Enhanced Platelet Accumulation onto Injured Carotid Arteries in Rabbits after Aspirin Treatment

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ABSTRACT Prostacyclin (PGI₂) is a powerful inhibitor of platelet aggregation, but its role in the pathogenesis of arterial thrombosis is uncertain. We have studied the thrombogenic effect of inhibiting PGI₂ production by aspirin (ASA) in carotid arteries of rabbits given 0, 3, 10, or 100 mg ASA/kg either 1, 3, 6, or 20 h beforehand. Platelet accumulation onto injured carotid arteries was enhanced with ASA in a dose of 10 mg/kg. A higher dose of ASA (100 mg/kg) had no further effect. The enhanced thrombogenic effect of ASA persisted for at least 20 h and was associated with a decrease in vessel wall PGI₂ production. There was a strong inverse correlation (r = -0.55, P < 0.01) between PGI₂ production and platelet accumulation. The findings suggest that the margin of safety in obtaining an antithrombotic effect of ASA and producing a potential thrombotic effect in arteries may not be as large as predicted by studies using cultured endothelial cells or experimentally induced thrombosis in veins.

INTRODUCTION

The production of thromboxane A₂ (TxA₂)¹ by platelets and of prostacyclin (PGI₂) by vessel-wall cells is thought to be important in the regulation of hemostasis and in the pathogenesis of thrombosis (1, 2). TxA₂ causes vasoconstriction and enhances platelet aggregation, whereas PGI₂ causes vasodilatation and inhibits platelet aggregation. Aspirin (ASA) inhibits TxA₂ formation by acetylated platelet cyclooxygenase (3). This effect lasts for the life span of the platelet and is produced by low concentrations of ASA, which have recently been shown to be effective in preventing thrombosis (4). Aspirin also inhibits PGI₂ formation by vascular wall cells by the same mechanism, but in contrast to platelets, its effect on cultured vessel wall cells has been reported to be relatively short-lived, presumably because the vascular wall cells have the capacity to resynthesize cyclooxygenase (5, 6).

Recently, we reported that the inhibitory effect of ASA on PGI₂ formation persists for up to 20 h in whole vessel wall preparations of carotid arteries, but is lost within 3–6 h in jugular veins in rabbits (7–9). To determine whether this prolonged effect of ASA on PGI₂ production in arteries is associated with a prolonged thrombogenic effect, we examined the relationship between the inhibitory effect of ASA on PGI₂ production and its enhancement of platelet accumulation onto injured carotid arteries in rabbits.

METHODS

Carotid injury model. Rabbits were injected with homologous ⁵¹Cr-labeled platelets. 20 h later, each animal was anesthetized with sodium pentobarbital (MTC Pharmaceuticals, Hamilton, Ontario) and both carotid arteries were isolated. Two clamps were applied 1 cm apart onto each artery for 10 min, during which time blood remained within the isolated segment. The clamps were then removed and blood flow restored. No macroscopic thrombi were ever seen to form in the segment during stasis. 1 h later, a 5-ml citrated blood sample was collected, and the rabbit was then heparinized (200 U/kg) and killed with an overdose of sodium pentobarbital. A standard length of each vessel, encompassing both clamp injury sites, was removed, rinsed in saline, and placed in a glass tube containing 1.5 ml saline. The vessel wall radioactivity was then determined in a gamma counter (model 1085, Nuclear-Chicago Corp., Des Plaines, Ill.). The vessel was then removed from the tube, slit longitudinally, laid flat, endothelial side down, on a transparent film of acetate, and photocopied. The area of the vessel wall was determined by cutting out the photocopy and weighing it. The weight was compared with the weight of an adjacent 1 cm square cut from the same piece of paper and was expressed as weight per unit area.

Platelet-rich plasma was prepared from the citrated blood sample collected before removing the injured vessels. Platelet counts and platelet radioactivity were determined, and
platelet specific activity calculated. The radioactivity of the vessel wall was then expressed as platelets/10 mm² of vessel wall.

Some animals were injected with both ³¹Cr-platelets and ¹²⁵I-fibrinogen, and their carotid arteries were then injured as described above. ³¹Cr-Platelet accumulation and ¹²⁵I-fibrinogen accretion on the injured vessels were determined using the method described elsewhere (9). In other animals, the injured vessels were removed and prepared for electron microscope examination.

