# ACTIVATION OF THE ANTICLOTTING SYSTEM BY PRETHROMBIN 2, A THROMBIN PRECURSOR

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UDC 612.115.3:612.111

Prethrombin 2, the direct precursor of thrombin, was obtained by restricted proteolysis of prethrombin 1 by trypsin or by active factor X. In the absence of enzymes, prethrombin 1 is not converted into prethrombin 2. Factor V was shown not to affect the rate of thrombin generation from prethrombin 2. It was shown that prethrombin 2 has no intrinsic coagulating or esterase activity. After intravenous injection of preparations of prethrombin 2, the plasma recalcification time was lengthened and total fibrinolytic activity and nonenzymic fibrinolysis were increased. A considerable increase also was found in the activity of the fibrinogen–heparin complex. These results are evidence of excitation of the anticlotting system by intravenous injection of small doses of prethrombin 2.

KEY WORDS: prethrombins 1 and 2; anticlotting system.

Investigations by Kudryashov and co-workers [3, 4, 9] have shown that not only thrombin, but also its precursor prethrombin 1, which has no coagulating activity, are full-fledged exciters of the reflex response of the second anticlotting system. Prethrombin 1 appears in the blood as a result of slow proteolysis of prothrombin by thrombin in the presence of a deficiency of the principal activator of the proenzyme — active factor Xa or its cofactors — factor V, phospholipids, and $Ca^{++}$ [12]. Further hydrolysis of prethrombin 1 by factor Xa leads to the appearance of prethrombin 2 — the direct precursor of thrombin. During rapid activation of prothrombin by factor Xa in the presence of cofactors, prethrombin 2 is formed immediately, but like prethrombin 1, it has no coagulating activity by itself. Thrombin activity appears as a result of proteolysis of prethrombin 2 by factor Xa [8, 10].

The role of prethrombin 2 in excitation of the function of the anticlotting system has not been studied. The object of the present investigation was to prepare prethrombin 2 free from thrombin and to study its effect on the state of the anticlotting system.

## EXPERIMENTAL METHOD

Experiments were carried out on 60 male albino rats weighing 180-200 g and on 50 male frogs (*Rana temporaria*). The substances for testing were injected into the jugular vein of the rats and into the frogs' heart. At definite time intervals after injection, blood samples were taken (1 ml from each rat, 0.1 ml from each frog), for determination of the total clotting time, plasma recalcification time, total fibrinolytic activity and nonenzymic fibrinolysis [2], and activity of the fibrinogen–heparin complex [1]. Physiological saline was injected into the control rats and Ringer's solution into the control frogs. Prothrombin and factor X were obtained from bovine blood [13]. Factor X was converted into the active form by passage through a column with immobilized trypsin [6]. Prethrombin 1 was obtained by proteolysis of prothrombin with thrombin and was purified [5]. Prethrombin 2 was obtained by restricted proteolysis of prethrombin 1 by trypsin immobilized on Sepharose 4B, or by active factor X (factor Xa) [11] in 25% sodium citrate solution. The prethrombin 2 was purified by chromatography on DEAE-cellulose. The relative molecular weight (mol. wt.) of the preparations was determined by electrophoresis in polyacrylamide gel in the presence of sodium dodecylsulfate [7]. Esterase activity was determined by the method described previously [14].

Laboratory of Physiology and Biochemistry of Blood Clotting, M. V. Lomonosov Moscow University. (Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 89, No. 3, pp. 270-272, March, 1980. Original article submitted April 13, 1979.

0007-4888/80/8903-0253$07.50  

TABLE 1. Changes in Plasma Recalcification Time (in sec) after Intravenous Injection of Products of Restricted Proteolysis of Prothrombin into Rats (M±m)

| Preparation injected | Protein concn., mg/ml | Time after injection of preparation, min | |
|---|---|---|---|
| | | 5 | 15 |
| Control: 0.85% NaCl solution | — | 95,0±2,8 (11) | 98,1±4,3 (11) |
| Experiment: | | | |
| prethrombin 1 | 0,40 | 110±3,75* (8) | 97,4±4,9 (7) |
| prethrombin $2_{tryp}$ | 0,16 | 120,0±6,6* (13) | 101,0±7,1 (11) |
| | 0,08 | 151,0±9,7* (10) | 138,0±14* (10) |
| prethrombin $2_{Xa}$ | 0,10 | 111,6±5,8* (6) | 92,5±6,6 (6) |
| | 0,05 | 136,0±7,0* (6) | 135,0±3,8* (6) |

Legend. Here and in Table 2: number of experiments shown in parentheses.
*P<0.05.

