THE INHIBITION OF ACTIVATED FACTOR XII (HAGEMAN FACTOR) BY ANTITHROMBIN III: THE EFFECT OF OTHER PLASMA PROTEINASE INHIBITORS[1]

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Summary

Human factor XII was activated by adsorption onto kaolin in the presence of high molecular weight kininogen. The washed kaolin-containing precipitates activate prekallikrein to kallikrein. When antithrombin III was added to the reaction mixture, the conversion of prekallikrein to kallikrein was inhibited, the degree of inhibition depending on the concentration of antithrombin and the time of incubation. Heparin had a slight enhancing effect with low concentrations of antithrombin and short incubation times. However, the inhibition of the generated kallikrein by antithrombin III was markedly enhanced by heparin. Antithrombin III inhibited also the effect of activated factor XII on the partial thromboplastin time, using factor XII-deficient plasma Of other plasma proteinase inhibitors used (α_1-antitrypsin, α_2-macroglobulin, $C\bar{1}$-inactivator) only $C\bar{1}$-inactivator inhibited activated factor XII.

Introduction

The contact phase of blood coagulation is intimately linked with the kinin- and fibrinolytic systems. There is a homeostatic balance between activation and inhibition of these systems, pathological states arising from excess activation or inadequate inhibition. In the cascade of the kinin system, formation of the vasoactive peptide bradykinin is the last step. This peptide is inhibited primarily by carboxypeptidase N (1). Bradykinin is cleaved from kininogen by plasma kallikrein. The intermediate step in the cascade is the generation of kallikrein from prekallikrein. This enzyme is readily inhibited by the plasma proteinase inhibitors α_2-macroglobulin, $C\bar{1}$-inactivator (2) and antithrombin III (3). The zymogen prekallikrein in turn is converted to its active form by activated factor XII, the latter being activated by negatively

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charged surfaces and requiring catalytic amounts of kallikrein and small quantities of high molecular weight kininogen for its full and rapid activation (4, 5, 6, 7, 8). The clot-promoting activity of factor XIIa is inhibited by $C\bar{1}$-inactivator (9). Fragmented factor XII or prekallikrein activator (PKA) is also inhibited by $C\bar{1}$-inactivator (10).

This paper describes the inhibition of contact activated factor XII by antithrombin III. The isolation of factor XII (11), kallikrein and high molecular weight kininogen (12) was described before and that of the inhibitor is described in the accompanying publication (13).

Materials and Methods

Activated factor XII was assayed by its capacity to activate prekallikrein to kallikrein and by the partial thromboplastin time (PTT), which measures the ability to correct the prolonged clotting time of factor XII-deficient plasma (8, 11). The activation of prekallikrein to kallikrein was determined indirectly. Activated factor XII converts the zymogen prekallikrein to its active form kallikrein and the latter, being an arginine esterase hydrolyses benzoyl arginine ethyl ester (BAEe). Briefly, 0.1 ml factor XII was incubated with 0.1 ml kaolin (10 mg/ml) for 2 minutes. To this mixture was added 0.1 ml of high molecular weight (HMW)-kininogen and incubated for 10 minutes. The reaction mixture was cooled to 4°, centrifuged at that temperature for 10 minutes at 3050g, then resuspended in cold 0.1 M Tris-HCl buffer (pH 8.0, containing 0.1 M NaCl) and recentrifuged. The kaolin-containing precipitates to which the activated factor XII was adsorbed (8) was added to 0.1-0.2 ml prekallikrein, incubated for 10 minutes, the precipitates removed by centrifugation and the supernatant (containing the kallikrein) added to 0.5 ml 3×10^{-3}M BAEe, made up to 3 ml with Tris-buffer and the extinction read at 253 nm at 2-minute intervals for 10 minutes. A mixture of 0.5 ml BAEe and 2.5 ml buffer served as the blank and the increase in absorbance was converted to nmoles of BAEe hydrolysed per ml of sample per minute, using a standard curve of known amounts of BA.

