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# The Effect of Trace Amounts of Tissue Factor on Thrombin Generation in Platelet Rich Plasma, its Inhibition by Heparin

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## Key words

Platelets – Tissue factor – Thrombin – Heparin

## Summary

Amounts of human brain thromboplastin that do not stimulate thrombin generation in platelet poor plasma, were shown to advance by about 4 min an explosive formation of thrombin that occurs after recalcification in the presence of blood platelets. This synergistic effect is inhibited by the specific thrombin inhibitor hirudin and mimicked by adding low concentrations (< 5 nM) of thrombin to platelet rich plasma. It is our conclusion that small amounts of thrombin, generated under the influence of thromboplastin induced procoagulant activity in the blood platelets. This activity is most likely mainly due to procoagulant phospholipids. Heparin inhibits this effect and retards the explosive thrombin formation. It does not, however, diminish the peak amount of thrombin eventually formed, because heparin neutralizing material released from the activated platelets quenches the heparin effect.

## Introduction

Thrombin formation and blood platelet reactions are intimately linked in haemostasis and thrombosis. Thrombin is the most potent biological platelet activator and activated platelets have important procoagulant properties. The concentration of thrombin necessary to trigger a half maximal platelet reaction is significantly lower than that of other physiological activators (ADP, thromboxane A<sub>2</sub>, PAF-acether) (1,2). The membrane of the intact platelets can undergo a transbilayer rearrangement of its phospholipids that makes procoagulant phospholipids (mainly phosphatidyl serine) available at the surface of the intact cell. In washed platelets at a concentration of 10<sup>6</sup> platelets/ml, thrombin plus collagen is the specific trigger for this process (2, 3). Activated platelets release a variety of substances, including coagulation factor V and platelet factor 4 (PF 4), a protein that neutralizes heparin (4–8). It also has been reported that ADP activated platelets trigger coagulation via a factor XII dependent pathway and that collagen activated platelets cause coagulation in a factor XI dependent pathway that bypasses factor XII (9, 10).

From these phenomena, observed mostly with isolated platelets, it is clear that in vivo there must exist several forms of close cooperation between the blood coagulation system and platelets. In vivo, haemostasis will usually be started by the release of tissue factor from wounded cells. Tissue factor is a lipoprotein, present in most mammalian cells but not in blood

platelets. It activates factor VII and thus starts the reaction sequence that leads to thrombin formation (11). In order to investigate the interplay between tissue factor, blood platelets and the plasmatic coagulation factors under in vitro conditions that approach the in vivo situation, we have compared the influence of human brain thromboplastin on thrombin generation in platelet rich and platelet poor plasma.

We found that concentrations of thromboplastin that are without effect on platelet poor plasma, significantly advance the generation of thrombin in platelet rich plasma. Unfractionated heparin inhibited the onset of thrombin generation but not the thrombin yield.

## Materials and Methods

Buffers: A: 0.05 M Tris, 0.1 M NaCl, pH 7.35. B: 0.05 M Tris, 0.1 M NaCl, 20 mM EDTA, 0.5 mg/ml ovalbumin, pH 7.9.

Calcium chloride 0.1 M adjusted to pH 7.35.

Chromogenic substrate, S2238 (Hd-Phe-Pip-Arg-p-nitroanilide) was supplied by AB Kabi Diagnostica (Stockholm).

Human brain thromboplastin was used as a source of tissue factor. It was prepared according to Owren and Aas (12), homogenized in a Potter Elvehjem homogeniser for 3 min, centrifuged at 2,000 g for 15 min and stored at –20°C in 0.1 ml aliquots. Before use it was thawed, diluted with 0.1 M calcium chloride adjusted to pH 7.35 and preincubated 1 hr at 37°C. Hirudin was obtained from Sigma (St. Louis, USA).

