BIOPHYSICS AND BIOCHEMISTRY

Effect of Iron Ions on Functional Activity of Thrombin

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The kinetics of thrombin inhibition by irons ions was studied in the thrombin time test with normal plasma. The kinetic and concentration characteristics for recovery of thrombin activity by desferal were evaluated at various periods of thrombin incubation with iron ions. The thrombin time test showed that incubation of thrombin with iron sulfate in a final concentration of 200 μM for 25-35 min is followed by the loss of thrombin activity. Pretreatment of iron-containing incubation system with desferal was shown to decelerate the process of thrombin inactivation. The kinetic characteristics for recovery of thrombin activity by 2 mM desferal were estimated at various periods after addition of iron sulfate in the inhibitory dose. The effect of reversibility was shown to depend on the time of thrombin preincubation with iron. Incomplete recovery of thrombin activity after increasing the time of incubation with iron (more than 30 min) was probably related to oxidative modification of thrombin.

Key Words: *reactive oxygen species; thrombin; iron ions; desferal*

The biological and physicochemical properties of fibrinogen and thrombin are evaluated in clinical, biochemical, and physiological studies. These major factors of hemostasis are involved in the final stage of thrombus formation. Previous experiments showed that iron ions have a direct inhibitory effect on functional activity of thrombin [7]. For example, iron ions prevent the formation of fibrin clot. Inhibition of thrombin activity is a reversible process. However, little is known about this problem.

One of the major causes of damage to biological molecules (*e.g.*, proteins) is their oxidation by free radicals during oxidative stress. Our previous studies showed that oxidation of fibrinogen has a strong effect on functional activity of this compound. Fibrinogen oxidation is followed by dysregulation of coagulation and changes in rheological characteristics of the blood [2,4,5]. Moreover, oxidized fibrinogen stimulates aggregation of platelets, activates leukocytes, and engages endothelial cells into the inflammatory process. It is related to the synthesis of a chemoattractant interleukin-8 by endothelial cells and increase in the expression of cell adhesion molecules (P-selectin and ICAM-1) [1,3,6].

Thrombin is inactivated by iron ions. The role of oxidative damage in the inhibition of thrombin activity by iron remains unknown.

Here we studied the kinetics of thrombin inhibition by iron ions in the thrombin time test with normal plasma. The kinetic and concentration characteristics for recovery of thrombin activity by desferal were evaluated at various periods of thrombin incubation with iron ions.

MATERIALS AND METHODS

Iron preparations were prepared from iron sulfate (ferrous ion) and iron chloride (ferric ion). The solutions

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were prepared *ex tempore* with distilled water. Each experiment was performed with freshly-prepared solutions. These solutions were added to the test samples. An equivalent volume of distilled water was added to control samples.

Lyophilized human thrombin (Trombin-test, Renam) was used as the source of thrombin. The solution of thrombin (6 U/ml) in 0.9% sodium chloride (with no buffer) was prepared and stored according to manufacturer's recommendations.

The coagulation time of citrate plasma after addition of thrombin (thrombin time test) was estimated by the clotting method on a Minilab-701 two-channel optical-mechanical coagulometer (Yunimed). The thrombin time test was conducted according to manufacturer's instructions.

Citrate plasma from several donors was divided into aliquots (1.2 ml) and frozen at -20°C. Immediately before the study, the plasma sample (1.2 ml) was defrosted in water and 37°C for 3 min and placed in an ice bath. The thrombin time of this plasma remained unchanged for 30-40 min. The next aliquot of blood plasma was defrosted and treated in a similar manner after 30 min. The thrombin time of control plasma samples in experiments with thrombin (6 U) was 9.5-11.5 sec.

Deferoxamine mesylate (desferal) was used for binding of iron ions.

Preliminary experiments showed that addition of iron sulfate (final concentration 200-5000 μM) or desferal (50-2000 μM) to the plasma immediately before heating (2 min at 37°C) and further treatment with additive-free thrombin have no effect on the clotting time (as compared to the plasma containing an equivalent volume of physiological saline).

Desferal in the specified doses (up to 2 mM, maximum final dose in the sample) did not modulate thrombin activity in the thrombin time test over several hours of incubation at room temperature.

RESULTS

In series I we selected the concentration of iron sulfate, which was reliable for studying the effect of thrombin inhibition. The time of fibrin clot formation was 9 sec. The thrombin time characterizes the final stage of blood coagulation (process of fibrin clot formation). It reflects the function of fibrinogen and presence of inhibitors and depends on functional activity of thrombin. Under control conditions, the formation of fibrin clot was observed for 9 sec (Fig. 1, curve *1*).

