Development of Nonthrombogenicity of Injured Rabbit Aortas Despite Inhibition of Platelet Adherence

Hallie M. Groves, Raelene L. Kinlough-Rathbone, and J. Fraser Mustard

After removal of the endothelium from normal rabbit aortas or after injury to the neointima, the injured surfaces rapidly become nonreactive to circulating platelets. Experiments were done to determine whether prevention of the initial interaction of platelets with the surfaces would influence the loss of vessel wall reactivity. Inhibition of platelet accumulation on the subendothelium by the infusion of PGI₂ (850 ng/kg/min) or the administration of dipyridamole (12.5 mg/kg initially followed by 5 mg/kg/hr) for periods of less than 8 hours inhibited platelet accumulation on the injured vessels during the infusion, but did not prevent the subsequent accumulation of platelets on the surfaces when the infusions were stopped. If the animals were treated for 8 hours, platelets did not accumulate on the surface when the drugs were discontinued. Thus, an injured vessel wall can develop a nonthrombogenic surface even when platelet adherence is prevented, although approximately 8 hours are required before the surface loses its ability to interact with platelets.


Platelets rapidly accumulate on the surface of freshly exposed subendothelium and release the contents of their granules, among which is a growth factor that stimulates smooth muscle cells to form a thickened neointima. After the initial accumulation of platelets on the subendothelium that is exposed following deendothelialization with a balloon catheter, the surface of the injured vessel loses its reactivity to circulating platelets within 30 minutes and few additional platelets accumulate. Within 2 to 4 days of deendothelialization, many of the adherent platelets are lost from the injured vessel, exposing a surface that appears morphologically similar to the subendothelium but which shows little reaction with circulating platelets. The factors responsible for injured vessels losing their ability to interact with circulating platelets are not understood, although there are several possibilities that could account for this.

The purpose of the present experiments was to determine whether the initial interaction of platelets with damaged vessel walls contributes to the subsequent loss of vessel reactivity. Rabbits were treated with dipyridamole or PGI₂, both of which inhibit platelet interaction with surfaces, including subendothelial structures, to determine whether vessels upon which few platelets accumulate retain their ability to attract circulating platelets.

From the Department of Pathology, McMaster University, Hamilton, Ontario, Canada.

Address for reprints: Dr. Raelene L. Kinlough-Rathbone, Department of Pathology, Room 3N22, McMaster University, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5 Canada.

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**Removal of Aortic Endothelium or Injury of Neointima with a Balloon Catheter**

The aortic endothelium was removed with a balloon catheter as previously described by using a 4F embolectomy catheter (Edwards Laboratories, Santa Ana, California). The neointima that had formed 7 days after deendothelialization was also damaged by the passage of a balloon catheter.

**Platelet Accumulation on Damaged Aortas In Vivo**

Platelets (8 ml of \(3 \times 10^9\) platelets per ml) labeled with \(^{51}\)Cr were injected into rabbits 18 hours before a balloon catheter was used to remove the aortic endothelium or damage the aortic neointima. The animals received an intravenous injection of heparin (1000 U) immediately before they were perfused through a carotid cannula with Locke’s-Ringer solution containing heparin (1 U/ml) followed by fixation with 4% paraformaldehyde in phosphate buffer. The radioactivity associated with the vessels was measured, and platelet accumulation was calculated as previously described. Calculations were based on the specific radioactivity of platelets in the circulation immediately after the removal of the balloon catheter.

**Administration of Dipyridamole**

Dipyridamole (a gift of Boehringer Ingleheim, Quebec) was dissolved in 0.1 N HCl, and the pH was adjusted to 3.0. An intravenous injection of dipyridamole (12.5 mg/kg) was given 10 minutes before the aorta was injured. To maintain the drug concentration in the plasma for longer periods, hourly injections of dipyridamole (5 mg/kg) were given for 3 or 7 hours through a cannula introduced into the superior vena cava. The accumulation of platelets on the surface of the injured aorta was measured at the end of the treatment period or 9 hours after the last injection of dipyridamole.

