THE POTENTIATION OF PLATELET AGGREGATION AND ADHESION BY HEPARIN IN VITRO AND IN VIVO

C. THOMSON, C. D. FORBES AND C. R. M. PRENTICE

University Department of Medicine, Glasgow Royal Infirmary

(Received 1 February 1973)

SUMMARY

1. Heparin has been shown to increase platelet aggregation by ADP and adrenaline and to enhance the platelet release reaction when tested in citrated platelet-rich plasma (P.R.P.). This activity is present when heparin is added to P.R.P. or when P.R.P. is prepared after intravenous injection of heparin, and when heparin is added to non-anticoagulated native P.R.P.

2. Retention of platelets by cellophane membranes within a specially designed test-cell was significantly increased when heparin was added to citrated whole blood.

3. Though aspirin blocks the release reaction with and without heparin, it does not prevent the potentiation of initial ADP or first wave adrenaline aggregation caused by heparin.

Key words: heparin, platelets, extracorporeal circulation.

Heparin is used as the initial therapy in many thrombotic disorders, and is the only anticoagulant suitable for extracorporeal circulations such as haemodialysis and cardiopulmonary bypass machines. In these situations, a fall in platelet count, and platelet/fibrin deposition on the artificial surface can occur in spite of adequate heparinization (Salzman & Britten, 1965; Lindsay, Prentice, Davidson, Burton & McNicol, 1972a) and it would appear that platelet adhesion to the artificial surface is important in initiating these changes (Lindsay, Prentice, Ferguson, Burton & McNicol, 1972b).

During a recent investigation of the problems of thrombus formation within cardiopulmonary bypass machines, we observed that therapeutic levels of heparin seemed to potentiate ADP-induced platelet aggregation. As there are conflicting reports of the effects of heparin on platelet function we have studied the action of heparin both in vitro and in vivo on platelet aggregation, and retention by cellophane membranes in a specially designed test-cell.

Correspondence: Dr C. Thomson, University Department of Medicine, Royal Infirmary, 86 Castle Street, Glasgow G4 0SF.
MATERIALS AND METHODS

Blood samples

Blood was taken in plastic syringes by clean venepuncture from healthy volunteers who had not ingested aspirin for 14 days before testing. Nine volumes of blood were added to 1 volume of 3.8\% (w/v) sodium citrate in plastic or siliconized glass tubes. In some experiments, native (non-anticoagulated) plasma was used.

Platelet rich plasma (P.R.P.) was obtained by centrifugation at 600 g for 5 min. Platelet poor plasma (P.P.P.) was obtained by centrifugation at 3000 g for 15 min. Plasma samples were kept in plastic or siliconized glass tubes at 37°C until needed; all were used within 60 min of sampling. Siliconized or plastic pipettes were used throughout.

Materials

- **Heparin.** Two brands were used; 'Pularin' (Evans Pharmaceuticals, Speke), batches 2F 957, mucous, both with and without preservative (0.15\% chlorcresol), and Weddel mucous heparin, with and without preservative (Weddel Pharmaceuticals Ltd, London, E.C.1, batches 412, 424E).
- **Adenosine diphosphate (ADP).** This was from Sigma Chemical Co., London.
- **Collagen.** Bovine Tendo-Achilles collagen (Sigma Chemical Co., London) was homogenized in 0.15 mol/l NaCl and coarse particles were removed by centrifugation. The supernatant was diluted to give a moderate response in control samples, and stored at 4°C.
- **Adrenaline.** Adrenaline injection B.P. (McCarthy's Ltd, Romford, Essex) was diluted in distilled water.
- **Adenosine.** This was from British Drug Houses Ltd, Poole, Dorset.
- **Barbitone buffer.** This was 0.028 mol of sodium barbitone/l and 0.126 mol of NaCl/l adjusted to pH 7.35 with HCl.
- **Dialysis membrane.** This was cellophane membrane 300PT (British Cellophane Ltd, Bridgewater, Somerset).

Platelet aggregation

Platelet aggregation was measured in P.R.P. by the turbidometric method of Born & Cross (1963) by using a Bryston aggregometer (Model AG2). The recorder was adjusted each time so that the difference between P.R.P. and P.P.P., which was designated 100\%, gave the same scale deflection. Test plasma (0.9 ml) was stirred in a siliconized glass cuvette at 37°C for 1 min in the machine before 0.1 ml of the aggregating agent was added. The final concentration of the aggregating agent was varied for each subject to give an intermediate response in the control sample but was approximately, 0.5 \( \mu \)mol of ADP/l and 4.5 \( \mu \)mol of adrenaline/l.

