Measurement of Thrombin Generation in Whole Blood -
The Effect of Heparin and Aspirin

Han Kessels*, Suzette Béguin, Harry Andree*, H. Coenraad Hemker

From the Cardiovascular Research Institute Maastricht, Dept. of Biochemistry, University of Limburg, Maastricht, The Netherlands

Summary

A technique has been developed to monitor the development of thrombin in freshly collected whole blood in the absence of anticoagulants. It is based on the centrifugal separation of the cellular components from subsamples of blood drawn from non-anticoagulated clotting whole blood which are diluted in buffer containing a chromogenic substrate.

It is shown that the burst of thrombin generation after triggering coagulation with trace amounts of tissue thromboplastin occurs sooner in non-anticoagulated whole blood than in citrated whole blood. Heparin is shown to prolong the lag-time of thrombin generation more in native blood than in recalciﬁed citrated blood.

It is also demonstrated that intake of 500 mg of aspirin signiﬁcantly delays and inhibits thrombin generation in non-anticoagulated, thromboplastin triggered whole blood, whereas it has no effect on the coagulation in citrated plasma. The effect of aspirin intake on thrombin generation in blood is roughly equal to that of 0.03 U/ml of unfractionated heparin. This demonstrates that platelet reactions and the coagulation system are closely linked processes. It further lends support to the hypothesis that inhibition of thrombin generation is a common denominator of antithrombotic therapy.

Introduction

Thrombin is the central enzyme in the process of haemostasis, being at the cross-roads of coagulation and platelet reactions. Measurement of thrombin generation in clotted plasma has provided much insight in the mechanisms of action of the coagulation system (1). Moreover, thrombin generation in platelet rich plasma (PRP) has proven useful in studying the interplay of the plasmatic and the cellular factors in coagulation (2). Measuring the course of thrombin generation is a better way to investigate generalised effects on coagulation than the measurement of clotting times. Clotting is an early event, occurring as soon as 10–20 nM of thrombin are present in the system. It requires less than 10% of the free thrombin that can be potentially generated in plasma (150–300 nM). One can imagine that the extra thrombin generated after coagulation in vivo will diffuse from the site of coagulation and act in a haemostatic and/or prothrombotic manner e.g. on platelets and other cells in the environment. Heparin e.g. has a well established antithrombotic action and yet will not or hardly prolong the thromboplastin time, i.e. the clotting time in the physiologically important extrinsic system. It will have, however, a large effect on the course of thrombin generation after clotting has occurred (3).

Thrombin generation in PRP is nearer to physiology than that in platelet poor plasma (PPP). Yet, PRP is prepared from blood which has been collected in citrate and is recalcified when coagulation is triggered. This process of citration and recalcification, however, may influence the function of platelets and the coagulation system. It is known e.g. that perfusing the isolated rat heart with citrate and then with a "recalciﬁying" Ringer solution is disastrous to heart cells (4). In an attempt to prevent the de- and recalcification step and to remain as close as possible to the physiological circumstances, we developed a technique for measuring thrombin concentrations in non-anticoagulat-ed whole blood. The clotting process between assays was synchronised by adding a very small amount of tissue thromboplastin. Erythrocytes were discarded by centrifugation of subsamples diluted in buffer containing a chromogenic substrate. A comparison was made between thrombin generation in untreated whole blood, and citrated and subsequently recalciﬁed blood.

The coagulation system and platelet activation are closely coupled. Activated platelets provide the negatively charged phospholipid surface necessary for various reactions of the coagulation system (5), and thrombin is the most potent physiological activator of platelets. Therefore, inhibition of platelet function may be expected to affect thrombin generation. Acetylsalicylic acid (aspirin) inhibits platelet function by irreversibly acetylating platelet cyclooxygenase (6). It causes a prolongation of the bleeding time (7) and has been reported to reduce the incidence of thromboembolic disease in several populations at risk (8–11). However, no inﬂuence of aspirin on thrombin generation has as yet been demonstrated. This nevertheless would be expected if indeed coagulation and platelet reactions are closely linked. We thought that this link might be severed by the preparation of PRP (e.g. de- and recalcification) and investigated the possible effect of aspirin on the coagulation system by measuring thrombin generation in non-anticoagulated whole blood. We compared the effect to that of heparin under the same circumstances.

It is our aim to present here the technique and to show that it may be useful in the domain of the study of cell-coagulation system interactions. Further studies on e.g. the aspirin effect will be necessary and are being carried out in our laboratory.