Preparation of tissues for scanning electron microscopy. Segments of injured carotid artery were fixed in 2% glutaraldehyde (0.1 M sodium cacodylate, 0.2 mM calcium chloride, pH 7.35) for 2 h at 4°C. The vessels were then postfixed in 1% aqueous osmium tetroxide for 1 h at 4°C, followed by dehydration in a series of graded ethanol. After dehydration, the vessels were critical-point dried from CO₂ in a Balzers critical point dryer (model SPC-900/EX, Tacoma, Wash.) and coated with gold (200 Å) using a Polaron sputter coater (Polaron Equipment Ltd., Watford, Herts, England). The vessels were viewed in a Phillips 501 scanning electron microscope (Phillips Electronic Instruments, Inc., Mahwah, N. J.). All chemicals for this procedure were obtained from Polysciences Inc., Warrington, Pa.

Measurement of PGI₂-like activity of carotid arteries. The effect of ASA on PGI₂ synthesis by the vessel wall was quantitated in two ways, using a radioimmunoassay for 6-keto-PGF₁α as described by Cavallin et al. (10) and by using a bioassay as described below. The antibody for the radioimmunoassay was kindly supplied by Dr. J. B. Smith, Temple University, Philadelphia, Pa. The sensitivity of the bioassay was 3 pmol, and the sensitivity of the radioimmunoassay was 30 pmol. The activity of the bioassay is referred to as “PGI₂-like” activity.

The basic method used to quantitate PGI₂-like activity of vessel walls is described in detail elsewhere (8, 9). In brief, rabbits were heparinized with 200 U/kg of heparin (Hepalecan, Harris Laboratories, Brantford, Ontario), and the carotid arteries were isolated and removed from the animal. The heparin was used to prevent the generation of thrombin during the isolation of arteries. We have previously demonstrated that the heparin was effectively removed from the vessel wall so that it does not interfere with the PGI₂-like activity or the PGI₂ assay procedure (11). Each vessel was flushed with calcium-free Tyrode solution (containing 0.35% albumin, bovine fraction V; Sigma Chemical Corp., St. Louis, Mo.) and then slit longitudinally to stimulate PGI₂ synthesis. It was immediately placed in 300 μl of Eagles’ medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.), containing 4% albumin, 5 mM Hepes buffer, pH 7.45 (Eagles’), and incubated at 37°C for 5 min. The vessel was then removed, and 7 μl of 1.0 M sodium carbonate was immediately added to the medium to raise the pH to 8.6 and so stabilize the PGI₂-like activity. The sample was stored at −70°C until testing. The area of the vessel wall was quantitated in the same manner as described above.

To test for the PGI₂-like activity of each test sample, the sample was thawed and 10 μl of 0.5 N hydrochloric acid was added to the sample to bring the pH back to 7.6. Immediately, the sample (300 μl) was added to 0.7 ml of ¹⁴C-serotonin-labeled platelet suspension which was being stirred in an aggregation module (Payton Associates, Scarboro, Ontario). 15 s later, imipramine (2 μM) was added to block reuptake of the ¹⁴C-serotonin and 15 s after that, a standard amount of thrombin (0.015–0.025 U, Parke, Davis & Co., Detroit, Mich.) was added to the sample. 60 and 120 s later, 100-μl aliquots were collected from the sample and added to 100 μl of 0.25% ice-cold paraformaldehyde to terminate the release reaction. The aliquots were then centrifuged for 60 s at 12,000 g and the amount of ¹⁴C-serotonin released into the supernate was determined. The percent inhibition of ¹⁴C-serotonin released by platelets was measured and the amount of PGI₂-like activity calculated from a dose-response curve that was standardized for each experiment, using PGI₂ obtained from Upjohn Co., Kalamazoo, Mich. There was no loss of PGI₂ activity when standards containing known amounts of PGI₂ were treated in an identical manner.

The recovery of PGI₂-like activity in the bioassay was measured by adding known amounts of PGI₂ to either Eagles’ medium or Eagles’ containing a vessel wall segment. In six experiments, the level of PGI₂-like activity for the vessel wall alone was 137±37 pg/100 μl Eagles’ (mean±SEM); for the vessel wall plus the PGI₂, 723±50 pg/100 μl Eagles’; and for the PGI₂ alone, 666±70 pg/100 μl Eagles’, giving a recovery rate of 87±3%.

The PGI₂-like activity had the following characteristics. Its activity was abolished at acid pH or after boiling, it rapidly decayed at neutral pH over time, it was totally inhibited by exposure of the vessel to ASA in vitro, it was inhibited by at least 90% after the animal was treated with ASA, and there was a similar pattern of inhibition of 6-keto-PGF₁α formation in animals treated with ASA. However, in these in vivo experiments there was no measurable 6-keto-PGF₁α for up to 6 h after ASA treatment even though there were measurable (although markedly reduced levels of PGI₂-like activity by the bioassay. Because of its increased sensitivity, the bioassay was used throughout these experiments. Because of its stability and recovery characteristics, its activity is referred to as PGI₂.

