Dose- and Shear Rate-dependent Effects of Heparin on Thrombogenesis Induced by Rabbit Aorta Subendothelium Exposed to Flowing Human Blood

Werner Inauen, Hans R. Baumgartner, Thomas Bombeli, André Haeberli, and P. Werner Straub

The effect of heparin on thrombogenesis induced by the subendothelium of rabbit aorta was investigated in 24 healthy volunteers after intravenous injection of different doses (0, 1000, 2500, and 5000 IU). By using an ex vivo perfusion chamber system, the interaction between flowing blood and exposed subendothelium was measured at low (50 s⁻¹), intermediate (850 s⁻¹), and high (2600 s⁻¹) wall shear rates. The low shear rate simulated blood flow in venous, the intermediate shear rate in arterial, and the high shear rate in small or stenosed arterial vessels. Deposition of fibrin, platelets, and platelet thrombi on vascular subendothelium (SE) was quantified by morphometrical and immunological techniques. Fibrin deposition prevailed at low shear rates and was only minimal at high shear rates (30±1% vs. 1±0.4% coverage of SE with fibrin, mean±SEM, p<0.001). In contrast, the interaction of platelets with SE was more intense at high compared to low shear rates, as indicated by higher platelet adhesion (54±5% vs. 4±1% coverage of SE with platelets, p<0.001) and platelet thrombus volumes (4.8±1.3 vs. 0.5±0.1 µm³/µm², p<0.001). Fibrin deposition on SE was inhibited by heparin in a dose-dependent manner and was abolished after high doses. In addition, high doses of heparin reduced the height and volume of platelet thrombi at low and intermediate wall shear rates, but no effect was found at the high shear rate. Our data show that heparin inhibits the formation of both fibrin and platelet thrombi on vascular subendothelium. The lack of effect of heparin on platelet thrombus formation at high shear rates indicates that thrombin modulates the growth rate and/or stability of platelet thrombi at low and intermediate shear rates, whereas additional factors may control platelet thrombus growth and stability at high shear conditions. (Arteriosclerosis 10:607-615, July/August 1990)

Heparin is an effective and widely used agent for the prophylaxis and treatment of thromboembolism. However, the guidelines for an optimal therapy with this antithrombotic drug have been debated for several decades, and there is still some controversy on the doses considered as effective for the prevention and treatment of thrombosis. Part of the dosage problem of heparin is due to the lack of detailed information concerning 1) the relationship between dose and antithrombotic effect and 2) the impact of local flow conditions on the efficacy of this drug.

In an attempt to bridge the gap between sophisticated test tube experiments in vitro and the complex situation in vivo, we used perfusion chambers, which expose vascular subendothelium to flowing blood directly withdrawn from the antecubital vein of human subjects. The chosen model permits a quantitative assessment of fibrin, platelets, and platelet thrombi deposited on the thrombogenic vascular surface. Variation of blood flow velocity and the use of chambers with different annular dimensions allows mimicking of shear conditions identical to the human vascular system. The exposure of vascular subendothelium to flowing blood has proven useful for the study of patients with coagulation and platelet defects as well as for the study of drugs inhibiting platelet aggregation and oral contraceptives. Therefore, we chose this method to evaluate the antithrombotic effect of heparin, testing the full dose range used clinically for the prevention and treatment of thrombosis. We further tested the impact of heparin for wall shear rates typically found in venous (50 s⁻¹), arterial (650 s⁻¹), and small or stenosed arterial (2600 s⁻¹) vessels.

