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ROLE OF THE ACTIVE CENTER OF ENZYMES IN TRIGGERING THE MECHANISM OF COMPENSATORY REACTION TO PLASMIN

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Plasmin (EC 3.4.21.7) is a serine proteinase whose function is to maintain the fluid state of the blood. Besides hydrolysis of fibrinogen and fibrin, plasmin also causes activation of high-molecular-weight kininogen, factors XII and VII, factors C1 and C3 of complement, and prorenin, and has a controlling influence on the level of plasma components of hemostasis, namely factors II, V, VIII, and XIII, and on platelet aggregation. The proteolytic activity of the enzyme is limited by the α_2-antiplasmin of the blood and through a physiological compensatory reaction leading to release of plasmin inhibitors and procoagulants into the bloodstream [11]. Plasmin binds highly specifically with the endothelium [7] and excites the receptor appartus of the vascular wall, with consequent activation of the sympathetic division of the autonomic nervous system [2]. Vasoactive reactions evoked by catecholamines lead to the release of procoagulants and of fibrinolysis inhibitors from the vascular wall into the bloodstream. Catecholamines also activate the contact phase of blood clotting [3] and stimulate platelet aggregation [10]. The structural features of the enzyme responsible for the triggering mechanism of the compensatory reaction are not yet known.

It was decided to study the role of the active center of the enzyme in realization of the triggering mechanisms of the compensatory reaction, using plasmin with a chemically modified active center.

EXPERIMENTAL METHOD

Plasmin was obtained by activation of human plasminogen (Leningrad Research Institute of Hematology and Blood Transfusion, USSR) and streptokinase (Streptase, Behringwerke, West Germany). Activation was carried out in the proportion of 33 U of streptokinase to 1 CTA U of plasminogen. The plasminogen was homogeneous on electrophoresis in polyacrylamide gel with SDS and had a molecular weight of 87 ± 2 kilodaltons. The caseinolytic activity of the plasmin was 14-16 CTA U/mg protein. The amidase activity of the plasmin preparation was determined spectrophotometrically from the rate of hydrolysis of valine–leucine–lysine paranitroanilide (S-2251, from "Kabi Diagnostica," Sweden), by the method in [13]. The plasminogen

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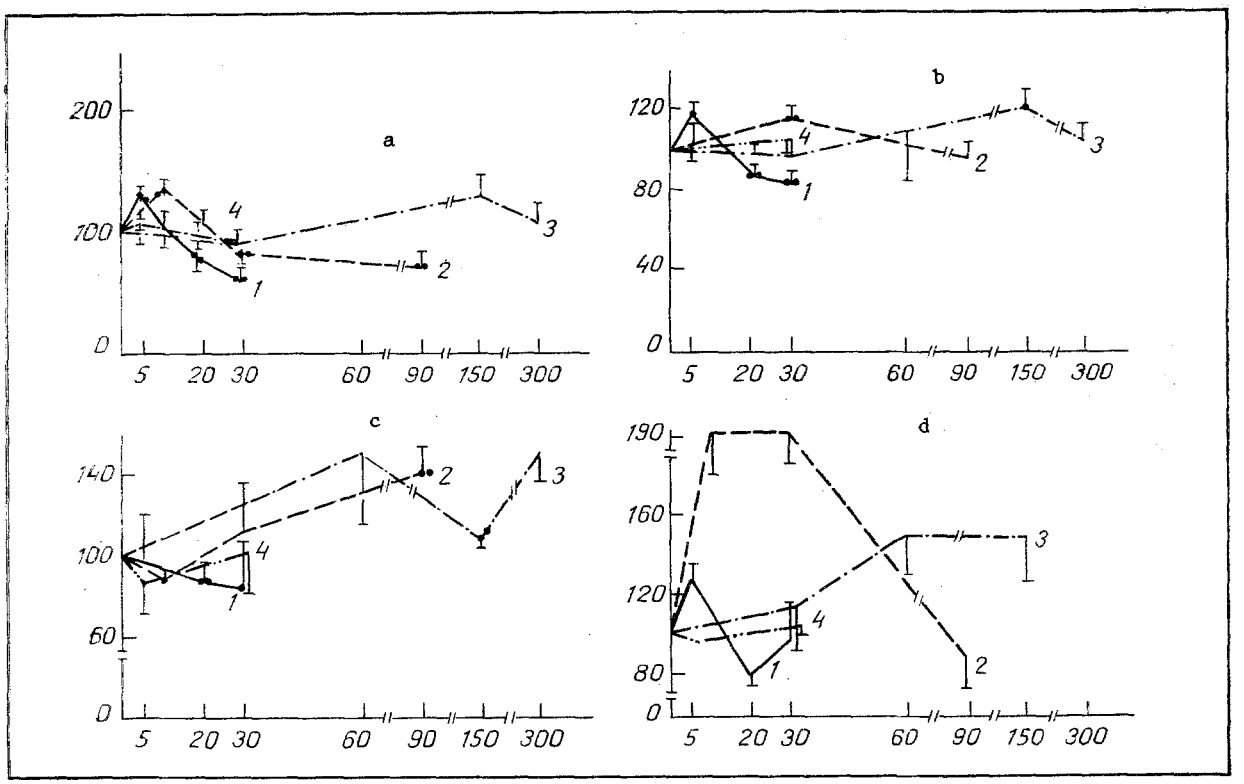