Materials and Methods

Materials

Buffers used were 50 mM Tris-HCl, 100 mM NaCl and 0.5 g/l bovine serum albumin, pH 7.9 (buffer B).

The 4th International standard heparin was obtained from the National Bureau of Standards and Control (London). Enoxaparin was from Rhône-Poulenc-Rorer (Paris). The stable synthetic prostacyclin analog ilomedine (ZK36374) was from Schering AG (Berlin).
Measurement of Thrombin Generation in Non-anticoagulated Whole Blood

The measurement of thrombin generation was carried out in five steps:

Step 1: Venipuncture and preparation of the sample.
Blood was obtained by antecubital venipuncture. The first 2 to 3 ml were discarded and then 4.5 ml were collected in a plastic tube on 0.5 ml buffer A, not containing an anticoagulant, and immediately transferred to a water bath at 37°C. The buffer A was added to obtain a dilution of blood equal to that of citrated blood.

Step 2: Synchronisation of coagulation.
Coagulation was triggered, or rather synchronised (see results) by addition of 0.25V_{final} of buffer A containing 1/600 diluted thromboplastin (final dilution 1/3600). This last addition was at exactly 1 min after collection of blood but may be done at any time within 5 min after venipuncture.

Step 3: Subsampling and dilution of subsamples.
At timed intervals 10 μl subsamples were taken from the whole blood mixture and transferred to tubes containing 190 μl buffer B with 200 μM of the chromogenic substrate S2238. Within 10 s a second dilution was made by transferring 40 μl of the obtained mixture to a microcentrifuge tube containing 760 μl buffer B, again containing S2238 (final concentration 200 μM). This amounts to an overall dilution of 1 in 400 of the original subsample. As soon as the clot tended to interfere with subsampling, it was wound on a plastic spatula and removed. This appeared not to influence thrombin generation (data not shown). The micro centrifugation tubes were kept on ice until subsampling had finished.

Step 4: Centrifugation. The tubes with the diluted samples were centrifuged for 1 min at high speed (Eppendorf centrifuge). Supernatants were transferred to disposable microcuvettes (0.5 ml) and prewarmed for at least 3 min at 37°C.

Step 5: Thrombin determination.
The rate of conversion of S2238 was determined photometrically at 405 nm by measuring optical density at two suitably spaced time points. The thrombin concentration in the whole blood sample was calculated, knowing that 1 pM thrombin causes 0.11 mOD/min, as was determined with active site-labelled pure human α-thrombin. The thrombin concentration was corrected for the void volume occupied by the erythrocytes in the original subsample. This void volume decreases during the course of blood clotting. This was shown (Fig. 1) to be due only to cells getting entrapped in the clot. Thus, it seemed appropriate to use the initial haematocrit values for samples until clot formation, and the final haematocrit values for samples starting from clot formation. The lag time of thrombin formation was determined as the time necessary to reach a level of 50 nM of thrombin, as determined by linear interpolation. The mean (± SD) lag time of thrombin formation was 335 ± 35 s (s.e.), the mean thrombin peak was 163 ± 25 nM (s.e.). Residual prothrombin was measured after coagulation by measuring the increase in amidolytic activity induced by adding an excess of staphylocoagulase (13).

Thrombin Generation in Whole Blood Collected in Citrate

The procedure was completely identical to that described above, except that 4.5 ml of blood were collected in 0.5 ml 0.13 M sodium citrate. Also the additions were made in 0.25 V

Step 4: Centrifugation. The tubes with the diluted samples were centrifuged for 1 min at high speed (Eppendorf centrifuge). Supernatants were transferred to disposable microcuvettes (0.5 ml) and prewarmed for at least 3 min at 37°C.

Step 5: Thrombin determination.
The rate of conversion of S2238 was determined photometrically at 405 nm by measuring optical density at two suitably spaced time points. The thrombin concentration in the whole blood sample was calculated, knowing that 1 pM thrombin causes 0.11 mOD/min, as was determined with active site-labelled pure human α-thrombin. The thrombin concentration was corrected for the void volume occupied by the erythrocytes in the original subsample. This void volume decreases during the course of blood clotting. This was shown (Fig. 1) to be due only to cells getting entrapped in the clot. Thus, it seemed appropriate to use the initial haematocrit values for samples until clot formation, and the final haematocrit values for samples starting from clot formation. The lag time of thrombin formation was determined as the time necessary to reach a level of 50 nM of thrombin, as determined by linear interpolation. The mean (± SD) lag time of thrombin formation was 335 ± 35 s (s.e.), the mean thrombin peak was 163 ± 25 nM (s.e.). Residual prothrombin was measured after coagulation by measuring the increase in amidolytic activity induced by adding an excess of staphylocoagulase (13).