Methods

Subjects

Twenty-four healthy members of the hospital staff (physicians and medical students; 19 men, five women) gave informed consent to a protocol approved by the local Human Subjects Committee. Their ages ranged from 23 to 43 years (mean 27.3 years). None had ingested any drugs for a period of at least 1 month. In a first group of 12 subjects, perfusion experiments at constant exposure times (5 minutes) and at a wall shear rate of 650 s⁻¹ were performed 20 minutes after an intrave-
nous bolus injection of: 1) the heparin solvent glucose 5% (control), 2) 1000 IU of heparin (Liquemin, Hoffmann-La Roche, Basel, Switzerland), 3) 2500 IU of heparin, and 4) 5000 IU of heparin, respectively. In a second group of six subjects, chamber perfusions were performed at different exposure times (3 and 10 minutes), starting with the perfusion 20 minutes after an intravenous application of: 1) glucose solution and 2) 5000 IU of heparin. In a third group of six volunteers, perfusion experiments were performed at low shear rates (50 s⁻¹, exposure time 5 minutes) and high shear rates (2600 s⁻¹, exposure time 2 minutes) after an intravenous application of: 1) glucose solution and 2) 5000 IU of heparin. In all groups, treatment was given in a randomized sequence, and there was an interval of at least 1 week between two experiments.

**Blood Values**

Blood was collected by an infusion set immediately before the start of each perfusion experiment. Platelet and leukocyte count, hemoglobin, and hematocrit were evaluated in ethylenediaminetetraacetic acid (EDTA) blood by an electronic counting device (Model S, Coulter Electronics, Hialeah, FL). Fibrinogen, partial thromboplastin time (PTT), thrombin time, and reptilase time were determined in citrated blood with standard methods. Fibrinopeptide A was measured immunologically; the normal range for standard collection by single needle was 0.5 to 2.0 ng/ml. Heparin activity was determined with a commercial anti-ilia system by using a chromogenic substrate (Chromozym-Th, Boehringer GmbH, Mannheim, FRG).

**Perfusion Procedure**

An annular perfusion chamber was used to expose subendothelium to whole blood under controlled flow conditions. The procedure has been previously described. Blood from the antecubital vein was drawn directly through an infusion set (Butterfly-19, Abbott Ireland, Sligo, Republic of Ireland) over subendothelium from everted rabbit aorta completely denuded of endothelial cells by balloon catheter injury and mounted on the rod of the annular perfusion chamber. Vessels were stored in 0.2 M Tris buffer at 4°C for 10 to 28 days before use. Blood was maintained at 37°C by a water bath and was not recirculated. Flow through the chamber was controlled by a peristaltic pump (Vario Perpex II, LKB Instruments, Littau-Luzern, Switzerland). After exposure, the vessel segments of 20 mm length were rinsed for 15 seconds with phosphate-buffered saline under perfusion conditions and were removed from the perfusion chamber and immediately cut into three pieces: the center piece of 10 mm in length was frozen for immunological determination of fibrin and fibrinogen; the adjacent pieces (each 5 mm in length) were fixed with glutaraldehyde for morphological evaluation.

**Perfusion Conditions**

Blood was withdrawn from an antecubital vein and was pumped through annular perfusion chambers for 2, 3, 5, and 10 minutes. If the blood flow was maintained constant at 10 ml/min, the resulting shear rate at the vessel surface was 50 s⁻¹ in the large and 850 s⁻¹ in the small chamber. A wall shear rate of 2600 s⁻¹ was produced by increasing blood flow to 40 ml/min in the small perfusion chamber. The dimensions of the chambers and calculations of the shear rates have been reported previously. A wall shear rate of 50 s⁻¹ corresponds to flow conditions in veins, a shear rate of 650 s⁻¹ to those in arteries, and a shear rate of 2600 s⁻¹ to flow in small or stenosed arteries.