Fig. 1. Changes in parameters of hemostasis system (M ± m, %) after injection of 1 μM plasmin, An-Pl, Gb-Pl, and DIP-plasmin. Abscissa, time after injection of preparations (in min); ordinate, changes in parameters of hemostasis system (in per cent of control level); values of parameters before injection of preparations taken as 100%. a) Total blood clotting time, b) thrombin time, c) fibrinogen concentration, d) fibrinolytic activity. 1) Plasmin, 2) An-Pl, 3) Gb-Pl, 4) DIP-plasmin. One filled circle on curve denotes $p < 0.05$, two – $p < 0.01$, three – $p < 0.001$.

and plasmin concentrations were calculated on the assumption that $A_{280}^{1\%} = 17.0$ [12]. Acyl substituents were introduced into the active center of plasmin by inactivation of the enzyme by acylating agents: the p-amidophenyl ester of p'-anisic acid (anisoyl-plasmin, An-Pl) and the nitrophenyl ester of p'-guanidinebenzoic acid (guanidinebenzoyl-plasmin, Gb-Pl) [1]. The deacylation constant (K_3) of An-Pl is $3.7 \cdot 10^{-4}$ sec^{-1}, and Gb-Pl $6.26 \cdot 10^{-5}$ sec^{-1}. The half-reactivation time of An-Pl was 30 min, and of Gb-Pl 180 min. Irreversible blockade of the active center of plasmin was carried out by the method [9], by treating $3 \cdot 10^{-5}$ M of the enzyme with di-isopropylfluorophosphate (DFP, from "Fluka," West Germany) in a final concentration of 100 μM. Experiments involving intravenous injection of plasmin and its modified forms were carried out on albino rats weighing 180-200 g. The preparations in a volume of 1 ml were injected into the jugular vein, and blood samples were taken from the opposite vein. The humorally isolated carotid sinus zone of a chinchilla rabbit, with its innervation intact was perfused by the Heymans–Anichkov method, as described previously [6]. The dissection was done under local anesthesia with 0.05% procaine solution. The carotid sinus zone was perfused at the rate of 4 ml/min. The concentration of the preparations in the perfusing solutions was 10^{-7} M. Blood was taken from the femoral vein before and at definite times after perfusion. The total blood clotting time, recalcification time, activated partial thromboplastin time, thrombin time, concentrations of fibrinogen and soluble fibrin-monomer complexes, enzymic fibrinolytic activity of the plasma, and activity of plasminogen activators were determined [5] in blood samples. Altogether 14 experiments were carried out on rabbits and 70 on rats. The experimental results were subjected to statistical analysis.

