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Mechanism of Clot Lysis by Streptokinase and Effects of Epsilon-Aminocaproic Acid.* (25374)

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In past years considerable experimental work, initiated in laboratories of W. S. Tillet (1), was undertaken to investigate the possibility of dissolving intravascular thrombi in humans by intravenous administration of streptokinase (SK). These and other investigators employed plasmin, prepared by activation of human plasminogen with SK, as the lytic agent, and recently reviewed by Sherry *et al.*(2). It is recognized that either form of treatment may cause certain undesirable side-effects(2) from induced hyperplasminemia, among which may be listed (a) destruction of fibrinogen and plasminogen, (b) destruction of some components involved in blood coagulation, (c) destruction of other physiologically active polypeptides and (d) elevation of antithrombin levels. Obviously it would be desirable to prevent or minimize the untoward systemic effects induced by hyperplasminemia, without interfering with dissolution of the thrombus. Epsilon-aminocaproic acid (ε-ACA), first reported to be a plasmin inhibitor (3), was recently shown by Ablondi and Hagan(4) to be a far more potent inhibitor of plasminogen activation. Subsequent studies confirmed and extended this observation to show that ε-ACA inhibited both SK and urokinase activation of plasminogen(5,6). Our more recent studies demonstrated that ε-ACA does not adsorb to a fibrin clot, a situation analogous to that found by Johnson, *vide infra,* for humoral inhibitors of fibrinolysis. It seemed worthwhile, therefore, to determine (a) the effect of ε-ACA on dissolution of a clot suspended in plasma and (b) its simultaneous effect on plasminogen activation in plasma. Our purpose here is to present data which demonstrate that, under certain conditions *in vitro,* ε-ACA can effectively inhibit activation of plasminogen by SK in plasma without markedly inhibiting the lysis of a clot immersed in such plasma.

Methods. I^{131}-labeled clots fortified with plasminogen were prepared as follows: Twelve cc of 0.9% saline were added to 1 cc aliquots of human plasma followed by 0.1 cc of a solution of I^{131}-labeled fibrinogen prepared by the method of Mihalyi and Laki(7). One-tenth cc human plasminogen containing 4000 units of proactivator(8), and prepared by the method of Kline(9), was then added. Clotting was effected at pH 7.4 by addition of 1.25 units of thrombin (Parke-Davis) in 0.1 cc volume. As clots formed they were collected by carefully winding onto glass capillary tubes (melting point tubes) to which they became tightly fixed. As much fluid as possible was squeezed from the adhered clot by pressing against inner wall of test-tube. The radioactive content of each clot was determined in a well type scintillation counter

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TABLE I. Inhibition of SK Activation of Plasminogen by ε-ACA in Plasma and Lack of Inhibition of Clot Lysis (Plasminogen Fortified Clot).

SK (u/ml)	ε-ACA conc. (M)	Clot dissolution: Clot radioactivity (cpm) at 0 time	Clot dissolution: Clot radioactivity (cpm) at 1 hr	Plasma radioactivity (cpm) at 1 hr	% of initial clot counts in plasma at 1 hr*	Fibrin plate assay zones of lysis† (d^2 in mm): Unheated	Fibrin plate assay zones of lysis† (d^2 in mm): Heated
0	0	15920	14220	1480	9.3	0	0
50	0	13770	10040	3820	28.2	64	9
100	0	14670	10040	4920	33.7	169	16
150	0	13220	8070	5290	39.5	225	25
200	0	12700	5780	6570	51.5	289	64
0	.025	17720	15660	1200	5.6	0	0
50	"	11730	9060	2535	21.6	0	0
100	"	13800	9520	5020	35.9	0	0
150	"	14000	8620	5520	39.2	0	0
200	"	15300	5940	10370	68.0	0	0

* Sum of counts on clot at 1 hr and in plasma at 1 hr equals 95-101% of initial clot count.
† .03 ml of each plasma reaction mixture was assayed by the procedure described by von Kaulla(10).

after which the clots were immersed in 10 cc 0.03 M phosphate buffer, pH 7.4 at 5°C until used. No radioactivity was washed from the clot during this period. Plasma incubation medium containing SK or SK plus ε-ACA was prepared as follows: Sodium chloride was added to an aliquot of human plasma to give a concentration of 0.025 M. ε-ACA was added to a second aliquot to give the same molar concentration. To 2 cc aliquots of plasma, containing either ε-ACA or NaCl, varying amounts of SK in 0.1 cc of buffer were added to give 0, 50, 100, 150 and 200 units of SK (Varidase®) cc. All reactants were maintained at 3-5°C to minimize instability of activator. Immediately before immersion of a radioactive clot 0.03 cc sample of each plasma reaction mixture was withdrawn and assayed for activator and plasmin activities using fibrin plate method of Astrup and Mullertz as modified by von Kaulla(10). Zones of lysis on the unheated plate served as a measure of activator and/or plasmin activity while zones of lysis on the heated plate were a measure of plasmin activity only. A prepared radioactive clot was then added to each plasma reaction mixture in a 75 × 10 mm Wassermann tube. The reaction mixtures were then warmed to 37°C in a constant-temperature water bath and incubated one hour. Then the clots were removed, squeezed free of plasma by pressing against the inner side of the tubes, and radioactivity determined in both the clots and plasma.