TABLE 2. Changes in Total Fibrinolytic Activity and Nonenzymic Fibrinolysis (in $mm^2$) after Intravenous Injection of Products of Restricted Proteolysis of Prothrombin into Rats (M±m)

| Preparation injected | Protein concn., mg/ml | Total fibrinolytic activity | | Nonenzymic fibrinolysis | |
|---|---|---|---|---|---|
| | | after administration | | | |
| | | 5 min | 15 min | 5 min | 15 min |
| Control: 0.85% NaCl solution | | 30±5,8 (7) | 26±6,8 (5) | 18±3,9 (8) | 10,5±2,4 (4) |
| Experiment: | | | | | |
| prethrombin | 0,40 | 91±11,6* (5) | | 41±5,8* (5) | |
| prethrombin $2_{tryp}$ | 0,16 | 72±5,8* (10) | 75±7,0* (10) | 24±4,3* (10) | 25±3,6* (10) |
| | 0,08 | 65±4,7* (10) | 85±6,7* (10) | 16,5±2,9* (10) | 17±2,9* (10) |
| prethrombin $2_{Xa}$ | 0,10 | 53±4,5* (6) | 73±4,8* (6) | — | — |
| | 0,05 | 60±5,8* (6) | 66±1,6* (6) | — | — |

## EXPERIMENTAL RESULTS

Prethrombin $2_{Xa}$ with mol. wt. 40,000±2000, free from contamination by thrombin and fragment 2, was obtained by restricted proteolysis of prethrombin 1 with mol. wt. 57,000±2000 by means of factor Xa. Proteolysis of prethrombin 1 on a column with immobilized trypsin yielded prethrombin $2_{tryp}$ with mol. wt. 46,000±2000. In the absence of enzymes proteolysis of prethrombin 1 in 25% sodium citrate solution was not observed.

The resulting prethrombin 2 preparations, after purification, had neither clotting, esterase, nor fibrinolytic activity. The study of the kinetics of activation of prethrombin 1 and 2 by a factor Xa in the presence of $Ca^{++}$ showed that addition of factor V to the system increased the rate of activation of prethrombin 1 considerably (by 3.5 times), without affecting the rate of activation of prethrombin 2. These results indicate that the preparations of prethrombin 2 did not contain fragment 2, which is responsible for binding the prothrombin molecule to factor V [8].

Intravenous injection of the preparations of prethrombin 2 into rats, just as in the case of prethrombin 1, gave rise to a strong response (Tables 1 and 2), characterized by an increase in the plasma recalcification time, and by a considerable (by 2-3 times) increase in total fibrinolytic activity and nonenzymic fibrinolysis. Substantial hypocoagulation shifts were observed after injection of small doses of prethrombin 2 (0.05 and 0.08 mg/ml). Activity of the fibrinogen–heparin complex was increased fivefold toward the 5th minute after injection of small doses of prethrombin 2 and still remained at a high level after 15 min. These results indicate excitation of the response of the anticlotting system after injection of prethrombin 2 — the direct precursor of thrombin, without coagulating activity of its own, into the bloodstream. Prethrombin $2_{tryp}$, incidentally, gave an effect similar to that of prethrombin $2_{Xa}$.

The course of development of the response of the anticlotting system to injection of prethrombin $2_{Xa}$ was studied in experiments on frogs. Injection of 0.1 ml prethrombin $2_{Xa}$ in a concentration of 0.02 mg/ml into the general circulation of the frogs induced an increase in the total clotting time by 31% after 3 min. The maximal response (by 79%) was observed 10 min after injection, in agreement with data on the effect of prethrombin 1 on the reaction of the anticlotting system [3].

The results are evidence that the mechanism of activation of the anticlotting system may be triggered even before the appearance of thrombin in the blood, with the formation of prethrombin 2 — the immediate precursor of thrombin. Prethrombin 2 appears in the body as a result of proteolysis of prothrombin by the principal physiological activator — factor Xa — in the form of a complex with factor V, phospholipids, and $Ca^{++}$. The formation of a structural analog of thrombin in the blood, differing from it only in preservation of the intact arginyl-isoleucine bond, gives a warning of the risk of thrombus formation and triggers the mechanism of activation of the anticlotting system. Further investigations will show whether this effect is due to the direct action of prethrombin 2 on chemoreceptors of the vascular system or whether it is mediated through thrombin generation.

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