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Research Article

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 ($r = 0.55$, $P$ less than 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.



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

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ABSTRACT Prostacyclin ($PGI_2$) 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 $PGI_2$ 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 $PGI_2$ production. There was a strong inverse correlation ($r = -0.55$, $P < 0.01$) between $PGI_2$ 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 $A_2$ ($TxA_2$)[1] by platelets and of prostacyclin ($PGI_2$) by vessel-wall cells is thought to be important in the regulation of hemostasis and in the pathogenesis of thrombosis (1, 2). $TxA_2$ causes vasoconstriction and enhances platelet aggregation, whereas $PGI_2$ causes vasodilatation and inhibits platelet aggregation. Aspirin (ASA) inhibits $TxA_2$ formation by acetylating 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 $PGI_2$ 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 $PGI_2$ 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 $PGI_2$ production in arteries is associated with a prolonged thrombogenic effect, we examined the relationship between the inhibitory effect of ASA on $PGI_2$ production and its enhancement of platelet accumulation onto injured carotid arteries in rabbits.

## METHODS

*Carotid injury model.* Rabbits were injected with homologous $^{51}Cr$-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 macroscope 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

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[1] *Abbreviations used in this paper*: ASA, aspirin; $PGI_2$, prostacyclin; $TxA_2$, thromboxane $A_2$.

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 $^{51}$Cr-platelets and $^{125}$I-fibrinogen, and their carotid arteries were then injured as described above. $^{51}$Cr-Platelet accumulation and $^{125}$I-fibrin 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 ethanols. After dehydration, the vessels were critical-point dried from $CO_2$ in a Bomar 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 $PGI_2$-like activity of carotid arteries.* The effect of ASA on $PGI_2$ synthesis by the vessel wall was quantitated in two ways, using a radioimmune assay for 6-keto-$PGF_{1\alpha}$ as described by Czervionke et al. (10) and by using a bioassay as described below. The antibody for the radioimmune assay 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 radioimmune assay was 30 pmol. The activity of the bioassay is referred to as "$PGI_2$-like" activity.

The basic method used to quantitate $PGI_2$-like activity of vessel walls is described in detail elsewhere (8, 9). In brief, rabbits were heparinized with 200 U/kg of heparin (Hepalean, 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 $PGI_2$-like activity or the $PGI_2$ assay procedure (11). Each vessel was flushed with calcium-free Tyrodes solution (containing 0.35% albumin, bovine fraction V; Sigma Chemical Corp., St. Louis, Mo.) and then slit longitudinally to stimulate $PGI_2$ 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 $PGI_2$-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 $PGI_2$-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 $^{14}$C-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 $^{14}$C-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 $^{14}$C-serotonin released into the supernate was determined. The percent inhibitoin of $^{14}$C-serotonin released by platelets was measured and the amount of $PGI_2$-like activity calculated from a dose-response curve that was standardized for each experiment, using $PGI_2$ obtained from Upjohn Co., Kalamazoo, Mich. There was no loss of $PGI_2$ activity when standards containing known amounts of $PGI_2$ were treated in an identical manner.

The recovery of $PGI_2$-like activity in the bioassay was measured by adding known amounts of $PGI_2$ to either Eagles' medium or Eagles' containing a vessel wall segment. In six experiments, the level of $PGI_2$-like activity for the vessel wall alone was 137±37 pg/100 μl Eagles' (mean±SEM); for the vessel wall plus the $PGI_2$, 723±50 pg/100 μl Eagles'; and for the $PGI_2$ alone, 666±70 pg/100 μl Eagles', giving a recovery rate of 87±3%.

The $PGI_2$-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-$PGF_{1\alpha}$ formation in animals treated with ASA. However, in these in vivo experiments there was no measurable 6-keto-$PGF_{1\alpha}$ for up to 6 h after ASA treatment even though there were measurable (although markedly reduced levels of $PGI_2$-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 $PGI_2$.

*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 (acetylsalicyclic 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 $^{51}$Cr-platelet accumulation (see Tables I and II; Figs. 2 and 3).