**Infusions of PGI2**

The PGI2 used in these experiments was a generous gift of the Upjohn Company, Kalamazoo, Michigan, and Salvador Moncada, Wellcome Research Laboratories, Beckenham, England. A polyethylene cannula (PE 190) was introduced through the left common carotid artery of an anesthetized rabbit and passed into the ascending aorta to within approximately 0.5 cm of the aortic valve. A solution of PGI2 in 0.1 M Tris buffer (pH 9.0) or a solution of Tris buffer (placebo) was infused with a Harvard pump (Model 927) calibrated to deliver 0.1 ml/min/kg body weight. In some experiments the infusion of PGI2 (850 ng/kg/min) was begun 30 seconds before the aorta was injured with a balloon catheter, and continued for 10 minutes after the injury. In other experiments in which PGI2 was infused for 8 hours, the infusion pump was calibrated so that the animal received the drug (850 ng/kg/min) or placebo solution at the rate of 1 ml/hr/kg. For these longer infusion periods, the cannula through which the drug was administered was passed subcutaneously from a scalp incision and inserted into the common carotid artery to within approximately 0.5 cm of the aortic valve. The infusion of PGI2 was begun 10 minutes before the aorta was injured with a balloon catheter. During the infusion, the syringes containing PGI2 were packed in ice to minimize degradation. After injury of the aorta with a balloon catheter, each animal was placed in an individual plastic basket at the same level as the pump and allowed to recover in a quiet, darkened room. The animals were monitored continuously during the remainder of the infusion period to ensure that their activity during and following recovery did not interfere with the continuous infusion of PGI2. Blood pressure was recorded during the infusion of PGI2 by a pressure-sensitive transducer placed in the right carotid artery and connected to a Mingograf 34 (Elma Schonander, Stockholm, Sweden). Shortly before the end of the infusion, the animals were anesthetized and prepared for perfusion-fixation.

**Scanning Electron Microscopy**

Segments of aorta approximately 2.5 cm long were dissected free of extraneous tissue and cut open to expose the luminal surface. The adventitial surface was attached to a coverglass with cyano-acrylic glue (Eastman 910). The tissue was kept moist with a buffer solution. The specimen was post-fixed in osmium tetroxide, dehydrated through graded ethanol, critical-point dried from CO2, mounted on a scanning electron microscope (SEM) stub with double-sided tape, and coated with gold. Specimens were examined in a Philips 501 SEM.

**Measurement of Plasma Dipyridamole**

Plasma dipyridamole was measured by the method of Zak et al.

**Platelet Aggregation**

The aggregation of platelets in citrated plasma was measured as previously described.

**Analysis of Data**

The individual values in Table 1 were logarithmically transformed before an analysis of variance was used to test the variation among the treatment groups. Student's t test was used to compare the values among the groups. For Tables 2–6, the significance of the difference among values was calculated by using untransformed data and Student’s t test.

**Table 1. Effect of a Single Injection of Dipyridamole on Platelet Accumulation on Subendothelium In Vivo**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time after injury</th>
<th>No. of rabbits</th>
<th>Platelet accumulation (no./mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>10 min</td>
<td>9</td>
<td>52,500 ± 8,400</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>10 min</td>
<td>9</td>
<td>16,900 ± 1,800</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>5</td>
<td>13,400 ± 1,500</td>
</tr>
<tr>
<td></td>
<td>1 hr</td>
<td>6</td>
<td>17,900 ± 2,700</td>
</tr>
<tr>
<td></td>
<td>2 hr</td>
<td>6</td>
<td>32,100 ± 5,200</td>
</tr>
<tr>
<td></td>
<td>4 hr</td>
<td>6</td>
<td>43,900 ± 8,100</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Rabbits were given an intravenous injection of dipyridamole (12.5 mg/kg) 10 minutes before the endothelium was removed from the aorta with a balloon catheter. The animals were killed by perfusion-fixation at the times indicated following injury and the accumulation of platelets on the surface of the vessels was measured. The significance of the differences in platelet accumulation among the groups was calculated as described in the Methods section and are as follows: A vs B = \(p < 0.0005\); B vs D = \(p < 0.005\); A vs F = \(p < 0.03\); D vs E = \(p < 0.005\).
Table 2. Effect of Administration of Dipyridamole Hourly for 4 Hours on the Accumulation of Platelets on the Subendothelium In Vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet accumulation (no./mm²) after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>38,100 ± 3,200</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>15,500 ± 5,400</td>
</tr>
</tbody>
</table>