EXPERIMENTAL RESULTS

In one series of experiments the state of the hemostasis system was analyzed after intavenous injection of 1 ml of 1 μM solution of plasmin and its modified forms. Allowing for dilution in the blood flow, the active concentration of the enzyme during the first minutes after injection was 10^{-7} mole/ml blood. It will be clear from Fig. 1 that plasmin evoked a characteristic biphasic reaction of the hemostasis systems, described previously [4]. Hypo-

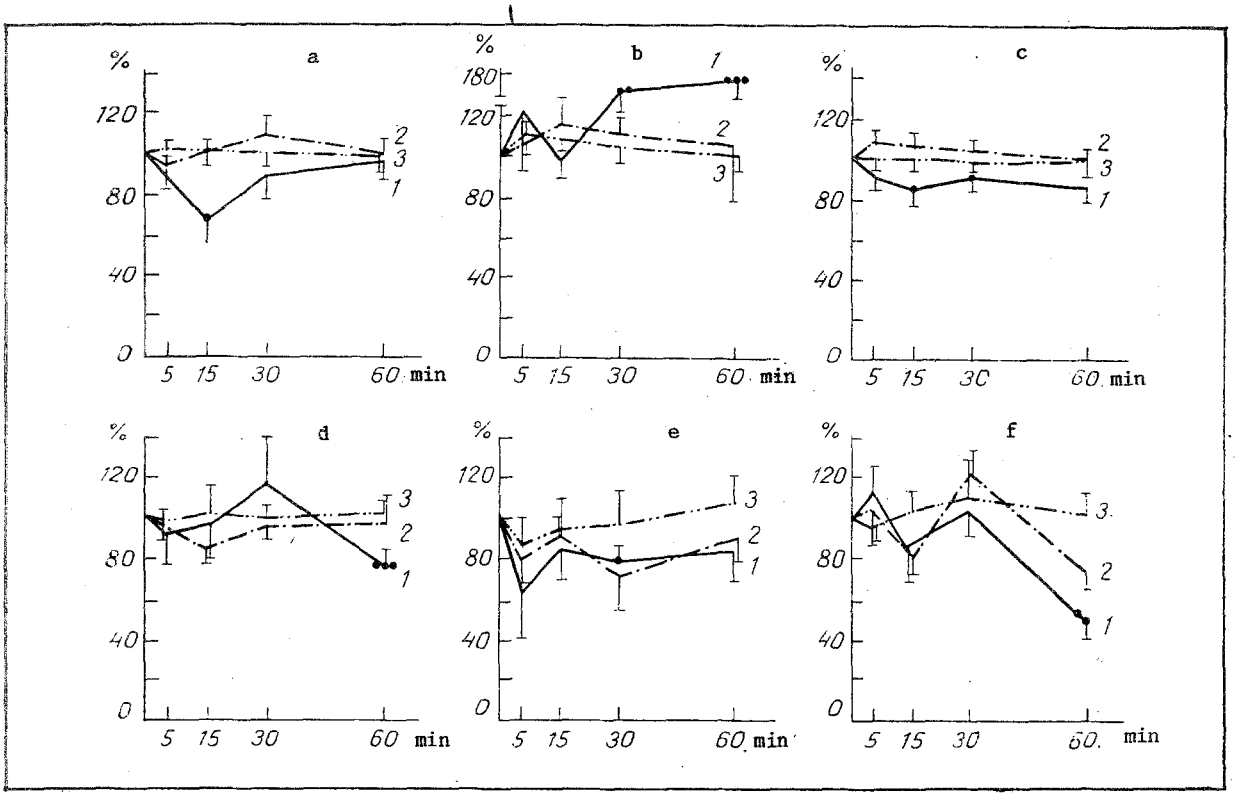

Fig. 2. Changes in parameters of the hemostasis system (M ± m, %) during perfusion of carotid sinus zone of a rabbit with $1 \cdot 10^{-7}$ M plasmin, An-Pl, Gb-Pl, and DIP-plasmin. Abscissa, time after beginning of perfusion (in min); ordinate, changes in parameters (in per cent of control level); values before beginning of perfusion taken as 100%. a) recalcification time, b) concentration of soluble fibrin-monomer complexes, c) thrombin time, d) fibrinolytic activity, e) activity of plasminogen activators, f) fibrinogen concentration. 1) Plasmin, 2) Gb-Pl, 3) DIP-plasmin. Remainder of legend as to Fig. 1.

coagulation was observed at the 5th minute, as shown by lengthening of the total blood clotting time and an increase in fibrinolytic activity. The first signs of a compensatory reaction were found by the 20th minute after injection, namely shortening of the total blood clotting time by 33%, reduction of anticoagulant activity by 15% (determined by the thrombin time), and inhibition of fibrinolytic activity by 19%. The rate of prothrombinase formation by the internal pathway, determined by lengthening of the activated partial thromboplastin time, was reduced by 25% ($p < 0.001$), and the fibrinogen concentration lowered by 13%. At the 30th minute, the signs of the compensatory reaction continued to increase.

