INHIBITION OF THROMBIN EXCITATION MECHANISM OF THE ANTICLOTTING SYSTEM BY ANTITHROMBIN III

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Thrombin (EC 3.4.21.5) is a serum proteinase which regulates hemostasis. It converts fibrinogen into fibrin, controls the activity of factors V and VIII and protein C of the blood cells and vascular wall, and excites the neurohumoral reflex reaction of the anticlotting system [1]. The main functions of thrombin are performed with a high degree of specificity, which has been the subject of intensive research [4]. The leading role of the recognition center of high-molecular-weight compounds has been demonstrated in specific interaction of thrombin with blood protein and in excitation of the function of the anticlotting system [1]. Different sites of the recognition center of high-molecular-weight compounds are responsible for binding with ligands [4]. It was shown previously that the β/γ-form of thrombin, modified with respect to the cationic site of the recognition center for high-molecular-weight compounds, is no longer able to excite the function of the anticlotting system, whereas blockade of the catalytic site of the active center by diisopropylfluorophosphate (DFP) does not deprive α-thrombin of this activity [8]. The question arises whether natural blockers of the active center of the enzyme affects the bioregulatory functions of thrombin. Among these substances an important role is played by the protein inhibitor antithrombin III, which is present in plasma in a fairly high concentration (0.15 g/liter). Interaction of arginine of the reactive site of antithrombin III with serine of the active center of the enzyme leads to the formation of a stoichiometric complex and to neutralization of activity of the thrombin [7]. Considering the possibility that a similar complex may arise during provocation of thrombin formation in the body, it can be postulated that thrombin, in the form of a complex with antithrombin III, blocking the active center, preserves its ability to activate the reaction of the anticlotting system. The investigation described below was carried out to test this hypothesis.

EXPERIMENTAL METHOD

Bovine α-thrombin was obtained by purification of a commercial preparation [2]. The α-thrombin was homogeneous on polyacrylamide gel electrophoresis in the presence of SDS and its molecular weight was 40 kilodaltons. The clotting activity of the preparation was 1500-2000

TABLE 1. Changes in Parameters of the State of the Anticlotting System after Intravenous Injection of α-Thrombin–Antithrombin III Complex (1 μmole) or of α-Thrombin (1 μmole)

Parameter of state of anticlotting system	Preparations		
	0.85% NaCl solution	α-thrombin	α-thrombin–antithrombin III complex
Thrombin time, sec	29,0±0,3 (15)	34,6±1,7 (15)	30,4±0,6 (15)
p		<0,01	>0,05
Total fibrinolytic activity, mm²	35,5±1,8 (15)	43,1±1,2 (15)	39,2±1,6 (15)
p		<0,001	>0,2
Nonenzymic fibrinolysis, mm²	32,1±1,6 (15)	39,1±2,1 (15)	36,9±2,3 (15)
p		<0,02	>0,1
Enzymic fibrinolytic activity, mm²	45,0±0,7 (10)	61,5±3,4 (10)	46,6±2,5 (10)
p		<0,001	>0,5

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TABLE 2. Changes in Parameters of State of Anticlotting System (M ± m, in percent of control level) after Perfusion of Carotid Sinus Zone of Rabbits with α-Thrombin–Antithrombin III Complex (1.25 μmole)

Parameter of state of anticlotting system	Time after perfusion (min)		
	5	15	30
Thrombin time, sec	101,3±3,6 (5)	99,9±1,8 (5)	98,4±1,0 (5)
p	>0,5	>0,5	>0,2
Total fibrinolytic activity, mm²	92,8±10,5 (4)	92,±7,5 (4)	95,5±10,6 (4)
p	>0,5	>0,2	>0,5
Nonenzymic fibrinolysis, mm²	97,8±5,8 (4)	101,7±13,1 (4)	98,5±10,2 (4)
p	>0,5	>0,5	>0,5
Enzymic fibrinolytic activity, mm²	112,5±6,9	109,3±6,5 (5)	108,3±5,7 (5)
p	>0,2	>0,2	>0,2

NIH units/mg protein, and its esterase activity, determined by hydrolysis of the methyl ester of N-benzoylarginine, was 11 μmoles substrate/min/mg protein. Antithrombin III was obtained by modification of methods [5, 6], as follows. Plasma freed from the prothrombin complex by adsorption on barium citrate was defibrinated by heat treatment and chromatographed on heparin-sepharose, with elution of antithrombin III in a linear ionic strength gradient, created by increasing concentrations of NaCl from 0.15 to 1.5 M in Tris-HCL buffer, pH 7.4, containing 10 mM sodium citrate. Preparations of antithrombin III with activity of 12 units/mg protein were used to obtain complexes with α-thrombin. The complex was obtained by incubation of the enzyme with the inhibitor for 15 min in a molar ratio of 1:1. The purity of the complex was determined by gel-filtration on "Toyopearl-HW-55" (Toyo-Soda, Japan) in 0.01 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl. The equimolar complex of α-thrombin with antithrombin III, not possessing clotting or esterase activity, was used in experiments on animals.