Significance of difference between means: p < 0.005

Values are means ± SEM for six rabbits. Dipyridamole (12.5 mg/kg) was administered intravenously 10 minutes before removal of the endothelium and was then given hourly at a concentration of 5 mg/kg for 3 hours following injury. The animals were killed by perfusion-fixation 4 or 12 hours following deendothelialization, and the accumulation of platelets on the surface was measured.

Experiments with Dipyridamole

A single injection of dipyridamole (12.5 mg/kg) 10 minutes before deendothelialization significantly reduced platelet accumulation on the subendothelium compared to the accumulation in placebo-treated animals; this effect was maximal during the first hour (Table 1). Four hours after this single injection of dipyridamole, platelet accumulation on the deendothelialized aortas was not significantly different from that in the placebo-treated group.

The administration of dipyridamole immediately before injury and at hourly intervals for 3 hours significantly reduced the number of platelets that accumulated on the deendothelialized aorta measured 4 hours after the injury. Nine hours after the last injection of dipyridamole (12 hours following injury) platelet accumulation was similar in dipyridamole- and placebo-treated animals (Table 2).

The administration of dipyridamole immediately before injury and hourly for 7 hours afterward reduced platelet accumulation measured 8 hours after injury, and during the following 8 hours (9 hours after the last injection of dipyridamole) additional platelets did not accumulate (Table 3). At the end of the administration of dipyridamole, the plasma concentration of the drug was 3.35 ± 0.18 µg/ml and 9 hours later was 0.002 ± 0.001 µg/ml, a concentration considerably lower than that required to inhibit platelet function. At 9 hours after the last injection of dipyridamole, there was no difference in the extent of platelet aggregation induced by collagen, ADP (5 µM) or sodium arachidonate (500 µM) compared with platelets from control animals.

Experiments with PG12

Because dipyridamole inhibited platelet accumulation on deendothelialized rabbit aortas by only about 60%, it is possible that platelets that interacted with the injured vessel might have been responsible for altering its surface properties. Therefore, PG12, a more potent inhibitor of platelet function, was used to inhibit platelet accumulation on the damaged vessels. When the infusion of PG12 was begun before the exposure of the subendothelium and maintained for 10 minutes after injury, platelet accumulation at the end of the infusion was reduced by more than 85%, as estimated by the accumulation of 51Cr (Table 4). However, 10 minutes after the end of the PG12 infusion, the number of platelets that accumulated on the subendothelium in vivo was reduced by more than 85%, as estimated by the accumulation of 51Cr (Table 4).

Table 3. Effect of Administration of Dipyridamole Hourly for 8 Hours on the Accumulation of Platelets on the Subendothelium In Vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet accumulation (no./mm²) after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>32,700 ± 6,800</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>12,700 ± 2,500</td>
</tr>
</tbody>
</table>

Significance of difference between means: p < 0.001

Values are means ± SEM for nine animals. Dipyridamole (12.5 mg/kg) was administered intravenously 10 minutes before removal of the endothelium, and thereafter at a concentration of 5 mg/kg hourly for 7 hours. The animals were killed by perfusion-fixation 8 or 16 hours following deendothelialization, and the accumulation of platelets on the surface was measured.

Table 4. Effect of Infusing PG12 for 10 Minutes on the Accumulation of Platelets on the Subendothelium In Vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet accumulation (no./mm²) after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no drug)</td>
<td>55,300 ± 5,500</td>
</tr>
<tr>
<td>PG12</td>
<td>7,300 ± 1,300</td>
</tr>
</tbody>
</table>

Significance of difference between means: p < 0.005

Values are means ± SEM for seven animals. The infusions of PG12 (850 ng/kg/min) were begun 1 minute before the aortas were deendothelialized and continued for 10 minutes thereafter. The animals were killed by perfusion-fixation at the end of the PG12 infusion 10 minutes after the end of the infusion, and the accumulation of platelets on the surface was measured.