Incubation of thrombin with iron sulfate in a final concentration of 200 μM for 25-35 min was followed by the loss of thrombin activity (Fig. 1, curve *2*). The kinetics of thrombin inactivation in the thrombin time test (increase in the time of fibrin clot formation in the control plasma sample in dependence on the period of thrombin incubation with iron sulfate) was analogous to the linear process. The maximum time of clot formation in citrate plasma could not exceed 100 min (peak sensitivity of the coagulometric method). Inactivation of thrombin was observed immediately after addition of iron (II) ions in high doses. After treatment with iron (II) ions in the lower dose (below 200 μM), the kinetics of a decrease in thrombin activity was described by a flat curve. This curve reached a plateau in the follow-up period. It should be emphasized that the ability to induce clot formation was persistent for a long time.

It remains unclear whether the inhibitory effect of iron on functional activity of thrombin is associated with the reversible binding of iron to certain sites on the thrombin molecule or irreversible inactivation of thrombin due to oxidative modification by iron ions. We studied the effect of desferal (chelating agent for iron (II) and iron (III) that prevents binding of iron ions to the thrombin molecule) on the thrombin time.

The protective effect of desferal was evaluated during inactivation of thrombin by iron sulfate in a concentration of 200 μM (Fig. 1). Pretreatment with desferal decreased the rate of inactivation (Fig. 1, curves *3-6*). At a 10-fold excess of desferal (relative to iron, desferal concentration 2 mM), activity of throm-

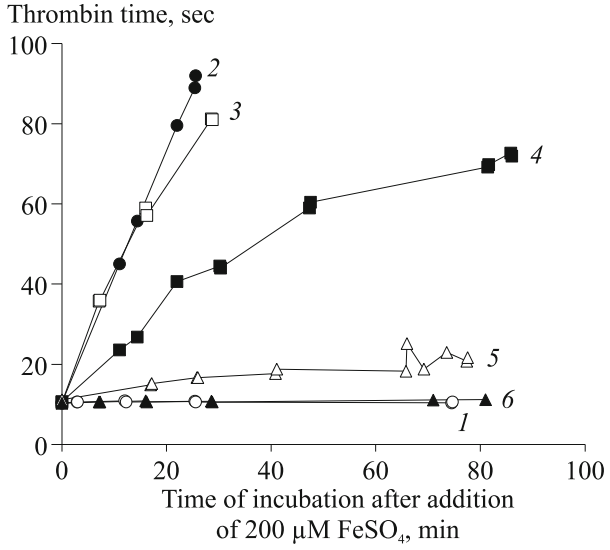

Fig. 1. Thrombin activity in the presence of iron ions and desferal. Incubation of thrombin with 200 μM $FeSO_4$. Addition of desferal (50-2000 μM) to thrombin before incubation with iron sulfate. Control thrombin (without 200 μM $FeSO_4$, *1*); control thrombin with 200 μM $FeSO_4$ (without desferal, *2*); thrombin with 200 μM $FeSO_4$+50 μM desferal (*3*); thrombin with 200 μM $FeSO_4$+100 μM desferal (*4*); thrombin with 200 μM $FeSO_4$+200 μM desferal (*5*); thrombin with 200 μM $FeSO_4$+2000 μM desferal (*6*).

bin remained unchanged and did not differ from that of additive-free thrombin (control thrombin). This effect was less pronounced at a lower concentration of desferal (Fig. 1, curves *3-5*).

In series II we studied the recovery of thrombin activity by desferal (final concentration 2 mM) after its inactivation by iron sulfate for various periods of time. The thrombin time in the control plasma sample was used as the test system in the measurement of thrombin activity (similarly to series I). Figure 2 shows the kinetics of recovery of thrombin activity by desferal at fixed time intervals after treatment with the inhibitory dose of iron sulfate (200 μM).

The loss of thrombin activity after addition of iron sulfate (200 μM) was described by a linear curve. Figure 2 shows that the critical value (100 sec) was achieved after 30 min.

Addition of desferal was followed by the recovery of enzyme activity. The thrombin time was reduced to the baseline level. However, the effect of reversibility depends on the time of thrombin preincubation with iron. Treatment with desferal (final concentration 2 mM) 5 min after addition of iron sulfate to the sample provided complete recovery of thrombin activity. Activity of thrombin returned to normal after addition of desferal by the 15th minute of incubation with iron. During this period, the curve of thrombin inactivation was at the "half-way point" (this presentation is conditional, because the thrombin time is not directly proportional to activity of thrombin or concentration of active thrombin).

Addition of desferal after 30-min incubation of thrombin with iron sulfate (complete inactivation of thrombin, according to the results of coagulometry) was not followed by complete recovery of thrombin activity. It was probably related to irreversible changes in some molecules of the enzyme (*e.g.*, oxidation).

Addition of desferal by the 60th or 120th minute of incubation with thrombin had the same effect. The curve for this parameter was practically similar on the 60th and 30th minutes. However, the recovery of thrombin activity was less pronounced on the 120th minute.