**Morphological Evaluation of Blood-Subendothelium Interactions**

Evaluation of fibrin and platelet interaction with subendothelium involved two separate microscopical screens, which were both performed on the same cross-sectional areas of the vessel segment. The proximal piece of 5 mm length was dehydrated and embedded in epoxy resin as previously described. Semi-thin sections ≈10 µm in thickness were prepared, and evaluations were determined at an axial position located ≈4 mm from the proximal end of the original segment, that is, adjacent to the upstream end of the center piece used for plasmin digestion (see below). In a first screen, the interactions of fibrin and platelets with the subendothelial surface were evaluated at 10 µm intervals. The values were expressed as the percentages of the total number of about 1000 evaluations per cross-section, that is, the percent coverage of the subendothelial surface with fibrin and adherent platelets, respectively. The identity of the fibrin was demonstrated by electron microscopy, which showed apparent banding of 230 Å of longitudinally cut fibrin strands. In a second screen, vessel sections were projected onto the recording plate of a microprocessor-linked manual optical picture analysis system (Kontron, Zürich, Switzerland) to evaluate more precisely the dimensions of the platelet thrombi. The total cross-sectional area of deposited thrombi was determined from the perimeter of each thrombus in the projected section. Normalization of the thrombus area by the vessel circumference (perimeter of the projected vessel section) resulted in the thrombus cross-sectional area per unit length of vessel segment circumference, which corresponds stereologically to the total volume of the thrombi associated with a unit surface area of subendothelium. Previous studies showed that the thrombus volume calculated in this manner was closely correlated with the mass of 51Cr-labeled platelets deposited on the surface. The maximum thrombus height was defined as the mean of the three highest thrombi on the subendothelium.

**Immunological Determination of Fibrin and Fibrinogen**

A reliable method for the quantitative determination of fibrin and fibrinogen in biological material has been described. In brief, the center piece of subendothelium with adhering thrombotic material was immersed into 1 ml of phosphate-buffered saline. One hundred microliters of a plasmin solution (80 µg plasminogen activated by 3000 IU of urokinase) was added, and the vessel segment was incubated for 48 hours at 37°C. In this way, the thrombotic material was degraded to the late soluble fibrin fragments D and E. Fragment E, which is more stable, was determined by radioimmunocassay with a
Table 1. Summary of Blood Values

<table>
<thead>
<tr>
<th>Determination</th>
<th>Control (x10^5/µl)</th>
<th>1000 IU</th>
<th>2500 IU</th>
<th>5000 IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count</td>
<td>220±7</td>
<td>219±9</td>
<td>224±10</td>
<td>224±7</td>
</tr>
<tr>
<td>Leukocyte count</td>
<td>5.4±0.3</td>
<td>5.4±0.4</td>
<td>5.5±0.4</td>
<td>5.9±0.3</td>
</tr>
<tr>
<td>Hemoglobin (g/100 ml)</td>
<td>14.7±0.3</td>
<td>14.3±0.4</td>
<td>14.4±0.4</td>
<td>14.5±0.3</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.44±0.01</td>
<td>0.42±0.01</td>
<td>0.43±0.01</td>
<td>0.43±0.01</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>1.9±0.1</td>
<td>2.0±0.1</td>
<td>2.1±0.1</td>
<td>1.9±0.1</td>
</tr>
<tr>
<td>Fibrinopeptide A (ng/ml)</td>
<td>2.9±0.6*</td>
<td>1.2±0.1</td>
<td>1.0±0.2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Partial thromboplastin time (sec)</td>
<td>46±1</td>
<td>67±5</td>
<td>172±13</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Thrombin time (sec)</td>
<td>12.3±0.2</td>
<td>25.4±2.7</td>
<td>&gt;300</td>
<td>&gt;300</td>
</tr>
<tr>
<td>Reptilase time</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
<td>normal</td>
</tr>
<tr>
<td>Heparin activity (USP-U/ml)</td>
<td>0</td>
<td>0.05±0.01</td>
<td>0.19±0.02</td>
<td>0.43±0.04</td>
</tr>
</tbody>
</table>

The values represent means±SEM. The number of evaluations was n=24 for control and heparin 5000 IU and n=12 for heparin 1000 and 2500 IU.