Platelet rich plasma was obtained by centrifugation (200 × g, 15 min) of freshly drawn citrated blood from a fasting donor; 1 volume trisodium citrate 0.13 M to 9 volumes blood. The platelet count was adjusted to 3 × 10<sup>8</sup>/ml with homologous platelet poor plasma (centrifuged for 15 min at 10,000 × g). Plastic tubes and pipettes were used throughout so as to minimize contact activation.

## Thrombin Generation

The reaction mixture consisted of 240 µl plasma and 60 µl of buffer A with or without heparin. The samples were kept at 37°C and magnetically stirred. Thrombin formation was started by addition of 60 µl of CaCl<sub>2</sub> (0.1 M) in which thromboplastin was diluted to the desired concentration.

In order to obtain comparable results independent of platelet aging, two to four experiments were run in parallel. At 10 sec intervals a 10 µl sample was taken from each of the mixtures and tested for thrombin. In this way the sampling interval for each mixture is 20–40 sec. A computerised registration of sampling times was used (13). The plasma could not be previously defibrinated as this would cause loss of the platelets. A plastic rod was placed in the samples and as soon as the clot was formed it was wound on the rod and removed. This did not interfere with the 10 sec sampling rhythm. The 10 µl samples were transferred to 490 µl of buffer B containing 0.2 mM S2238. After about 2 min the reaction was stopped with 300 µl of concentrated acetic acid. The time of stopping the reaction was again recorded by the computer. Optical densities (O.D.) were measured in an LKB Ultrospec II spectrophotometer at 405 nm. From the optical density the recorded incubation interval and the proportionality factor (1 O.D. unit/min = 725 nmol thrombin/l) the computer calculated the thrombin content of the samples.

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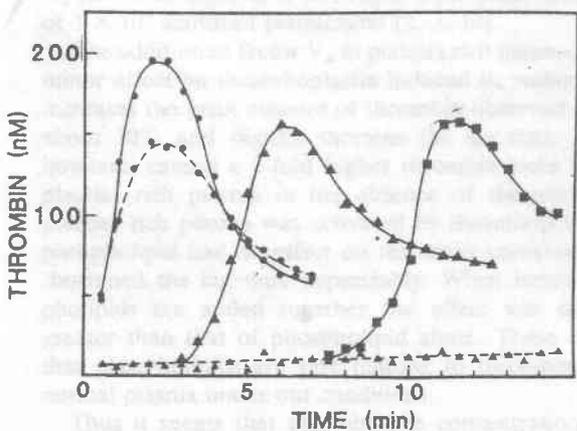


Fig. 1 The influence of thromboplastin on thrombin generation in platelet rich plasma (PRP) and in platelet poor plasma (PPP). Thrombin generation in PRP (solid line) and PPP (broken line) at a final dilution (v/v) of 1/240 (●) or 1/2,400 (▲) and  $\text{Ca}^{2+}$  (25 mM) or 25 mM  $\text{Ca}^{2+}$  alone (■). Experimental conditions as described in Materials and Methods

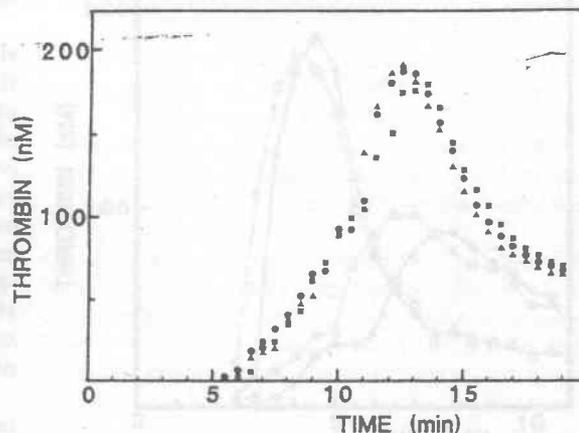


Fig. 2 Thrombin generation in congenital factor VII deficient platelet rich plasma. Symbols used: (●), 1/240 diluted thromboplastin; (▲), 1/2,400 diluted thromboplastin and (■), no thromboplastin. See Materials and Methods for experimental details

## Results

First we determined what dilution of thromboplastin would, in platelet poor plasma, cause less than 10 nM of thrombin to be formed in the course of 20 min. With our thromboplastin preparation this turned out to be a 1:2,000 final dilution. We decided to use a somewhat lower amount (1:2,400) in our experiments in platelet rich plasma.