The PTT assay was based on the procedure described by Ratnoff and Davie (14). The kaolin precipitates described above, to which the activated factor XII was adsorbed, was resuspended in 0.1 ml of Tris-buffer containing 0.1 M NaCl (pH 8.0). This was incubated in duplicate with 0.1 ml crude soybean phospholipid (Centrolex P, Chemurgy Div., Central Soyal, Chicago, Ill.) for 2 minutes at 37° in polystyrene test tubes (8 mm internal diameter). To this reaction mixture 0.1 ml factor XII-deficient plasma (15) was added, immediately recalcified with 0.1 ml 0.02 M $CaCl_2$ and the time required for a solid clot-formation was ascertained.

Inhibition of activated factor XII was carried out by incubating the kaolin precipitates to which factor XII and HMW-kininogen were adsorbed with either increasing concentrations of antithrombin III (with or without heparin) for 30 minutes or with a constant amount of inhibitor for varying lengths of time. Several preparations of antithrombin were assayed in this manner. One experiment was carried out using α_1-antitrypsin, α_2-macroglobulin and $C\bar{1}$-inactivator.

Having observed differences between the effect of heparin in earlier studies (3, 16) and those described below, we compared the effect of heparin in one experiment, in which the inhibitor was aded to the kallikrein-containing supernatant. As described above the activated factor XII when added to prekallikrein converts it to kallikrein.

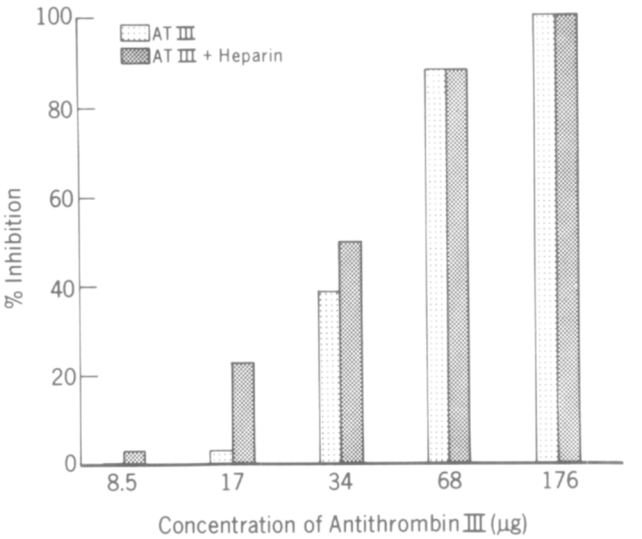

Figure 1. One tenth ml (10 mg/ml) kaolin was incubated with 0.1 ml factor XII (1.5 μg) 0.1 ml HMW-kininogen (0.45 μg; 4.8 ng bradykinin equivalents) and increasing amounts of antithrombin III (0.1 ml) with or without heparin (4 units). In the control the antithrombin was replaced with 0.1 M Tris-HCl (pH 8.0), containing 0.1 M NaCl. As described in the Materials and Methods the conversion of prekallikrein to kallikrein by the kaolin-containing precipitates was assayed as BAEe hydrolysis. In the control 218 nmoles per ml per minute of ester was hydrolysed. The incubation with antithrombin was for 30 minutes. The data are expressed as percent inhibition.