When plasmin whose activity center was reversibly blocked by an acyl group was used, signs of the compensatory reaction appeared during reactivation of the enzyme in the bloodstream. For instance, after injection of An-Pl the first signs of the reaction, expressed as shortening of the total clotting time by 15%, were not found until the 30th minute after injection of the preparation. Maximal manifestations of the compensatory reaction were observed at the 90th minute of the experiment.

Injection of Gb-Pl, an acylated enzyme with a slower rate of reactivation than An-Pl, caused hypocoagulation and hyperfibrinolysis starting with the 60th minute of the experiment. The changes observed were due to the fact that the acylated enzyme binds highly specifically with fibrinogen and fibrin [8]. In the course of its deacylation, leading to proteolysis of the substrate, free plasmin is released into the bloodstream and is inhibited by α_2-antiplasmin. Later, due to exhaustion of the blood α_2-antiplasmin, plasmin which accumulates in relative excess induced a compensatory reaction, the initial manifestations of which did not appear the 5th hour of the experiment.

Di-isopropylphosphoryl-plasmin (DIP-plasmin), which has no catalytic activity, had no significant effect on the parameters of hemostasis studied. It should be noted that the doses of the acylated derivatives of plasmin which were used, although large enough to increase ac-

tivity, did not induce significant fibrinogenolysis, due to the greater affinity of the acylated derivatives for fibrin [8]. The results suggest that the triggering mechanism of the compensatory reaction to plasmin is effected only when the active center of the enzyme is intact. The reaction is probably triggered by proteolytic activation of receptors in the vascular wall.

This hypothesis was tested in another series of experiments involving perfusion of the humorally isolated carotid-sinus zone of rabbits by preparations of plasmin and its modified forms. It will be clear from Fig. 2 that plasmin, in direct contact with the vascular wall, induced only the characteristic compensatory reaction in the systemic circulation, or interaction between the enzyme and blood proteins and cells was excluded. From the 3rd-15th minute after perfusion an increase in procoagulants and decrease in anticoagulant activity of the plasma was observed, as shown by shortening of the recalcification time by 44% and of the thrombin time by 15%. By the 30th minute the concentrations of molecular markers of thrombin production (soluble fibrin-monomer complexes) was increased by 66%, and activity of plasminogen activators was reduced by 14%. Subsequent stimulation of thrombin formation, determined by the increase in concentration of soluble fibrin-monomer complexes, 1 h after perfusion led to a decrease in the fibrinogen concentration by 49%. As a result of the release of antiplasmins into the bloodstream, fibrinolytic activity was reduced by 23%.

In the next series of experiments involving perfusion of the carotid sinus zone of rabbits with plasmin preparations with reversibly (Gb-Pl) and irreversibly (DIP-plasmin) blocked active center, no significant deviations of the parameters characterizing the state of the hemostasis system could be found from the control level (before perfusion) in the course of observation lasting 60 min. Perfusion of the carotid sinus zone by Gb-Pl, after its partial (for 5 h) reactivation in vitro, led to the development of a compensatory reaction at the same times and with the same manifestations as perfusion with native plasmin.

In the control series of experiments with perfusion of the carotid sinus zone with 10^{-7} M albumin, no significant changes were found in the hemostasis system.

For a compensatory reaction to arise, besides those structures of the molecule by which plasmin binds specifically with the endothelium, an intact active center of the enzyme is thus necessary. Plasmin evidently induces proteolytic activation of vascular wall receptors responsible for its realization. This is not observed during irreversible blockade of the catalytic center of the enzyme by DFP. In the case of its reversible blockade by an acyl group, manifestations of a compensatory reaction are delayed in time and weaker. The time required for development of the reaction to acylated derivatives of plasmin is proportional to the rate of their reactivation.

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