In platelet rich plasma without thromboplastin, a burst of thrombin formation is seen after a lag time of about 9 min. This lag time is shortened to 4 min in the presence of 1:2,400 diluted thromboplastin. At higher thromboplastin concentrations (1:240) the difference between platelet rich- and poor plasma vanishes (Fig. 1).

The shortening of the lag time is dependent upon thromboplastin-factor VII interaction because it is not seen in congenital factor VII deficient platelet rich plasma (Fig. 2). Thrombin generation in the absence of thromboplastin must be mainly caused by contact activation because it is much retarded in platelet rich plasma from a congenital factor XII deficient patient (Fig. 3).

These results suggest that a common product of extrinsic and intrinsic coagulation is responsible for the thrombin burst in

platelet rich plasma. If hirudin, a specific inhibitor of thrombin is added to the mixture, the lag phase in the presence of thromboplastin is prolonged in a dose dependent way (Fig. 4). As 1 U/ml of hirudin neutralizes 8–10 nM of thrombin, the shortening of the lag time seems to be caused by low concentrations of thrombin produced under the influence of thromboplastin. If thromboplastin is replaced by nanomolar amounts of thrombin, the lag phase is shortened dose dependently (Fig. 4). When the same experiments were performed with platelet poor plasma, preincubation with thrombin had no effect on the thrombin generation curve. These findings suggest that low concentrations of thrombin, either generated during the initial stage of the coagulation process or added to plasma, activate platelets. As a result platelet procoagulant activity is generated and causes an explosive thrombin generation.

We tried to determine the nature of the platelet procoagulant activity that might be responsible for the effect observed. There are two obvious candidates, platelet factor V and procoagulant phospholipids. We mimicked the effect of either of the two by adding 10 nM factor  $\text{V}_a$  or 40  $\mu\text{M}$  phospholipid (20% phosphatidylserine and 80% phosphatidylcholine) to the platelet rich plasma. These amounts of procoagulant phospholipid and factor

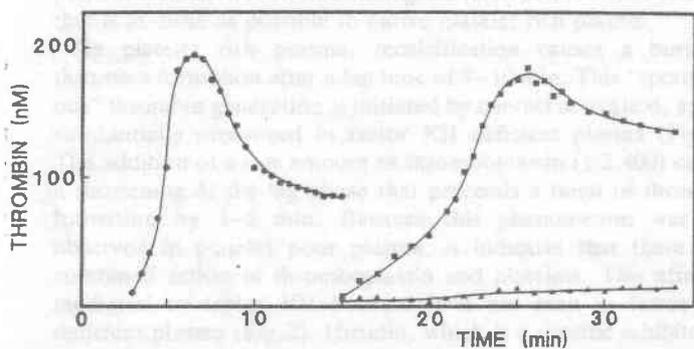


Fig. 3 Thrombin generation in congenital factor XII deficient platelet rich plasma. Symbols used: (●), 1/2,400 diluted thromboplastin; (■), no thromboplastin and (▲), no thromboplastin in platelet poor plasma. See Materials and Methods for experimental details

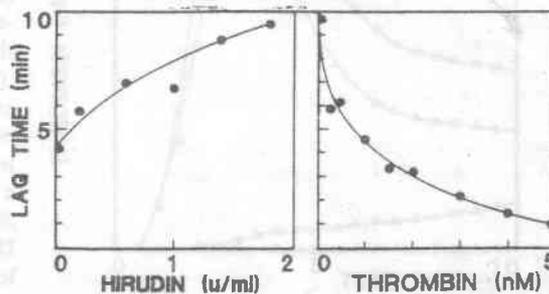


Fig. 4 The effect of hirudin and thrombin on the lag time of thrombin generation in platelet rich plasma. The thromboplastin dilution was 1/2,400 and experimental conditions as in Fig. 1. Hirudin and thrombin were added at zero time to obtain the final concentrations indicated. The lag times were measured at the point where the concentration of generated thrombin (i. e. total thrombin minus added thrombin) exceeded 20 nM

$V_a$  can be considered to be roughly equivalent to the contribution of  $3 \times 10^8$  activated platelets/ml (2, 3, 14).