Table 5. Effect of Infusing PG12 for 8 Hours on the Accumulation of Platelets on the Subendothelium In Vivo

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Platelet accumulation (no./mm²) after injury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no drug)</td>
<td>36,600 ± 4,100</td>
</tr>
<tr>
<td>Placebo</td>
<td>30,700 ± 4,200</td>
</tr>
</tbody>
</table>

Significance of difference between means: p < 0.005

Values are means ± SEM for four animals. The infusions of PG12 (850 ng/kg/min) or Tris buffer were started 1 minute before the aortas were deendothelialized and continued for 8 hours following injury. The animals were killed by perfusion-fixation at the end of the PG12 infusion or 30 minutes after the end of the infusion and the accumulation of platelets on the surface was measured.

The significance of the difference in platelet accumulation between the control group (no drug) and the PG12-treated group perfused at 8 hours following injury.

pg12
Figure 1.  A. Scanning electron micrograph of the surface of a normal rabbit aorta subjected to injury with a balloon catheter 10 minutes previously. The surface is covered with a layer of platelets (PLT). B. Scanning electron micrograph of a normal rabbit aorta subjected to injury with a balloon catheter. The aorta was perfusion-fixed at the end of a 10-minute infusion of PGI2. Platelets are not present on the subendothelium (SE). C. Scanning electron micrograph of a normal rabbit aorta subjected to injury with a balloon catheter. The aorta was perfusion-fixed 10 minutes after completion of a 10-minute infusion of PGI2. The surface is covered by a layer of platelets (PLT). Bar = 10 μm.

Figure 2. A. Scanning electron micrograph of the surface of a normal rabbit aorta subjected to injury with a balloon catheter 8 hours previously. The surface is covered with a layer of platelets (PLT). B. Scanning electron micrograph of a normal rabbit aorta subjected to injury with a balloon catheter. The aorta was perfusion-fixed at the end of an 8-hour infusion of PGI2. Very few platelets are present on the subendothelium (SE). C. Scanning electron micrograph of a normal rabbit aorta subjected to injury with a balloon catheter. The aorta was perfusion-fixed 30 minutes after completion of an 8-hour infusion of PGI2. Only a few platelets are associated with the subendothelium. Bar = 10 μm.
Platelet accumulation at the end of the 8-hour infusion of PGI₂ was approximately 25% of the control value. Again, few platelets were detectable on the surface by SEM (Figure 2). However, in contrast to the results following short-term PGI₂ infusion, additional platelets did not accumulate during the 30-minute period after the infusion was stopped (Table 5). Infusion of PGI₂ for 6 hours did not prevent subsequent platelet accumulation (data not shown).

Injury to the neointima leads to platelet accumulation through a process involving connective tissue and thrombin. In two experiments, we examined the effect of PGI₂ infusion on platelet accumulation on the injured neointima. When PGI₂ was infused for 8 hours after injury to the neointima, few platelets accumulated on the injured aorta, and during the 30 minutes after the end of the infusion, no additional platelets accumulated (Table 6, Figure 3).

**Discussion**

The results from these studies confirm those of earlier experiments in which it was shown that dipyridamole inhibits platelet adhesion to the subendothelium. As previously shown by Adelman and his co-workers, these results also demonstrate that the administration in vivo of high concentrations of PGI₂ strongly inhibits platelet adhesion to the subendothelium; the concentrations of PGI₂ required to inhibit platelet adhesion to the subendothelium caused a fall in blood pressure.

A single infusion of dipyridamole significantly reduced platelet accumulation on the subendothelium over a period.

