), due to rapid clearing by the liver. The disappearance of activator from the blood is very similar for the intact one-chain form and for its proteolytically degraded two-chain form and blocking of the active site of the enzyme does not change its turnover rate. These findings suggest that application of plasminogen activator for thrombolysis in vivo will probably require its continuous infusion.

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