Platelet adhesiveness

Platelet retention by cellophane dialysis membranes was measured in a test-cell designed for this purpose, as described by Lindsay, Prentice, Ferguson, Muir & McNicol (1973). The test-cell consists of two perspex blocks clamped together with an intervening gasket to provide a thin compartment lined by dialysis membrane. Citrated blood (10 ml) is introduced through a port in to this compartment, and the test-cell rocked uniformly for 10 min, after which time the blood is removed. Either 0.1 ml of heparin (final concentration 4 units/ml) or 0.1 ml of 0.15
mol of NaCl/l were added to the blood before its introduction into the test-cell, and the fall in platelet count after exposure to the membranes was expressed as a percentage of the initial count.

Platelet counts

Platelet counts were carried out by the method of Dacie & Lewis (1970) on coded samples to eliminate bias in the observer.

Addition of heparin

For testing the effect of heparin in vitro on platelet aggregation, 1 volume of heparin or saline was added to 9 volumes of citrated P.R.P. to give the final desired heparin concentration. For testing the effects in vivo, seven volunteers were fasted overnight and given 10 000 units of heparin intravenously after initial samples had been taken. A second sample was taken from the opposite arm 15 min after the injection. The effects in vitro of heparin were also tested before and 2 h after the subject had ingested 600 mg of aspirin. Finally, the effect of adenosine added to the citrate anticoagulant to give a final concentration of 100 mmol of adenosine/l was studied.

Analysis of results

The terms used are defined below.

Degree of aggregation. The difference in light transmission between P.R.P. and P.P.P. is called 100%. The difference in light transmission between the P.R.P. baseline and any point on the aggregation curve is called the degree of aggregation, and is expressed as a percentage.

Maximum rate of aggregation. A line was drawn tangentially to the steepest part of the ADP and first and second wave adrenaline curves, and the slope of this line was expressed as the degree of aggregation in unit time.

Reaction time. The time taken from the addition of collagen to the beginning of aggregation.

Results were analysed by comparing the maximum rate of aggregation by ADP, maximum rate of first and second wave adrenaline aggregation, and the degree of aggregation 2 min after the addition of ADP and adrenaline. For studies with collagen, the reaction time and the degree of aggregation 3 min after the addition of collagen were used. Statistical significance was evaluated by a paired Student's t-test.

RESULTS

Effect of different heparin concentrations

The effects of varying amounts of heparin in vitro on ADP aggregation in citrated P.R.P. are shown in Fig. 1. Potentiation of the response to 0.5 μmol of ADP/l was present at heparin concentrations between 0.25 and 3.0 units/ml. An increase of heparin concentration did not further potentiate the response. As a result of this experiment, a heparin concentration of 3 units/ml was used in the remainder of the aggregation tests in vitro.

Effects of heparin with varying ADP concentrations

Typical dose–response curves in one subject’s citrated P.R.P. with added heparin or saline, to two different concentrations of ADP are shown in Fig. 2. With 0.25 μmol of ADP/l, platelet aggregation in the presence of saline (curve 1) is considerably less than in the presence of
heparin (curve 3). Similarly with 0·5 μmol of ADP/l the control platelet aggregation response (curve 2) is less than after the addition of heparin (curve 4). This pattern of response was seen consistently in each of four subjects. Fig. 2 also shows that secondary aggregation occurred in the heparin sample at ADP concentrations that were insufficient to cause this in the control.

As can be seen from Fig. 3, there was a linear relationship between the rate of maximum aggregation and the logarithmic ADP concentration, both in the control samples, and in the heparin samples at low ADP concentrations. Aggregation was potentiated in the heparin samples, the maximum effect occurring at an ADP concentration of 0·8 μg/ml (1·5 μmol/l). At higher ADP levels the heparin response was not linear and approached the control values.

![Graph showing the potentiating effect of increasing heparin concentrations on aggregation by ADP.](image)

**Fig. 1.** The potentiating effect of increasing heparin concentrations on aggregation by ADP. The heparin concentration in units per ml is indicated in parentheses for each curve. The arrow shows when 0·5 μmol of ADP/l was added.

**Effects of heparin in vitro on ADP, collagen and adrenaline aggregation**

The effect of heparin *in vitro* at 3 units/ml on ADP, collagen and adrenaline aggregation in the citrated P.R.P. from seven subjects is shown in Table 1. The mean (±1 SD) of the differences between the control and heparin values are tabulated. Both the maximum rate, and the degree of ADP and adrenaline aggregation (first and second wave) were increased by heparin in every subject; these changes were statistically significant. The reaction time after addition of collagen was significantly shortened. Although the mean degree of aggregation by collagen after heparin showed an increase, this change was not statistically significant.
Effect of heparin in vivo on ADP, collagen and adrenaline aggregation

The pattern of ADP aggregation in vivo was similar to that noted when heparin was added in vitro, in that both the rate and degree of aggregation were significantly increased by heparin. Table 1 shows the means (±1 SD) of the differences in ADP, collagen and adrenaline aggregation in the citrated P.R.P. from seven volunteers before and 15 min after the injection of heparin.