The isolation of factor XII was described before (11). This procedure was modified by using Sephadex G-200 instead of G-100 and having used a larger starting volume of plasma (8 liters), the partially purified factor XII obtained by CM-Sephadex was rechromatographed on the same cation exchanger. The concentration of factor XII and other proteins was determined by the method of Lowry et al. (17); that of factor XII being 15 μg/ml. HMW-kininogen (85-132 μg/ml) and prekallikrein (85.5 μg/ml) were purified as reported earlier (12). The prekallikrein was adjusted so that on conversion it hydrolysed 200-300 nmoles of BAEe per minute per ml. The antithrombin was prepared as described in detail in the accompanying paper (13), which also describes the isolation of α_1-antitrypsin. To obtain α_2-macroglobulin the procedure of Harpel (18) was followed. Partially purified C$\bar{1}$-inactivator was a by-product of the purification of HMW-kininogen (12) and a highly purified preparation was supplied by Dr. Jack Pensky (Case-Western Reserve University). The inhibitors were quantitated by radial immunodiffusion (M-Partigen, Behringwerke, Hoechst Pharmaceuticals, Montreal, Que.) and by their ability to inhibit certain enzymes (thrombin, trypsin,kallikrein) (see Ref. 13).

Results

As indicated, incubation of kaolin with factor XII slowly converts the latter into its activated form and addition of increasing concentrations of HMW-kininogen markedly enhances the

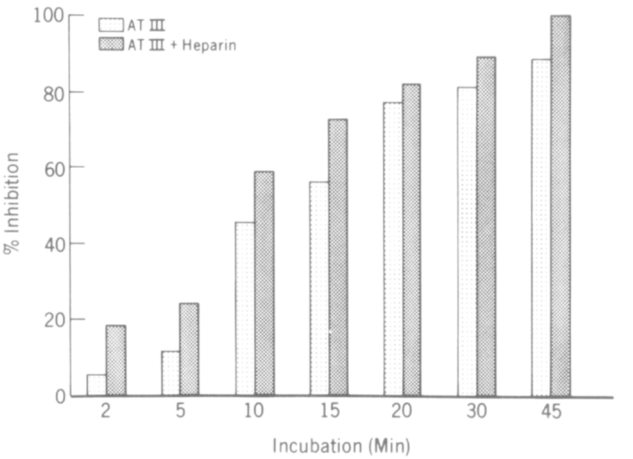

Figure 2. Conditions as in Figure 1, except varying the time of incubation as shown. The antithrombin concentration was 68 μg.

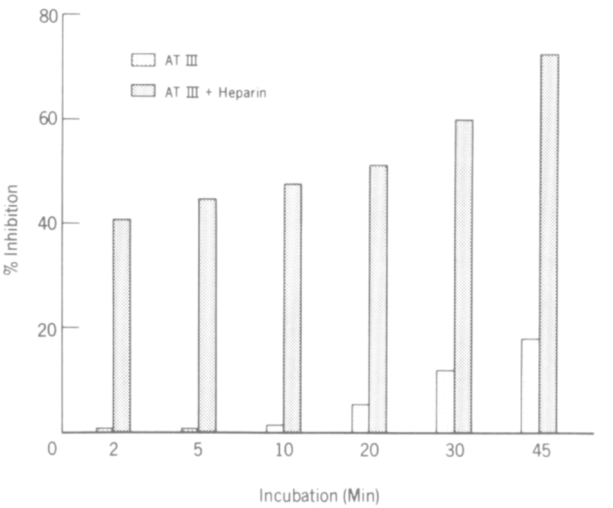

Figure 3. Inhibition of kallikrein by antithrombin III. The inhibitor with or without heparin (see Fig. 1) was added to the kallikrein-containing supernatant. The kallikrein without inhibitor hydrolysed 218 nmoles BAEe/min/ml. Note that unlike with activated factor XII heparin had a marked enhancing effect on the inhibition. Concentration of antithrombin was 68 μg.

rate of this reaction (8). When the kaolin-factor XII-HMW-kininogen reaction mixture is incubated with increasing concentrations of antithrombin III there is gradual inhibition of the activated factor XII, which no longer can convert prekallikrein to kallikrein (Fig. 1). With lower concentrations of antithrombin III heparin enhances the inhibition. With fixed amounts

TABLE 1

Effect of Antithrombin III on Activated Factor XII as Measured by the Partial Thromboplastin Test with Factor XII-Deficient Plasma

Antithrombin III (μg)	PTT (sec.)	% Inhibition
0	124	0
8.5	141	14
17.0	150	19
34.0	154	22
68.0	190	38

The PTT was carried out in duplicates as described in Materials and Methods. When the PTT was done by the conventional method (incubation of factor XII with kaolin for 2 minutes, followed by incubation with factor XII-deficient plasma for 8 minutes, followed by addition of $CaCl_2$) a solid clot formed in 91 seconds. The factor XII-deficient plasma without any additive clotted in 564 seconds. Time of incubation with antithrombin III was for 20 minutes.