Experimental design. Experiments were performed on rabbits either 1, 3, 6, or 20 h after treatment with 0, 3, 10, or 100 mg/kg of ASA given intravenously. The ASA (acetylsalicylic acid, Sigma Chemical Co., St. Louis, Mo.) was solubilized by first adding the crystals to water and then slowly adding sodium carbonate until the crystals dissolved. During this procedure, the pH remained below 7.0, so hydrolysis of the ASA was minimized. The ASA solutions were made up in concentrations of 3, 10, and 100 mg/ml (16.5, 55, and 550 mM, respectively).

The data were analysed using analysis of variance and multiple comparison procedures. Preliminary analysis of the data obtained in the animals treated with increasing doses of ASA showed that the standard deviation increased as the mean platelet accumulation increased, and hence a logarithmic transformation was performed on all data, to meet standard statistical requirements.

RESULTS

Electron microscopic examination of injured carotid arteries. Examination of the carotid artery by scanning electron microscopy 1 h after the stasis/injury showed that the endothelial surface was covered by a fine mesh of fibrin to which platelets adhered (Fig. 1). A control vessel is shown for comparison. The quantity of fibrin accreted onto the injured vessels did not differ between control animals and those given 100 mg/kg ASA 1, 6, or 20 h beforehand (Table I). In contrast, the number of platelets accumulated onto the injured carotid arteries in the ASA-treated rabbits was significantly increased, as is indicated by the measurement of ³¹Cr-platelet accumulation (see Tables I and II; Figs. 2 and 3).
Effect of ASA on platelet accumulation onto injured carotid arteries. The observed platelet accumulation onto the injured arteries in each of the four treatment groups is shown in Fig. 2. An analysis of variance of the log platelet accumulations in the three ASA-treated groups revealed no significant changes with time. Therefore, the results over time within each ASA-treated group were averaged. These mean values, as well as that for the control group, are shown in Table II. The observed differences among the means for the four groups were statistically significant ($P < 0.001$). Using an appropriate multiple comparison procedure

<table>
<thead>
<tr>
<th>ASA treatment</th>
<th>1 h</th>
<th>6 h</th>
<th>20 h</th>
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<tbody>
<tr>
<td>Control</td>
<td>56±6</td>
<td>52±6</td>
<td>66±7</td>
</tr>
<tr>
<td>100 mg ASA</td>
<td>52±6</td>
<td>66±7</td>
<td>65±7</td>
</tr>
<tr>
<td>50 mg ASA</td>
<td>1.00±0.1</td>
<td>2.10±0.25</td>
<td>2.51±0.21</td>
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There was no significant difference in fibrin accretion between control and ASA-treated animals. In contrast, platelet accumulation was significantly enhanced ($P < 0.002$) in all ASA-treated groups.
There was no effect of ASA on platelet accumulation with time; therefore, all of the data obtained at each time interval for each treatment group were pooled and analyzed as four treatment groups. The data were transformed to log values for data handling. The table represents the antilogs of the mean.

(Newman-Keuls), it was shown that there was no significant difference between 0 (control) and 3 mg/kg ASA, or between 10 and 100 mg/kg ASA. However, there was a highly significant difference between 10 and 100 mg/kg ASA, compared with the control and 3 mg/kg groups ($P < 0.01$).

**Correlation between inhibition of PGI$_2$ production and enhanced platelet accumulation.** To determine whether there was a correlation between inhibition of PGI$_2$ production and enhanced thrombogenesis, PGI$_2$ production was measured in one carotid artery, and platelet accumulation onto an injured vessel was measured in the other carotid artery of the same animal. The measurements were determined from carotid arteries obtained from rabbits either 3 or 20 h after treatment with 0, 5, or 10 mg/kg of ASA. There was a significant negative correlation between PGI$_2$ production by the normal vessel wall and the log platelet accumulation onto the injured vessel wall on the opposite side. The correlations between PGI$_2$ production and log platelet accumulation in the 0, 5, and 10 mg/kg ASA treatment groups were $r = -0.66, n = 30, P < 0.01$; $r = -0.61, n = 27, P < 0.01$; and $r = -0.39, n = 32, P < 0.05$, respectively. There was no evidence that this relationship differed among these three groups. Therefore, all the data were combined and are plotted in Fig. 3. The overall correlation coefficient was $r = -0.55, n = 89, P < 0.01$.