Thrombin Generation in Platelet Rich Plasma

The procedure has been described in detail previously (2). Briefly, platelet rich plasma (PRP) was prepared from citrated whole blood by centrifugation (10 min, 1000 rpm). To 240 μl of PRP were added 60 μl of buffer A, and 60 μl of 1/600 diluted thromboplastin (final dilution 1/3600) in 100 nM CaCl₂ (final added concentration 16.7 mM). Thrombin generation was then monitored by diluting timed subsamples in buffer B containing 200 μM S2238, and assessing the S2238 conversion rate by an end-point measurement of optical density.

Determination of Calcium Dependency of Clotting Times

Blood was collected in an empty PVC tube and immediately placed in a waterbath. At registered times within 2 min after collection, 500 μl were added to a tube containing 55.6 μl of trisodium citrate (0.13 M), 0.25V_{final} of a
Rationale of the Method

The optimal amount of CaCl₂ for recalcifying citrated whole blood was determined by measuring the clotting times of citrated whole blood recalcified with different CaCl₂ concentrations, in the presence and absence of standard heparin (0.2 U/ml, final concentration). Fig. 4 shows the existence of a relatively broad CaCl₂ optimum. The figure also shows that no citrate "calcium" ratio can rival non-anticoagulated whole blood with regard to sheer speed of clotting. Both in citrated and in non-citrated blood the residual prothrombin concentrations as measured with the aid of staphylococagulase was less than 0.5%, i.e. significantly less than the 10% of residual prothrombin that is usually observed when PPP is clotted with thromboplastin (13).

Treatment of the Subsamples

Upon subsampling thrombin formation is halted by the presence of EDTA in the buffer. At the same time the chromogenic thrombin substrate S2238 competitively inhibits the calcium-independent binding of thrombin to protease inhibitors. We measured that the rate of
chromogenic substrate conversion remains constant until the absorbancy at 405 nm exceeds 0.9 OD units. Under our conditions this allows for a centrifugation step before measuring amidolytic activity. With a 400-fold dilution, as used during our experiments, one typically obtains a maximum amidolytic activity of no more than 30 mOD/min, which means that we have about 30 min of constant reaction rate.

**Effects of Heparin**

We investigated whether the effect of unfractionated heparin and a low molecular weight heparin (Enoxaparin) would be the same in native blood and in recalcified citrated blood. The results are shown in Fig. 5. In the upper panel, we show three pairs of generation curves in which we compare the effect of 0, 0.1 and 0.2 U/ml of unfractionated heparin in citrated (open symbols) and native whole blood (closed symbols). Blood was collected three times from the same volunteer, each time in parallel in citrate and in buffer A. The lower panel of Fig. 5 shows a similar experiment, performed with and without 1 µg/ml of enoxaparin. The lag phase between triggering and the burst of thrombin generation was always shorter in untreated whole blood than in citrated whole blood. Increasing concentrations of heparin cause a lag time that is progressively longer in citrated whole blood than in non-citrated whole blood. The heparin induced inhibition of the peak

**Table 1. Effect of aspirin on thrombin generation**

<table>
<thead>
<tr>
<th>Lag-time of thrombin generation (s ± s.e.m.)</th>
<th>before aspirin</th>
<th>after aspirin</th>
<th>paired t-test (two-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>335 ± 11</td>
<td>372 ± 13</td>
<td>t = 4.53, P &lt; 0.005</td>
<td></td>
</tr>
<tr>
<td>Thrombin peak (nM ± s.e.m.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>163 ± 8</td>
<td>147 ± 6</td>
<td>t = 2.46, P &lt; 0.05</td>
<td></td>
</tr>
</tbody>
</table>

The time course of thrombin concentrations (n = 10) was measured in non-anticoagulated whole blood, 5 min before and 1.5 h after intake of 500 mg acetylsalicylic acid. The lag of thrombin generation defined as the exact time when 50 nM of thrombin had formed, was determined by linear interpolation from surrounding data points. Data were evaluated using Student’s t-test for paired observations (two-tailed).