TABLE 2

Effect of Antithrombin III on Activated Factor XII as Measured by the Partial Thromboplastin Test with Factor XII-Deficient Plasma

Incubation with Antithrombin III (min.)	PTT (sec.)	% Inhibition
Control	124	0
5	150	18
10	161	24
20	186	35
30	208	41

Conditions were identical to those in Table 1. The concentration of antithrombin III was 68 μg.

of activated factor XII and antithrombin, increasing the time of incubation enhances the inhibition (Fig. 2). Here too with shorter times of incubation heparin further enhanced the inhibition. When the antithrombin was added to the kallikrein that had been generated, heparin had marked enhancing effect on the inhibition (Fig. 3).

TABLE 3

Effect of Plasma Proteinase Inhibitors on Activated Factor XII as Measured by its Ability to Convert Prekallikrein to Kallikrein

Inhibitors	BAEe-hydrolysis (nmoles/min/ml)	% Inhibition
α_1-antitrypsin (275 µg)	282	0
α_2-macroglobulin (532 µg)	285	0
$C\bar{1}$-inhibitor (84 µg)	157	45
Buffer	285	0

One tenth ml of inhibitor was added to the activated factor XII-containing kaolin precipitates, incubated for 30 minutes at 37° and the washed precipitates added to prekallikrein as described in Materials and Methods and the BAEe-hydrolysing activity of the generated kallikrein assayed.

Using the modified PTT, as described in Materials and Methods, increasing concentrations of antithrombin (in the absence of heparin) lengthened the PTT as shown in Table 1. With a constant amount of antithrombin, the PTT was lengthened proportionally to the time of incubation of antithrombin and activated factor XII (Table 2).

Factor XII was inhibited also by $C\bar{1}$-inactivator, but not by α_1-antitrypsin and α_2-macroglobulin (Table 3).

Discussion

Antithrombin has been shown to be probably the principal inhibitor of thrombin, a process markedly enhanced by heparin (19, 20, 21), although α_2-macroglobulin likewise inhibits thrombin (22, 23). Since antithrombin III has become available in highly purified form (24, 25, 26, 27) it has been shown to inhibit also plasmin (16, 28), plasma kallikrein (3, 16, 29, 30) and certain clotting factors, namely activated factor XI (31), factor IX (32, 33) and factor X (33).

There seems to be a definite difference in the co-factor effect of heparin in the inhibition

of the above enzymes. Whereas the inhibition of thrombin, plasmin, kallikrein and of the activated clotting factors XI and IX is markedly augmented by heparin, this co-factor has little effect on the inhibition of activated factor X, and as shown in this paper, also on the inhibition of activated factor XII. The reason for these differences are presently unknown, but at least some of the enzymes on the inhibition of which heparin has an effect (thrombin, plasmin, kallikrein) hydrolyse arginine esters.

In addition to antithrombin III other proteinase inhibitors were tested. Of these only $C\bar{1}$-inactivator inhibited activated factor XII, which is in keeping with earlier observations (9, 10).

The nature of the activated factor XII adsorbed to kaolin remains to be ascertained. Preliminary attempts to study complex formation between factor XII activated by ellagic acid and antithrombin III failed. There is however, a recent communication, reported in abstract form (34), that both intact activated factor XII and fragmented factor XII or prekallikrein activator form complexes with antithrombin III, as shown by SDS-disc gel electrophoresis.

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