Blood was collected by an infusion set 20 minutes after intravenous administration of glucose 5% solution (control) and heparin doses of 1000, 2500, and 5000 IU immediately before starting with the perfusion experiments. *p<0.05 vs. heparin treatment (paired t test).
Table 2. Effect of Wall Shear Rate and Heparin on Thrombogenesis

<table>
<thead>
<tr>
<th>Determination</th>
<th>50 s⁻¹</th>
<th>650 s⁻¹</th>
<th>2600 s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface coverage with fibrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (%)</td>
<td>76.0±3.4</td>
<td>33.7±5.1</td>
<td>0.9±0.4</td>
</tr>
<tr>
<td>Heparin 5000 IU (%)</td>
<td>0†</td>
<td>0†</td>
<td>0*</td>
</tr>
<tr>
<td>Surface coverage with platelets (platelet adhesion)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (%)</td>
<td>11.0±2.3</td>
<td>34.6±1.7</td>
<td>54.1±4.8</td>
</tr>
<tr>
<td>Heparin 5000 IU (%)</td>
<td>17.5±2.61</td>
<td>51.9±4.0†</td>
<td>49.1±8.2</td>
</tr>
<tr>
<td>Platelet thrombus volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (µm³/µm²)</td>
<td>1.2±0.2</td>
<td>4.4±0.6</td>
<td>4.8±1.3</td>
</tr>
<tr>
<td>Heparin 5000 IU (µm³/µm²)</td>
<td>0.2±0.07†</td>
<td>2.4±0.4†</td>
<td>4.6±1.5</td>
</tr>
<tr>
<td>Maximum thrombus height</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (µm)</td>
<td>33.3±6.2</td>
<td>44.3±2.7</td>
<td>51.5±11.6</td>
</tr>
<tr>
<td>Heparin 5000 IU (µm)</td>
<td>13.5±2.8†</td>
<td>34.0±2.7†</td>
<td>40.2±5.1</td>
</tr>
<tr>
<td>Perfusion time (min)</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

The values represent means±SEM.

Rabbit aorta subendothelium was exposed to flowing human blood at the indicated shear rates and perfusion times. Coverage of the subendothelium with fibrin and platelets and the dimensions of platelet thrombi were determined by morphometrical methods. Maximum thrombus height indicates the mean of the three tallest platelet thrombi on subendothelium. Control indicates experiments performed 20 minutes after intravenous injection of the drug solvent (glucose 5% solution). Heparin 5000 IU indicates experiments 20 minutes after intravenous injection of heparin.

*p<0.05, **p<0.01, ***p<0.001 vs. control (paired t test).

and fibrinogen of both nonperfused and buffer-perfused vessel segments gave only traces of fibrin and fibrinogen.

Statistics

All values are given as means±SEM. The significance of paired and unpaired data was determined by Student's t test. Significance levels are indicated only for the major variables of interest, namely, in the morphological data (subendothelial coverage with fibrin and platelets, thrombus dimensions), to avoid spurious significance due to comparison of multiple measurements.

Results

Blood Values

Platelet and leukocyte count, hemoglobin, hematocrit, and fibrinogen were not changed by the treatment with heparin (Table 1). Due to blood collection through the tube of an infusion set, slightly elevated values of fibrinopeptiade A were found in the control experiments. Heparin led to a dose-dependent prolongation of PTT and thrombin time, whereas reptilase time was within the normal range. Heparin activity was identical in blood samples taken immediately before and after each experiment, indicating a constant activity during the whole perfusion experiment. A positive linear correlation was found between the administered heparin doses and plasma heparin activity (r=0.98, p<0.001, n=48).