The addition of factor  $V_a$  to platelet rich plasma has a relatively minor effect on thromboplastin induced thrombin generation. It increases the peak amount of thrombin observed in the burst by about 10% and slightly shortens the lag time. Phospholipids, however, caused a 2-fold higher thrombin yield when added to platelet rich plasma in the absence of thromboplastin. When platelet rich plasma was activated by thromboplastin, additional phospholipid had no effect on thrombin generation (Fig. 5) and shortened the lag time appreciably. When factor  $V_a$  and phospholipids are added together the effect was not significantly greater than that of phospholipid alone. These results indicate that phospholipids are rate limiting to thrombin generation in normal plasma under our conditions.

Thus it seems that thrombin, in concentrations of less than 5 nM, under the influence of small amounts of thromboplastin during the lag time provokes a procoagulant reaction in blood platelets that enables the plasmatic clotting factors to produce a burst of thrombin formation of 100–200 nM. Of the known procoagulant functions of the platelets the exposure of procoagulant phospholipids is the most likely to produce this effect.

#### Effect of Heparin

When heparin is added to platelet rich plasma in concentrations of up to 0.3 U/ml the lag phase is prolonged but, surprisingly, hardly any decrease is seen of the amount of thrombin eventually produced in the burst (Fig. 6). In contrast, heparin, when added to platelet poor plasma in these concentrations causes an important inhibition (more than 90%) of the amount of thrombin formed (15). As can be seen from Fig. 7 this difference between the action of heparin on platelet rich plasma and on platelet poor plasma can be explained by the release of heparin neutralizing material (in all probability platelet factor 4) from the triggered platelets. Heparin (0.5 U/ml) will cause only 20% inhibition of the peak of thrombin formation in the presence of platelet release product(s).

#### Discussion

The procoagulant effect of blood platelets is known since the earliest days of coagulation research. It was indeed postulated by Bizzozero in his first description of the blood platelets (16). Many later observations confirmed the role of platelets in coagulation (17).

We wanted to investigate what the relative importance of platelet activation is on thrombin generation in a reaction medium that is as close as possible to native platelet rich plasma.

In platelet rich plasma, recalcification causes a burst of thrombin formation after a lag time of 8–10 min. This "spontaneous" thrombin generation is initiated by contact activation, as it is substantially prolonged in factor XII deficient plasma (Fig. 3). The addition of a low amount of thromboplastin (1:2,400) causes a shortening of the lag phase that precedes a burst of thrombin formation by 3–5 min. Because this phenomenon was not observed in platelet poor plasma, it indicates that there is a combined action of thromboplastin and platelets. This effect is mediated by factor VII because it is not seen in factor VII deficient plasma (Fig. 2). Hirudin, which is a specific inhibitor of thrombin without known effect on platelets per se is able to prolong the lag time (Fig. 2). Thrombin on the other hand shortens the lag time (Fig. 3). These observations indicate that the effect of thromboplastin on the lag time is mediated by thrombin. The effect of traces of thrombin in advancing the appearance of a thrombin burst in whole blood has been reported as early as 1953