Male albino rats weighing 180-211 g and rabbits of both sexes weighing 2-3 kg were used. The preparations were injected into rats into the jugular vein in a volume of 1 ml, and blood was taken from the same vein for analysis. The carotid sinus zone of the rabbit, isolated from the systemic circulation but with its innervation intact, was perfused by the Heymans–Anichkov method with Ringer–Locke solution, pH 7.4, containing 0.4% glucose at 37°C. A constant rate of perfusion was maintained with a peristaltic pump. The Ringer–Locke solution and the test solutions were continuously aerated. At the beginning of each experiment the carotid sinus zone was perfused for 40 min with Ringer–Locke solution, after which the test solution was perfused in a volume of 21 ml, and this was followed by perfusion with the solvent. After definite time intervals blood was taken from the femoral vein for determination of the thrombin time, total fibrinolytic activity, and nonenzymic and enzymic fibrinolysis [8]. Altogether 11 experiments were carried out on rabbits and 50 on rats. The results were subjected to statistical analysis by the Fisher–Student method.

EXPERIMENTAL RESULTS

In the experiments of series I the state of the anticlotting system was analyzed after intravenous injection of preparations of the α-thrombin–antithrombin III complex or of α-thrombin into rats. It will be clear from Table 1 that parameters reflecting the state of the anticlotting system (thrombin time, total fibrinolytic activity, nonenzymic fibrinolysis, and enzymic fibrinolytic activity), determined 5 min after injection of 1 μmole of the α-thrombin–antithrombin III complex into the rats did not differ significantly from the control level. Injection of 1 μmole of α-thrombin led to a statistically significant increase in all parameters.

The results suggested that antithrombin III prevents interaction of α-thrombin with the vascular wall receptors responsible for excitation of the anticlotting system, and prevents manifestation of the effector act of the system. This hypothesis was tested in a series of experiments with perfusion of the humorally isolated rabbit carotid sinus zone, with its nervous connections with the rest of the body preserved, with preparations of the α-thrombin–antithrombin III complex. Values of total fibrinolytic activity, nonenzymic fibrinolysis, thrombin time, and enzymic fibrinolytic activity, recorded in the systemic circulation during perfusion of the carotid sinus zone of the rabbits with preparation of the α-thrombin–antithrombin III complex (1.25 μmole), are given in Table 2. It will be clear from Table 2 that parameters reflecting the state of the anticlotting system did not differ significantly from the control level (before perfusion with the complex) for 30 min after perfusion. In the

series of experiments with perfusion of the rabbit carotid sinus zone without α-thrombin, in a concentration close to its content in the complex (1 μmole), a significant change was observed in the parameters reflecting the state of the anticlotting system: nonenzymic fibrinolysis was increased by 33.6% ($p < 0.001$), whereas total fibrinolytic activity was increased by 30.4% by the 5th minute of the experiment.

Antithrombin III thus blocks the mechanism of excitation of the anticlotting system by thrombin. It has been suggested that the conformational change in the enzyme molecule observed previously [9] during the formation of the complex of antithrombin III with α-thrombin disturbs the structure of the recognition subcenter for high-molecular-weight compounds, which binds the cell receptors. As a result of this conformational change, interaction of α-thrombin with receptors responsible for excitation of the anticlotting system evidently is disturbed. In the light of the facts described above, the results of the present investigation can be understood as a consequence of high lability of the sites of the thrombin molecule responsible for interaction with receptors of the anticlotting system. Evidence in support of this view is given by data in the literature [3] on the marked lowering of the chemotaxic activity of α-thrombin by antithrombin III.

This suggests that the development of ways of obtaining low-molecular-weight inhibitors of the catalytic center of α-thrombin, which do not modify the recognition center of high-molecular-weight compounds and receptors, may enable preparations highly effective for exciting the anticlotting system to be created.

LITERATURE CITED

1. B. A. Kudryashov and L. A. Lyapina, Lab. Delo, No. 6, 326 (1971).
2. B. A. Kudryashov and S. M. Strukova, Usp. Sovrem. Biol., 97, 193 (1984).
3. E. F. Luk'yanenko, S. M. Strukova, E. G. Kireeva, et al., Biokhimiya, 50, 1433 (1985).
4. T. N. Mitrochina, S. M. Strukova, B. A. Umarova, et al., Vopr. Med. Khim., No. 5, 68 (1982).
5. A. A. Sereiskaya and S. M. Strukova, Dokl. Akad. Nauk SSSR, 282, 481 (1985).
6. S. M. Strukova, O. A. Semenova, and E. G. Kireeva, Biokhimiya, 45, 738 (1980).
7. S. M. Strukova, B. A. Umarova, E. G. Kireeva, et al., Biokhimiya, 43, 708 (1978).
8. T. Astrup and S. Mullerts, Arch. Biochem., 40, 346 (1952).
9. R. Bizios, L. Lai, J. W. Fenton II, et al., Thromb. Res., 38, 425 (1985).
10. J. W. Fenton II, Cell Proliferation: Recent Advances (A. L. Boynton and H. L. Leffert, eds.) Vol. 2, New York (1985), p. 4.
11. F. Markwardt and H.-P. Klocking, Haemostasis, 6, 370 (1977).
12. S. Radoff and J. Danishefsky, Thromb. Res., 22, 353 (1981).
13. R. Warren and A. Wysocki, Surgery, 44, 435 (1958).