Although the mean reaction time after addition of collagen was shortened after heparin injection, this was not statistically significant, and the degree of aggregation by collagen did not vary significantly. The rate of first wave adrenaline response was significantly greater after heparin in all seven subjects. The rate of second wave response could not be compared, as the adrenaline level had to be decreased in the heparin samples to give a clear first and second wave, and at this low level, the control samples did not always undergo a measurable secondary aggregation.

Effect of heparin in native P.R.P.

It was found that by careful handling and thorough siliconizing of the glass cuvettes, native plasma could be prepared and used for one or two aggregation experiments before clotting took place. Smaller concentrations of ADP and adrenaline were required to induce aggregation.
Fig. 3. Maximum rate of initial aggregation (%/min) is plotted against log of ADP concentration giving a linear relationship. Potentiation by heparin (final concentration 3 units/ml) is maximal at 0·8 µg/ml of (1·5 µmol/l) ADP after which the relationship is non-linear tending toward control values. ●, Heparin; □, control.

Table 1. Results of tests *in vitro* and *in vivo* in seven subjects. Mean and standard deviation of the differences from control values caused by heparin are tabulated.

<table>
<thead>
<tr>
<th></th>
<th>ADP</th>
<th>Collagen</th>
<th>Adrenaline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate (%/min)</td>
<td>Degree (%)</td>
<td>Reaction time (s)</td>
</tr>
<tr>
<td><em>In vitro</em></td>
<td>Mean</td>
<td>+23</td>
<td>+22</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>±9·8</td>
<td>±19</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0·001</td>
<td>0·0125</td>
</tr>
<tr>
<td><em>In vivo</em></td>
<td>Mean</td>
<td>+20</td>
<td>+23</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>±7·2</td>
<td>±6·8</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0·001</td>
<td>0·001</td>
</tr>
</tbody>
</table>
**FIG. 4.** ADP aggregation in native P.R.P. is potentiated by the addition of heparin (final concentration 3 units/ml) immediately before the aggregating agent. The arrow marks when 0·125 μmol of ADP/l was added to the plasma.

**FIG. 5.** The potentiation of initial ADP and first wave adrenaline aggregation by heparin is still present after ingestion of aspirin, even though this blocks secondary aggregation. The arrows indicate where (a) 4·5 μmol of adrenaline/l and (b) 0·5 μmol of ADP/l was added to the plasma.
in native plasma than in citrated plasma. Fig. 4 shows the effect of adding heparin (final concentration 3 units/ml) or saline to native plasma immediately before the aggregating agent. Potentiation of both ADP and adrenaline aggregation by heparin occurred in all of the four subjects tested.

Effect of inhibitors of aggregation

Ingestion of 600 mg of aspirin completely inhibited the second wave of ADP and adrenaline aggregation in citrated P.R.P., but failed to abolish the potentiation of the initial response by heparin (Fig. 5). The presence of adenosine at 100 mmol/l concentration completely inhibited aggregation by all agents, with no difference between heparin and control.

Platelet retention by cellophane membranes

A significant increase in platelet retention within the cellophane membrane test-cell was produced by the addition of heparin (final concentration 4 units/ml) to the citrated whole blood of seven volunteers. The mean difference was +34% (SEM ±3.52; t, 9.66; P<0.001).

Effect of heparin on platelet count

The mean platelet count of twelve volunteers before 10 000 units of heparin intravenously was 204 000/mm^3 (1 SD ± 28 000). After heparin (15 min) the counts were not changed significantly (205 000/mm^3, 1 SD ± 30 000).

Throughout the experiments, the different makes and batches of heparin all appeared to potentiate platelet aggregation to a similar extent.

DISCUSSION

Since its introduction, reports of the action of heparin on platelet function have been conflicting. Best (1938) showed that heparin did not prevent ‘white thrombus’ formation in cellophane arteriovenous shunts in rabbits, but did so in the dog, monkey and cat, indicating some species difference. Earlier workers (Fidlar & Jaques, 1948; Gollub & Ulin, 1962) found a fall in platelet count after heparin injection, though our present study and those of Quick, Shanberge & Stefanini (1948) and Davey & Lander (1968) did not confirm this.