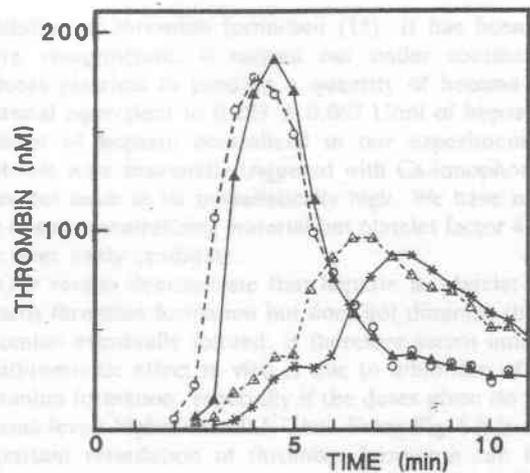


Fig. 5 The effects of phospholipid and factor  $V_a$  on thrombin generation in platelet rich plasma. Symbols used: (▲), 40  $\mu$ M phospholipid (20% phosphatidylserine/80% phosphatidylcholine) and no thromboplastin; (○), 40  $\mu$ M phospholipid (20% PS/80% PC) and 1/2,400 diluted thromboplastin; (Δ), 10 nM factor  $V_a$  and no thromboplastin and (★), no addition

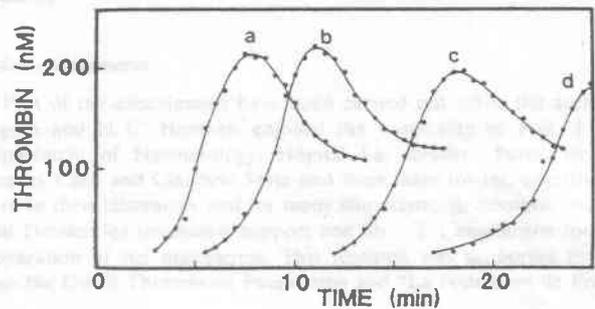


Fig. 6 The influence of unfractionated heparin on thrombin generation in platelet rich plasma. Thrombin generation was started by the addition of thromboplastin (1/2,400) in the presence of (a), no heparin; (b), 0.1 U/ml heparin; (c), 0.2 U/ml heparin and (d), 0.3 U/ml heparin

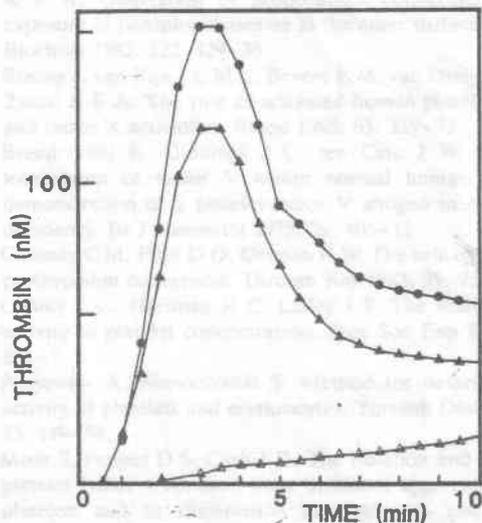


Fig. 7 Neutralization of heparin by activated platelet by 5  $\mu$ M Ca-ionophore (AZ 23187). (●) Plasma obtained by centrifugation of platelet rich plasma activated by Ca-ionophore, to which 0.1 unit/ml heparin has been added. (▲) 0.4 unit/ml heparin. (Δ): control with non activated platelet poor plasma with addition of 5  $\mu$ M Ca-ionophore and 0.1 U/ml heparin

y Biggs and Macfarlane. The mechanism behind this phenomenon has remained obscure, however (17). The occurrence of low concentrations (0.1–1 nM) of thrombin in blood before a thrombin burst occurs that leads to coagulation has been demonstrated by Shuman and Majerus (18).

Two products from activated platelets can contribute directly to thrombin generation: phospholipids and factor V. We added procoagulant phospholipid or activated factor V in quantities that are roughly equivalent to the maximal contribution of  $3 \times 10^8$  platelets/ml. The effect of factor V<sub>a</sub> appeared to be minor compared to that of phospholipids (Fig. 5). The combined effect of phospholipid and factor V<sub>a</sub> was hardly greater than that of phospholipid alone; the lag time was shortened by 30 sec compared to the curves with phospholipid alone and the peak activity was not significantly increased. In order not to overload Fig. 5 these data are not shown. We conclude that it is probably the phospholipid produced by the platelets that enhances thrombin formation.