It may be suggested that thrombin includes the easily-oxidized fractions differing by the degree of oxidative resistance. Otherwise, complete loss of enzyme activity in one molecule of thrombin due to chemical modification requires simultaneous oxidation in several sites. Progressive oxidation of these sites is followed by gradual loss of thrombin activity.

Our results indicate that inactivation of thrombin under the influence of iron (II) (in iron sulfate solution) is mediated by several mechanisms. Hence, it was interesting to study the interaction of iron (III) with thrombin.

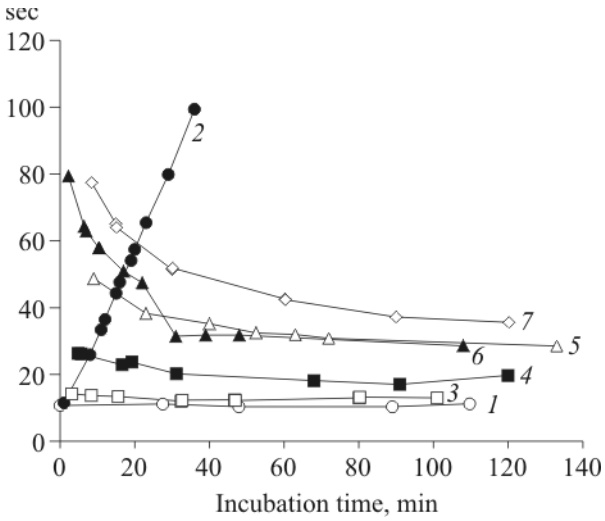

Fig. 2. Effect of desferal on the recovery of thrombin activity after incubation with iron sulfate. Ordinate, time of fibrin clot formation. Control, thrombin without iron (*1*); kinetics of thrombin inactivation by 200 μmM iron sulfate (thrombin time test, *2*). Kinetics of the recovery of thrombin activity in dependence on the time of incubation with 200 μM $FeSO_4$ and further treatment with 2 mM desferal (*3-7*): 5-min incubation with 200 μM $FeSO_4$ before addition of 2 mM desferal (*3*); 15-min incubation with 200 μM $FeSO_4$ before addition of 2 mM desferal (*4*); 30-min incubation with 200 μM $FeSO_4$ before addition of 2 mM desferal (*5*); 60-min incubation with 200 μM $FeSO_4$ before addition of 2 mM desferal (*6*); 120-min incubation with 200 μM $FeSO_4$ before addition of 2 mM desferal (*7*).

Figure 3 shows the kinetic dependence of thrombin inhibition by iron chloride (final concentration 400 μM) and effect of treatment with desferal (2 mM) on the 38th minute of incubation. The influence of iron (III) (solution of $FeCl_3$) on thrombin was similar to that of iron sulfate.

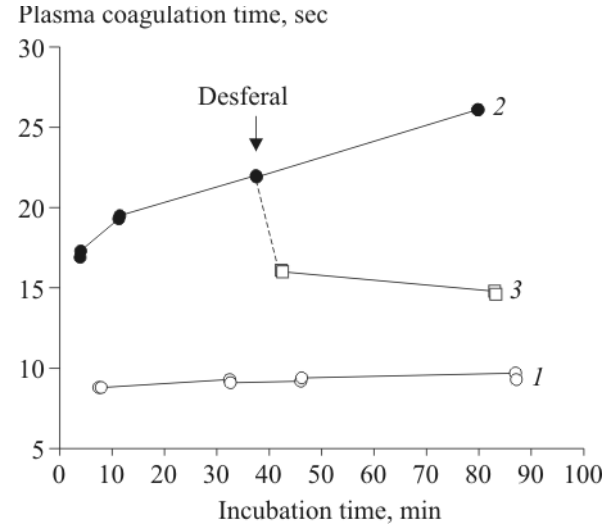

Fig. 3. Effect of Fe(III) ions (final concentration 400 μM) and desferal (2 mM) on thrombin activity (6 U). T_0, addition of Fe(III) ions. Control thrombin (*1*); thrombin+Fe(III) (*2*); thrombin+Fe(III)+desferal (*3*).

Iron (II) and iron (III) suppress the ability of thrombin to form fibrin clot (after addition to the plasma). Thrombin activity is partially or completely normalized after treatment with chelating agents (desferal or EDTA). Incomplete recovery of thrombin activity by desferal is probably related to oxidative modification of thrombin.

We conclude that iron ions have an inhibitory effect on the ability of thrombin to form fibrin clot in blood plasma. It is associated with reversible binding of iron ions to the thrombin molecule and irreversible oxidative modification of thrombin. When the blood accumulates an excess of iron ions, the impairment of coagulation and fibrin clot formation is related to binding of iron ions by thrombin.

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