Effect of Different Perfusion Times on Thrombogenesis

Figures 1 and 2 summarize the effect of different perfusion times on thrombogenesis induced by subendothelium at a shear rate of 650 s⁻¹. If the vessel segments were

![Figure 3. Effect of wall shear rate and heparin on subendothelial coverage with fibrin (A) and platelets (B). Rabbit aorta subendothelium was exposed to flowing human blood at the shear rates indicated. Values are extrapolated for a perfusion time of 2 minutes. Control indicates experiments performed at 20 minutes after intravenous injection of the drug solvent (glucose 5% solution). Heparin indicates experiments at 20 minutes after intravenous injection of heparin (5000 IU). The values represent means±SEM. The number of evaluations was n=12 for 650 s⁻¹ shear rate and n=6 for 50 and 2600 s⁻¹ shear rates. *p<0.05, **p<0.01, ***p<0.001 vs. control (paired t test).](http://atvb.ahajournals.org/)

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EFFECT OF HEPARIN ON THROMBOGENESIS

40 to 100
80
40
0

Figure 4. Effect of wall shear rate and heparin on platelet thrombus dimensions. Rabbit aorta subendothelium was exposed to flowing human blood at the shear rates indicated. Values are extrapolated for a perfusion time of 2 minutes. A shows the mean height of the three tallest platelet thrombi (maximum thrombus height). B shows the morphometrically determined volume of platelet thrombi. Control indicates experiments performed at 20 minutes after intravenous injection of the drug solvent (glucose 5% solution). Heparin indicates experiments at 20 minutes after intravenous injection of heparin (5000 IU). The values represent means±SEM. The number of evaluations was n=12 for 650 s⁻¹ shear rate and n=6 for 50 and 2600 s⁻¹ shear rates. **p<0.01 (paired t test).

Exposed to normal nonanticoagulated blood (control experiments), the coverage of subendothelium with fibrin increased from 3 to 5 minutes of perfusion time, without further increase from 5 to 10 minutes (Figure 1A). In contrast, platelet adhesion (coverage of the subendothelial surface with platelets, Figure 1B), as well as the height and volume of platelet thrombi (Figure 2), showed a continuous increase up to 10 minutes of perfusion time. A positive linear correlation was found between thrombus volume and perfusion time (r=0.89, p<0.001, n=24). Fibrin coverage of subendothelium was completely abolished by heparin (5000 IU) in the experiments with 3 and 5 minutes of perfusion time, and only traces were found after an exposure time of 10 minutes (Figure 1A). The heparin-induced inhibition of fibrin formation increased the available surface for platelet-subendothelium interaction, resulting in enhanced platelet coverage (Figure 1B). At a wall shear rate of 650 s⁻¹, heparin (5000 IU) reduced the dimensions of platelet thrombi independent from exposure time (Figure 2B).

Effect of Different Shear Rates on Thrombogenesis

The effect of different shear rates on the interaction of fibrin and platelets with subendothelium is summarized in

Figure 5. Effect of different heparin doses on thrombogenesis induced by vascular subendothelium. Rabbit aorta subendothelium was exposed to flowing human blood during 5 minutes at a shear rate of 650 s⁻¹. Experiments were performed at 20 minutes after intravenous injection of the drug solvent (glucose 5% solution) and 1000, 2500, and 5000 IU of heparin, respectively. A shows the results for subendothelial coverage with fibrin and platelets. B shows the mean height of the three tallest platelet thrombi (maximum thrombus height) and the morphometrically determined volume of platelet thrombi. The values represent means±SEM. The number of evaluations was n=12 for each dose. *p<0.05, **p<0.01, ***p<0.001 vs. control (paired t test).

Figure 6. Effect of different heparin doses on subendothelial deposition of fibrin and fibrinogen. Rabbit aorta subendothelium was exposed to flowing human blood during 5 minutes at a shear rate of 650 s⁻¹. Experiments were performed at 20 minutes after intravenous injection of the drug solvent (glucose 5% solution), and 1000, 2500, and 5000 IU of heparin, respectively. Fibrin and fibrinogen were determined in plasmin-digested thrombi attached to the center piece of the vessel segment by using specific antibodies against fibrin fragment E and fibrinopeptide A. The values represent means±SEM. The number of evaluations was n=12 for each dose. *p<0.001 vs. control (paired t test).