**DISCUSSION**

ASA has been reported to be an effective antithrombotic agent in a number of clinical trials (4, 12–14). It has been suggested that the effectiveness of this drug might be reduced if the dosage regimen used also inhibits PGI$_2$ synthesis by vascular wall cells (15–16). Differences have been reported in the inhibitory effect of ASA on prostaglandin synthesis in platelets and vascular wall cells. Baenzinger et al. (17) reported that the concentration of ASA required in vitro to inhibit platelet cyclooxygenase is at least 1/25 of the concentration required to inhibit prostaglandin

**TABLE II**

<table>
<thead>
<tr>
<th>ASA treatment (mg/kg)</th>
<th>Mean platelet accumulation ($\times 10^4$/10 mm$^3$)</th>
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<tbody>
<tr>
<td>0</td>
<td>0.84</td>
</tr>
<tr>
<td>3</td>
<td>0.79</td>
</tr>
<tr>
<td>10</td>
<td>1.24</td>
</tr>
<tr>
<td>100</td>
<td>1.32</td>
</tr>
</tbody>
</table>

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**Figure 2**. Log platelet accumulation (mean±SEM) onto injured carotid arteries in rabbits that were given 3, 10, or 100 mg ASA/kg i.v. either 0, 1, 3, 6, or 20 h beforehand. Control animals injected with an equivolume of suspending vehicle were included in each time-frame. Since there was no difference between control groups, these data were pooled and are shown as one control group in this figure. Each solid dot represents the mean value of both carotid arteries in any given animal.
platelet accumulation may be results with control animals. ASA-treated carotid and increased carotid in arteries effect of ASA is treatment of thrombogenic vessel wall its with accretion high doses of effect by inhibit platelet glandin synthesis by Jaffe contrast, demonstrated that muscle cells. These and other synthesis, have previously reported that the association between PGI2 production and log platelet accumulation in the control and 5 or 10 mg/kg ASA-treated animals (r = -0.66, P < 0.01; r = -0.61, P < 0.01, and r = -0.39, P < 0.05, respectively).

We have previously reported that the inhibitory effect of ASA on PGI2 synthesis is more prolonged in arteries than in veins. We now report that ASA, in a dose of 10 mg/kg i.v., inhibits PGI2 production in carotid arteries and that this is associated with an increased accumulation of platelets onto injured rabbit carotid arteries, an effect that lasts for at least 20 h. A significant correlation was found between PGI2 production and platelet accumulation in not only the ASA-treated animals but also in the control animals. The results with control animals suggest that there may be an association between PGI2 production and platelet accumulation onto the injured vessel wall, but do not provide conclusive proof of a causal relationship.

A number of investigators have reported that PGI2 does not influence platelet adhesion onto vascular wall cells. Curwen et al. (19) reported that the adhesion of platelets to cultured endothelial cells in vitro was not increased when PGI2 production was inhibited by ASA (19). Dejana et al. (20) reported that ASA treatment did not influence the adherence of 51Cr-platelets to ballooned or undamaged aortae in rabbits. In contrast, Czervionke et al. (5) found that the exposure of cultured endothelial cells to ASA increased the adhesion of thrombin-induced platelet aggregates to the cells. In our experiments, ASA increased the adhesion of platelets to damaged vessel walls. Electron microscope examination revealed that the underlying endothelial surface was intact and that the platelets adhered to a fibrin mesh that covered the endothelial cells. It is likely, therefore, that thrombin was generated during stasis, which resulted in both fibrin formation (Fig. 1) and PGI2 production (21), and that treatment with ASA enhanced platelet adhesion to the fibrin mesh by inhibiting PGI2 production. These findings suggest that PGI2 may be particularly important in contributing to the thromboresistance of the intact vessel wall when it is covered with fibrin and that ASA may enhance thrombosis under these conditions by inhibiting PGI2 synthesis.

Although recommendations about the therapeutic

![Figure 3](image-url)
use of ASA can only be derived from results of clinical trials, our results raise the possibility that the margin of safety between obtaining an antithrombotic effect of ASA and producing a potential thrombogenic effect may not be as large as indicated by studies using cultured endothelial cells or injured jugular veins. The findings therefore support the suggestion that, when administered as an antithrombotic agent, ASA should be used in the lowest daily dose required to inhibit platelet TXA2 production (4, 17).

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REFERENCES