**Figure 3.** A. Scanning electron micrograph of the surface of a rabbit aorta that had been deendothelialized with a balloon catheter 7 days previously, 8 hours after a second injury with a balloon catheter. Platelets (PLT) adhere to the damaged neointima. CT = connective tissue. B. Scanning electron micrograph of the damaged neointima that had formed 7 days after the aorta was deendothelialized with a balloon catheter. The aorta was perfusion-fixed at the end of an 8-hour infusion of PGI₂. There are few platelets on the surface. SMC = smooth muscle cells. C. Scanning electron micrograph of the damaged neointima that had formed 7 days after the aorta was deendothelialized with a balloon catheter. The aorta was perfusion-fixed 30 minutes after the end of an 8-hour infusion of PGI₂. Few platelets are present on the surface. Bar = 10 μm.
of 3 hours, but when the blood levels of dipyridamole decreased, platelets accumulated on the subendothelium. However, when dipyridamole was given for at least 8 hours, platelets did not reaccumulate on the denuded surface.

PGI₂ was a more effective inhibitor of platelet accumulation on the subendothelium than dipyridamole. The results of these experiments with PGI₂ indicate that when platelet interaction with the subendothelium is extensively inhibited, about 8 hours are required before the surface becomes nonreactive to platelet accumulation. Infusions of PGI₂ for shorter periods of time did not inhibit subsequent platelet accumulation when the infusion was stopped. Adelman and co-workers18 also found that, with short-term PGI₂ infusions, platelet accumulation on the subendothelium was restored when the infusion was stopped. In the experiments with dipyridamole and PGI₂, platelet adherence after these treatments was measured when the circulating levels of these drugs were very low; for dipyridamole this was 9 hours after the last injection and for PGI₂ it was 30 minutes after the infusion was stopped.

Thus, it seems reasonable to conclude that the loss of reactivity of the subendothelium to platelet accumulation after 8 hours is not a result of platelet interaction with the injury site. It is also unlikely that loss of vessel wall reactivity can be attributed to plasma proteins or other plasma factors masking the sites on the subendothelium with which platelets interact, since deposition of plasma proteins should take place almost immediately. It is probable that there is a change in the vessel wall that accounts for this effect. PGI₂ formation by the vessel wall is one factor that could inhibit platelet adhesion to the subendothelium. In earlier studies, we found that inhibition of PGI₂ production by damaged vessels did not cause a further increase in platelet accumulation, indicating that PGI₂ production was not likely to be responsible for the loss of vessel wall reactivity.18 Eldor and his colleagues demonstrated that PGI₂ production by denuded vascular surfaces is essentially diminished; this observation provides additional evidence that increased PGI₂ production by injured vessels probably does not account for the loss of reactivity.

The two experiments involving the injured neointima show that the PGI₂-sensitive platelet accumulation follows a similar pattern to that observed for the subendothelium. However, with the injured neointima both thrombin generation and platelet-connective tissue interaction are involved in the initial platelet accumulation.3 The combination of heparin and PGI₂ has been shown to be required to produce maximum inhibition of platelet accumulation resulting from damage of the neointima.21 Although our experiments do not provide any information about the coagulation component, Piepgras and his colleagues22 found that after endarterectomy in cats, an infusion of heparin for a period of 6 hours prevented thrombus formation and resulted in a surface that was nonreactive to the formation of platelet-fibrin thrombi when heparin was discontinued.

In contrast to the results of the present experiments in which platelet adherence was inhibited and approximately 8 hours were required before the injured surface became nonreactive to platelets, we have previously demonstrated that if platelet adherence is not inhibited, the monolayer of platelets that immediately covers the injured vessel wall provides a surface to which other platelets do not adhere.2,6

The results from our experiments indicate that within 6 to 8 hours after injury with a balloon catheter, the aortas of rabbits become nonreactive to circulating platelets. If drugs that inhibit platelet adherence are administered for a sufficient period of time, the injured vessel wall loses its reactivity to circulating platelets. Inhibition of platelet adherence to the subendothelium by the infusion of PGI₂ has been shown to prevent the accumulation of released platelet proteins in the vessel wall.18 Thus, it is possible to prevent the initial delivery to the injured vessel of the platelet factor that is mitogenic for smooth muscle cells, and if the therapy is continued until the vessel wall becomes nonreactive, it may be possible to stop the therapy with little subsequent platelet interaction with the damaged vessel surface.

References

Index Terms: thrombogenicity of vessels • platelet adherence • PGI₂ • vessel injury • dipyridamole • platelet adhesion
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