Bevers et al. (2) have shown that isolated platelets are capable of producing procoagulant phospholipids at the outside of their membrane ("flip-flop"). Under these experimental conditions ( $10^6$  platelets/ml) thrombin (2 nM) needs the simultaneous presence of collagen in order to provoke the reaction. Rosing et al. (3) have shown that at higher platelet concentrations thrombin alone is an efficient trigger. At the platelet concentration we used ( $3 \times 10^8$  platelets/ml) the requirement for collagen is abolished. Indeed, the addition of collagen in our system, either with or without stirring had no effect on thrombin generation in the presence and in the absence of thromboplastin (results not shown).

We can summarize our view of the course of events as follows. In platelet rich plasma, when coagulation is triggered by Ca<sup>2+</sup> and a low concentration of thromboplastin, the system immediately after recalcification can be regarded as platelet poor plasma, in which inactive platelets are suspended. Thromboplastin causes small amounts of thrombin to be formed. These induce a procoagulant reaction in the platelets that starts an explosive thrombin formation. From the known procoagulant activities of the platelets, the production of procoagulant phospholipids is the most likely candidate for the explanation of this effect. The factor V contributed by the platelets seems less important for thrombin generation than the phospholipid is. An additional effect of the thrombin on the activation of factor V and VIII during the lag time cannot be excluded. Biggs and Nossel (19) and Josso and Prou-Wartelle (20) described that at low thromboplastin concentrations the factor VII dependent activation of factor IX contributes to thrombin formation. Østerud and Rappaport proved the existence of this mechanism without reasonable doubt (21). We could show (experiments to be reported separately) that in our system, dilutions of thromboplastin of 1:500 and higher cause an increasingly important contribution of the VII → IX → X pathway. This means that we cannot exclude the possibility of an additional contribution of factor VIII activation to the phenomena observed. The explosion of thrombin is limited by exhaustion of the prothrombin available.

The effect of unfractionated heparin on thrombin generation in platelet rich plasma is significantly different from that in platelet poor plasma. In platelet rich plasma the main feature of heparin addition is a prolongation of the lag time of thrombin formation whereas in platelet poor plasma the decrease of the peak amount of thrombin is the predominant phenomenon (15).

From Fig. 7 it follows that platelets at a physiological concentration contain amounts of heparin neutralizing material that are capable of virtually complete neutralization of 0.5 U/ml of heparin. This represents a heparin concentration that in rigorously platelet and PF 4 free plasma will cause more than 95%

inhibition of thrombin formation (15). It has been shown that mere venipuncture, if carried out under routine conditions, induces platelets to produce a quantity of heparin neutralizing material equivalent to  $0.183 \pm 0.067$  U/ml of heparin (22). The amount of heparin neutralized in our experiments, in which platelets were maximally triggered with Ca-ionophore, therefore does not seem to be unrealistically high. We have not identified the heparin neutralizing material but platelet factor 4 seems to be the most likely candidate.

Our results demonstrate that heparin in platelet rich plasma retards thrombin formation but does not diminish the amount of thrombin eventually formed. It therefore seems unlikely that its antithrombotic effect in vivo is due to inhibition of the bulk of thrombin formation, especially if the doses given do not result in plasma levels higher than 0.5 U/ml. From Fig. 6 it is clear that an important retardation of thrombin formation can follow from heparin concentrations as low as 0.10–0.15 U/ml. We postulate that this retardation is instrumental in obtaining an antithrombotic effect with low doses of heparin. On basis of the data of van Putten et al. (22) we can assume that a circulating heparin concentration of 0.1–0.2 U/ml will hardly be detected by routine laboratory methods. This accounts for the difficulty in finding a laboratory correlate to the antithrombotic action of low dose heparin.

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