**Fig. 6. Influence of Ilomedine on thrombin generation in non-anticoagulated whole blood and platelet rich plasma. Upper panel: Whole blood. Blood from a healthy donor was collected both on Ilomedine (C) (100 µM, 9 vol blood on 1 vol of Ilomedine) and in an empty plastic tube (O), and placed in a water bath at 37°C. Clotting was triggered by addition of thromboplastin (1/3600 final dilution) exactly at 1 min after collection. Lower panel: PRP. Blood from the same donor was collected both on citrate (C), and on citrate/ILOmedine (O). PRP was prepared as described in the materials and methods section, and placed in a water bath at 37°C. Clotting was triggered by addition of thromboplastin (1/3600 final dilution). In both cases thrombin concentrations were measured as described in the Materials and Methods section.
amount of thrombin appears more important in non-anticoagulated blood than in recalcified blood.

The Effect of Aspirin on Thrombin Generation

The effect of intake of a single dose of 500 mg of aspirin on thrombin generation in whole blood was investigated in 10 healthy male volunteers, none of whom had taken any medication for at least one week. Samples for determination of thrombin generation in non-anticoagulated whole blood, were taken 5 min before, and 1.5 h after aspirin ingestion. Thrombin generation appeared to be both delayed and diminished 1.5 h after aspirin intake (Table 1). The average increase of the lag time, 37 s ± 8 (SEM), was statistically significant (p < 0.005, t-test for paired observations, two tailed). Inhibition of thrombin peak values of thrombin generation after aspirin was significant as well (16 nM ± 6, p < 0.05).

In order to determine the maximal effect on thrombin generation in whole blood that can be obtained by inhibiting blood platelets, we collected blood in 10 µM (final concentration) of the stable synthetic prostacyclin analog Ilomedine (ZK36374). Platelets from blood collected in Ilomedine did not aggregate after addition of collagen. Blood was also collected in citrate and Ilomedine to assess the effect of Ilomedine in platelet rich plasma. In five different experiments we saw that both in non-anticoagulated whole blood and in platelet rich plasma Ilomedine caused a small (2 min) delay of thrombin generation. In non-anticoagulated blood the amount of thrombin generated was reduced by about 15% whereas the inhibition in PRP was much larger. Fig. 6 shows a typical experiment.

Discussion

It is common practice to study the thrombin generating system in platelet poor plasma. This indeed is the best way to investigate the interplay of factors in the soluble plasmatic coagulation system. In the blood however this system does not act in splendid isolation, but in the presence of blood cells. It becomes more and more clear that there are many connections between the thrombin-generating system of plasma and blood platelets and other blood cells. It therefore may be useful to compare thrombin generation in PPP to thrombin generation in whole blood. If citrated blood is used, then the platelets and other cells go through a period of low ambient Ca²⁺ concentration. It has been shown that this procedure in the isolated perfused rat heart induces important changes in cell reaction and viability (see 4 and references therein). One would therefore like to study whole blood in a setup where no transient changes of Ca²⁺ concentration have occurred. This amounts to investigate freshly drawn blood. Such blood must be thought to be triggered immediately upon venipuncture. It is evident from our results that the addition of a fixed low concentration of tissue factor at between 1 and 5 min after venipuncture makes it independent of suboptimal triggering occurring upon venipuncture and synchronises the clotting process in different experiments.

Apart from developing the experimental system, in this study we did preliminary experiments to see whether the effect of heparin and platelet inhibitors would be different in non anticoagulated whole blood and in citrated and recalcified whole blood.

Heparin

Comparison of thrombin generation in the absence and presence of heparin in citrated whole blood with thrombin generation in platelet rich plasma in a similar experimental system, as measured by Bégün et al. (2) showed little differences both in lag times and in peak thrombin concentrations (results not shown). In contrast to this, we observed that the burst of thrombin generation occurs earlier after triggering in blood without added anticoagulant. Furthermore, the influence of heparin on the lag time of thrombin generation is considerably smaller in untreated whole blood than in recalcified citrated whole blood but the inhibition of the peak amount of thrombin appears larger. Fig. 4 shows that this effect is not due to a suboptimal citrate to calcium ratio in the case of citrated whole blood. It may be related directly to the presence of citrate.

There are several conceivable effects of citrate on the clotting process. Citrate is a weak Ca²⁺ buffer, since it binds Ca²⁺ with a relatively low affinity (Kd = 0.25 mM, 14). Moreover, Mg²⁺ is bound by citrate with equal affinity, resulting in a decreased concentration of free Mg²⁺ after recalcification, which may affect platelet function. Also, citrate may slightly increase the pH of the reaction mixture. Finally, a direct effect of citrate on platelet procoagulant or heparin scavenging functions may not be excluded. It is therefore conceivable that the presence of citrate modifies thrombin generation when platelets participate in the process.