Table 2 and Figures 3 and 4. Since there is a linear increase of platelet thrombus dimensions with time and this also approximately holds for subendothelial coverage with fibrin and platelets during the initial 5 minutes of perfusion, the data shown in Figures 3 and 4 are extrap-
The observation that fibrin deposition prevails at low shear conditions and is drastically reduced at high shear rates (2600 s⁻¹) consisted almost exclusively of fibrin, the thrombi formed at intermediate shear rates (650 s⁻¹) contained significant amounts of both fibrin and platelets. These findings indicate that thrombin activity is an important determinant for the formation of fibrin thrombi because they prevail at low or venous shear rates. A major and novel finding of this study is that high doses of heparin (5000 IU) also reduce the dimensions of platelet thrombi and that this effect is confined to low and intermediate shear rates (Figure 4B). Therefore, thrombin appears to modulate the growth and/or stability of platelet thrombi at low and intermediate shear rates, whereas an alternative, perhaps physically initiated, pathway may be important for platelet thrombus formation under high shear conditions. Since the high doses of heparin, which were necessary to inhibit platelet thrombus dimensions at low and intermediate shear rates, virtually abolished fibrin formation (Figures 5A and 5B), the lack of fibrin formation might have affected the growth and/or stability of platelet thrombi. Findings in patients with a fibrinogenemia studied under identical experimental conditions, however, argue against a major role of fibrin in platelet thrombus formation, since these patients had normal platelet thrombus dimensions. To further differentiate between platelet thrombus growth and stability at high shear rates, perfusion times longer than 2 minutes would be of interest. The chambers used in this study, however, do not allow longer perfusion times at the high shear rate, since it is critical to draw a blood volume of 40 ml/min from human antecubital veins for longer periods.

The reported data on the effects of heparin on platelets are controversial. The presence of heparin has been shown to enhance, reduce, or have no influence on platelet aggregation when investigated under various in vitro conditions. At first glance, our findings that high doses of heparin increase platelet adhesion (Figures 1B and 5) but reduce platelet thrombus dimensions (Figures 2 and 5) appear controversial, as well. Therefore, we want to point out that the observed heparin-induced increase of platelet adhesion is an effect of the substantially reduced fibrin deposition, which in turn increased the available surface for platelet-subendothelium interaction. Although our study cannot resolve the controversial in vitro findings, our results, which were obtained without additives and with minimal blood manipulation, demonstrate that high heparin doses exert an inhibitory effect on the growth and/or stability of platelet thrombi under low and intermediate shear conditions.

In our perfusion experiments, we used segments of rabbit aorta that were denuded of endothelial cells.
Therefore, the observed blood-subendothelium interactions reflect thrombogenesis on a severely damaged vessel surface. This surface is highly thrombogenic, possibly due to tissue factor and connective tissue present in the subendothelium. Although a significant loss of endothelial cells is unlikely to occur in veins, venous vascular endothelial cells have been shown to express tissue factor after stimulation. With regard to the arterial part of the vascular tree, the stimulus for activating the coagulation mechanism provided by a ruptured atherosclerotic plaque or severe atherosclerosis may appear different to that provided by our ex vivo model. However, in both instances, subendothelial connective tissue is exposed to the flowing blood, and our data at shear rates of 650 s⁻¹ and 2600 s⁻¹ might reflect thrombogenesis in arteriosclerotic vessels or at sites where hemodynamic forces cause repeated endothelial injury. In addition, a study on the effects of heparin on platelet accumulation and formation of platelet-fibrin thrombi on injured neointima of rabbit aorta showed a comparable heparin-induced inhibition of blood interaction with the vessel wall.

The clinical extrapolation of our findings is that prevention of fibrin-rich thrombi as they prevail at the low shear conditions in the venous circulation can be achieved by relatively low heparin doses. High doses of heparin are, however, necessary to inhibit thrombogenesis at the intermediate shear rates in arterial vessels. Finally, the thrombi formed at high shear rates, which consist almost exclusively of platelets, are relatively resistant against anticoagulation with heparin.

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