The effect of heparin on platelet retention when blood is passed through columns filled with glass beads is also not clear. There are reports of decreased (Moolten, Vroman, Vroman & Goodman, 1949), increased (O’Brien, Shoobridge & Finch, 1969; Bowie, Owen, Thompson & Didisheim, 1969) and unchanged (Salzman, 1963) retention in the presence of heparin. These variations reflect probably both the number of techniques for measurement of platelet retention, and the effect of batch variation amongst different brands of heparin.

In this study, we found that heparin causes potentiation of platelet aggregation by ADP and adrenaline, and increases platelet retention within a test-cell lined by cellophane membranes. Heparin also appeared to lower the threshold at which ADP-induced aggregation goes on to the secondary, irreversible phase. We have shown that these changes can be induced consistently by plasma levels of heparin which are achieved by normal therapeutic dosage.

This effect was seen with two brands of heparin widely used in the United Kingdom and did not appear to be dependent on the presence or absence of preservative. Though variation in
batches of heparin is known to occur (Jaques, 1972) all of the batches tested gave the same kind of response.

The effect of ionized Ca\(^{2+}\) concentration on platelet function in blood anticoagulated by heparin or citrate must be considered. Heparin is known to bind only small amounts of ionized Ca\(^{2+}\) as shown by Tidball (1967), and the lowered ionized Ca\(^{2+}\) concentration in citrated plasma compared to heparinized plasma could account for the potentiation of platelet aggregation in heparin-anticoagulated plasma noted previously by Spaet & Zucker (1964) and O'Brien et al. (1969). The greater sensitivity to ADP which we observed in native P.R.P. compared with citrated P.R.P. would seem to be a reflection of the greater availability of ionized Ca\(^{2+}\). Nevertheless by keeping the ionized Ca\(^{2+}\) concentration constant, by use of citrated or native P.R.P., we were able to study changes due solely to heparin on platelet aggregation and found that it potentiated platelet aggregation both in the presence or absence of citrate.

O'Brien et al. (1969) reported that the second wave of adrenaline aggregation was poorer in heparinized P.R.P. than in citrated P.R.P., and felt that heparin inhibited the platelet release reaction. The responses in citrated and heparinized plasma are not comparable, as already discussed because of the different ionized Ca\(^{2+}\) levels. In citrated plasma, we found (Fig. 2) that heparin lowered the ADP concentration needed for the release reaction (as judged by progression to secondary aggregation) and thus appeared to enhance the release reaction. It was not possible to show this phenomenon in native plasma as dose–response curves could not be done before clotting occurred. There is additional evidence that heparin enhances the release reaction. Zucker (1971) showed that nucleotide release from platelets by centrifugation was greater when heparin was added to the P.R.P. Also, Eika (1972) has shown that, in washed human platelets, heparin can cause aggregation after a lag period owing to release of endogenous ADP. The shortening of the reaction time after addition of collagen which we observed in the heparin samples also suggests that heparin enhances the release reaction. The effects of heparin cannot be completely explained by enhancement of the release reaction since, when this was blocked by aspirin, we found that potentiation of the initial ADP response and first wave adrenaline response still persisted. It is noteworthy that heparin carries a strong negative charge, and agents such as unsiliconized glass or kaolin, which can induce platelet release, also have negative surface charges. Possibly the effect of charged heparin complexing with the platelet membrane could potentiate platelet reactions.

The potentiating effect of heparin on platelet aggregation and retention of platelets by cellophane membranes may be of clinical importance. When heparinized blood is passed through an extracorporeal circulation, thrombocytopenia and consumption of clotting factors frequently occurs (Salzman & Britten, 1965) and in haemodialysis machines, thrombus formation on the dialysis membrane can occur (Lindsay et al., 1972a). These changes can contribute to the bleeding problem after cardiopulmonary bypass. A recent trial of both aspirin and the anti-platelet RA 233 showed that these agents significantly decreased the thrombocytopenia and blood loss which occurred during dialysis (Lindsay et al., 1972b). The increased platelet retention by cellophane membranes, induced by heparin, suggests that although providing anticoagulation, heparin may exacerbate the thrombocytopenia. Williams (1971) has described neurological deficits, and retinal vessel occlusions following bypass, and suggests that these are caused by platelet emboli.

It is possible that problems of thrombocytopenia with post-operative bleeding, and the
neurological sequelae from platelet microemboli, which are associated with cardiopulmonary bypass, may be aggravated by heparin anticoagulation because of its effect on platelets. Similarly, these problems may be helped by the use of an antiplatelet agent, as well as by attempts to make improved non-thrombogenic surfaces.

ACKNOWLEDGMENT

C. R. M. Prentice acknowledges gratefully the support of the Wellcome Trust.

REFERENCES