It must be stressed that our results pertain to heparin added to the blood after venipuncture. It is planned to determine the effects of the administration (IV and SC) of (unfractionated- and low molecular weight-) heparin to volunteers. The main effect to be expected is that in low molecular weight heparins the very low molecular weight material that is resistant to pf 4 action will be relatively enriched in the later hours of heparin action, due to the longer half life time of these heparin molecules (15).

Aspirin

Classically a distinction is made between arterial thrombosis and primary haemostasis which are viewed as predominately platelet mediated processes, and venous thrombosis and secondary haemostasis where the coagulation system plays the major role. Yet recent insights tend to stress the importance of thrombin in the pathogenesis of arterial thrombosis (16-17) and major clinical trials show that anticoagulant drugs such as heparin and oral anticoagulants, are effective anti-thrombotics not only in the venous but also in the arterial circulation (18-20). This suggests that affecting the concentration of free thrombin may be a shared mechanism of action of anti-thrombotic drugs. On the other hand aspirin has been reported to reduce the incidence of thromboembolic disease in several populations at risk (8-11). Its mode of action is understood to be the irreversible acetylation of platelet cyclooxygenase. The blood coagulation system has not generally been thought to be involved in its antiplatelet and bleeding time prolonging effect.

This might be thought to refute the hypothesis of a central role of thrombin. In the body however thrombin generation and platelet activation are strongly interlinked. It therefore is a logical question to ask whether, in a more integrated system such as whole blood, platelet inhibition and thrombin generation are coupled or not.

Our observation that aspirin intake delays and inhibits thrombin generation in non-anticoagulated whole blood opens the possibility that aspirin indeed (partially) acts via its influence on thrombin generation. The two explanations are not mutually exclusive because inhibition of cyclooxygenase may be the cause of the anticoagulant effect and/or may have additional effects independent of coagulation. Anyhow the cyclooxygenase inhibition must be thought to be maximally present 1.5 h after aspirin ingestion, i.e. the moment that we took the second
blood sample (21). The extent of the effect is comparable to that of a moderate concentration (0.03 IU/ml) of unfractionated heparin. We found no effect of aspirin in citrated blood or platelet rich plasma. A similar effect of aspirin on coagulation in non-anticoagulated whole blood in an otherwise completely different experimental system was recently observed by Basic-Micic et al. (22). Furthermore, Kyrie et al. showed that fibrinogen-free plasma after bleeding thrice was decreased after aspirin treatment (23).

An estimate of the maximal effect on thrombin generation in whole blood and platelet rich plasma that can be caused by platelet inhibition was obtained by collecting blood on Ilomedine (final conc. 10 μM). This substance in this concentration is known to completely inhibit platelet activation by thrombin (24). Iomedine causes a relatively small delay of thrombin generation (about 2 min). Both in PRP and in whole blood, a reduction of the amount of generated thrombin was observed. The effect was much more important in PRP than in whole blood, which may possibly be attributed to the presence of erythrocytes. Anyhow, addition of the leucocyte fraction to PRP had no effect on thrombin generation and its inhibition by Iomedine (results not shown).

We must conclude that inhibiting platelets is a relatively inefficient way to inhibit overall thrombin generation in experimental systems such as the one we used. The effect of aspirin must be considered as being of the same order as that of Iomedine. The modest maximal effect attainable by platelet inhibition will make that the influence of aspirin on thrombin generation is easily overlooked.

The observation that inhibitors of platelet function, such as aspirin, do affect thrombin generation in non-anticoagulated whole blood, lends support to the notion of a common pathway for the various sorts of anti-thrombotic therapy. Reduction of thrombin concentrations, then, may be achieved by inhibition of thrombin formation (e.g. oral anticoagulation), by increase of thrombin inactivation (e.g. heparin) or by inhibition of platelet function (e.g. aspirin). We do not claim that the observed inhibition of thrombin generation completely explains the thienopyridone action of aspirin but we want to suggest that it contributes to it. Further studies e.g. on dose dependency, duration of the effect etc. will be necessary.

In conclusion, a precise chromogenic measurement of thrombin generation in whole blood samples is feasible using the technique presented here. Collection of blood in citrate appears to cause a delay and diminution of thrombin formation and causes the effect of heparin to be overestimated. In addition a delaying and inhibiting effect of aspirin ingestion on thrombin generation can be demonstrated in whole blood only, stressing the linkage between platelet reactions and coagulation. We are aware of the fact that the reported phenomena pose more questions than they answer and that much more work has been done to precisely define the pharmacokinetics of this aspect of heparin and aspirin action.

Acknowledgements

We thank “De Broeders van Den Beiaard” for their generous donations of blood.

References