Results are shown in Table I. Little inhibition of clot lysis occurred in the presence of ε-ACA although activation of plasminogen was completely inhibited in the plasma incubation mixtures as measured by the fibrin plate method. Essentially similar results were obtained when the concentration of ε-ACA was increased to 0.05 M (data not shown).

It was of further interest to repeat the experiment with clots which were not fortified with plasminogen. Radioactive clots were prepared as described above, except that no exogenous plasminogen was added before clotting. Since rate of clot lysis was much slower with clots prepared in this manner, incubation time was extended to 2½ hours. The results are shown in Table II. They again demonstrate a complete inhibition by ε-ACA of plasminogen activation in plasma. However, partial inhibition of clot lysis is also apparent.

Discussion. These experiments demonstrate that clot lysis can occur in plasma in which no detectable plasmin is present because of inhibition of its formation by an inhibitor of plasminogen activation, ε-ACA. In agreement with the findings of Alkjaersig *et al.*(11) the data also demonstrate that plasminogen is adsorbed (or entrapped within) a clot as it forms and that the rate of clot lysis induced by SK is a function of clot plasminogen concentration.

Much attention has been devoted to the apparently greater fibrinolytic rather than fibrinogenolytic activity of plasmin. Studies

TABLE II. Inhibition of SK Activation of Plasminogen in Plasma by ε-ACA with Partial Inhibition of Clot Lysis (Unfortified Clot).

		Clot dissolution					
		Clot radioactivity (cpm) at		Plasma radioactivity (cpm) at 2½ hr	% of initial clot counts in plasma at 2½ hr*	Fibrin plate assay zones of lysis† (d^2 in mm)	
SK (u/ml)	ε-ACA conc. (M)	0 time	2½ hr			Unheated	Heated
0	0	12870	12550	215	1.5	0	0
50	0	11300	9270	2000	17.7	64	16
100	0	12230	9090	2860	22.8	196	16
150	0	11600	8810	2510	21.8	256	36
200	0	11450	8270	3090	26.4	361	81
0	.025	11900	11050	190	1.7	0	0
50	"	11750	11450	610	5.2	0	0
100	"	12210	11300	910	7.2	0	0
150	"	11750	11450	1060	9.1	0	0
200	"	12700	11050	1130	9.2	0	0

* Sum of counts on clot at 2½ hr and in plasma at 2½ hr equals 95-101% of initial clot count.

† .03 ml of each plasma reaction mixture was assayed by the procedure described by von Kaulla(10).

suggest that this phenomenon is due to adsorption of plasmin on fibrin as it forms, thereby resulting in a concentraion of enzyme on the substrate to be digested(12). Results of our experiments are in agreement with this interpretation (Unpublished). Of further interest, in this connection, is the observation of Johnson that inhibitors of fibrinolysis are not adsorbed to fibrin (personal communication). Indeed, Astrup(13) suggested that the presence of inhibitors of plasmin in blood interferes with splitting of soluble proteins, including fibrinogen. However, an effect of plasmin on fibrin is still exerted by virtue of its adsorption to fibrin. He states "we can but admire the ingenious way Nature has solved the extremely difficult problem of providing blood with a proteolytic system which exerts its effect only when and where it is needed."

A final speculation on the clinical application of these findings appears pertinent. It is possible that advantage would be gained by the simultaneous administration of an inhibitor of plasminogen activation along with an activator of plasminogen for treatment of thromboembolic disease. It might then be possible to inhibit or minimize the systemic formation of plasmin and thus avoid harmful effects of hyperplasminemia without significantly inhibiting clot dissolution. Experiments to test this hypothesis are underway in experimental animals.

Summary. The effects of ε-ACA, a potent inhibitor of plasminogen activation, on *in vitro* pre-formed clot lysis and plasma plasminogen activation by SK have been studied. Complete inhibition of plasma plasminogen activation by ε-ACA has been demonstrated to occur with little concomitant effect on the dissolution of a clot immersed in plasma. Rate of clot dissolution by SK is shown to be a function of clot plasminogen concentration. Potential clinical applications are discussed.

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