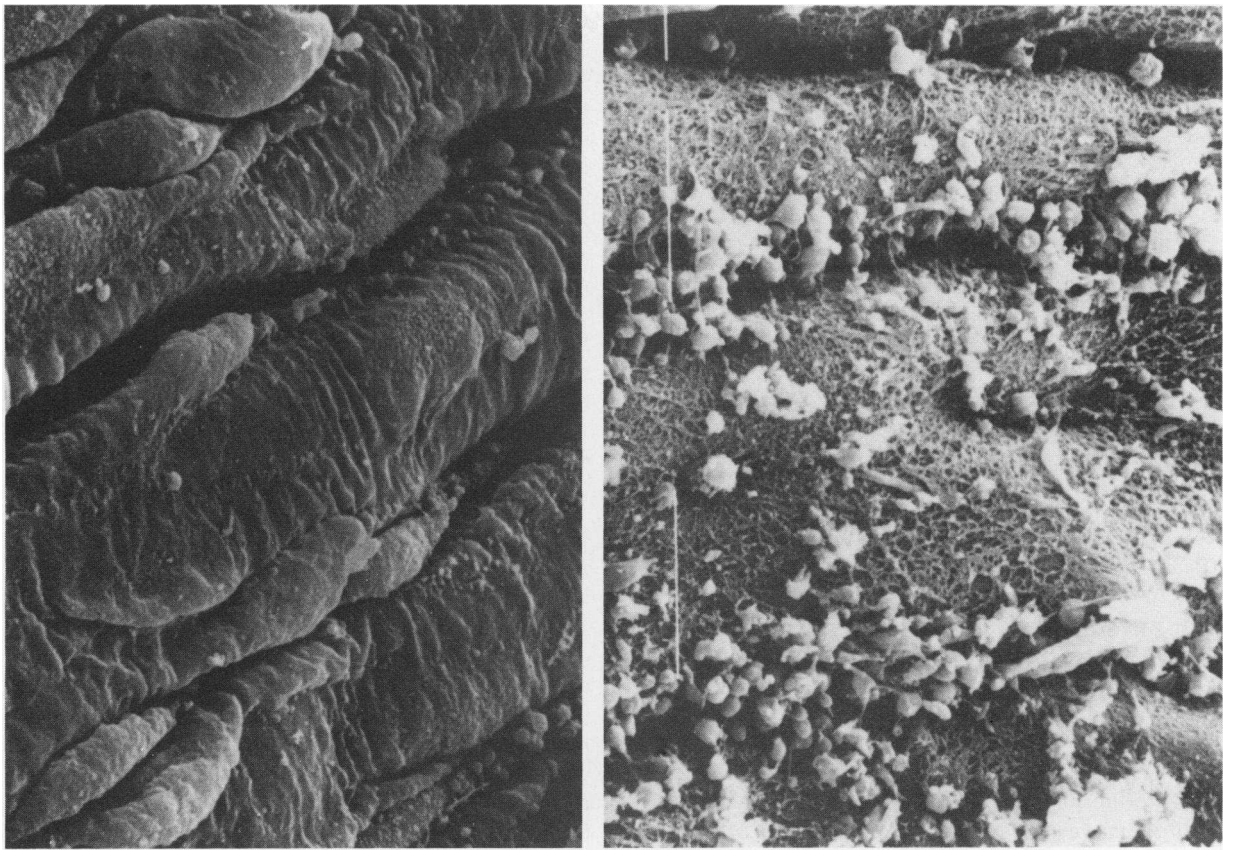

FIGURE 1 A scanning electron micrograph of a carotid injury 1 h after restoration of flow. The endothelial surface is covered with a mesh of fibrin to which numerous platelets and platelet aggregates have adhered (right side). A control vessel is shown for comparison (left side).

*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 I

*Mean $^{125}$I-Fibrin Accretion and Mean $^{51}$Cr-Platelet Accumulation onto Injured Carotid Arteries in Rabbits Given 0 or 100 mg/kg ASA i.v. either 1, 6, or 20 h Beforehand*

| | | ASA treatment | | |
|---|---|---|---|---|
| | Control | 1 h | 6 h | 20 h |
| $^{125}$I-Fibrin accretion, $\mu g/10\ mm^2$ *vessel wall* | 56±6 | 52±6 | 66±7 | 65±7 |
| $^{51}$Cr-Platelet accumulation, $\times 10^6/mm^2$ *vessel wall* | 1.00±0.1 | 2.10±0.25 | 2.51±0.21 | 1.92±0.23 |

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.

TABLE II
*Mean Platelet Accumulation onto Injured Carotid Arteries of ASA-treated Rabbits*

| ASA treatment | Mean platelet accumulation |
|---|---|
| *mg/kg* | *$\times 10^6$/10 mm²* |
| 0 | 0.84 |
| 3 | 0.79 |
| 10 | 1.24 |
| 100 | 1.32 |

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

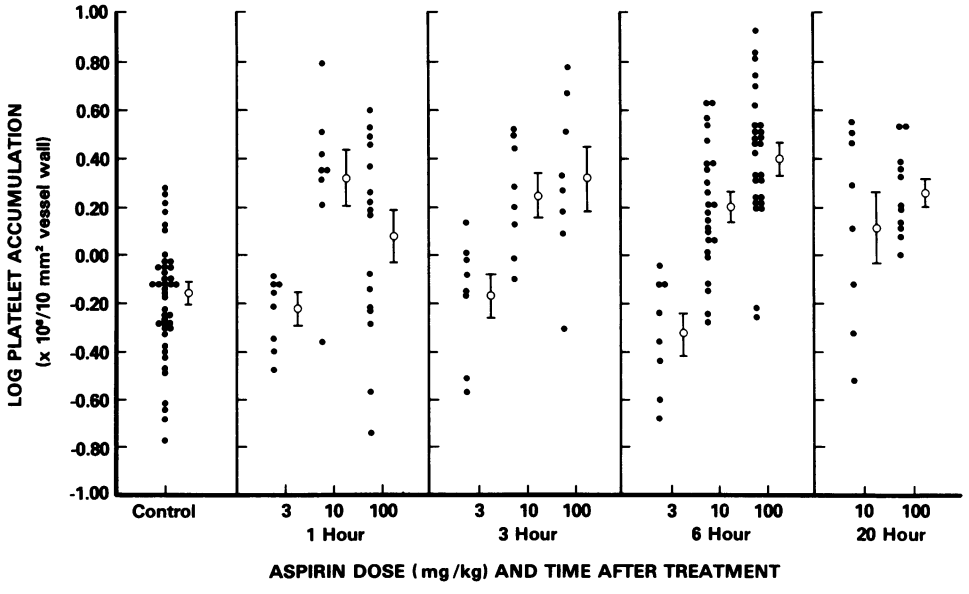


FIGURE 2 Log platelet accumulation (mean±SEM) onto injured carotid arteries in rabbits that were given 3, 10, or 100 mg ASA/kg i.v. either 0, 1, 3, 6, or 20 h beforehand. Control animals injected with an equivolume of suspending vehicle were included in each time-frame. Since there was no difference 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.

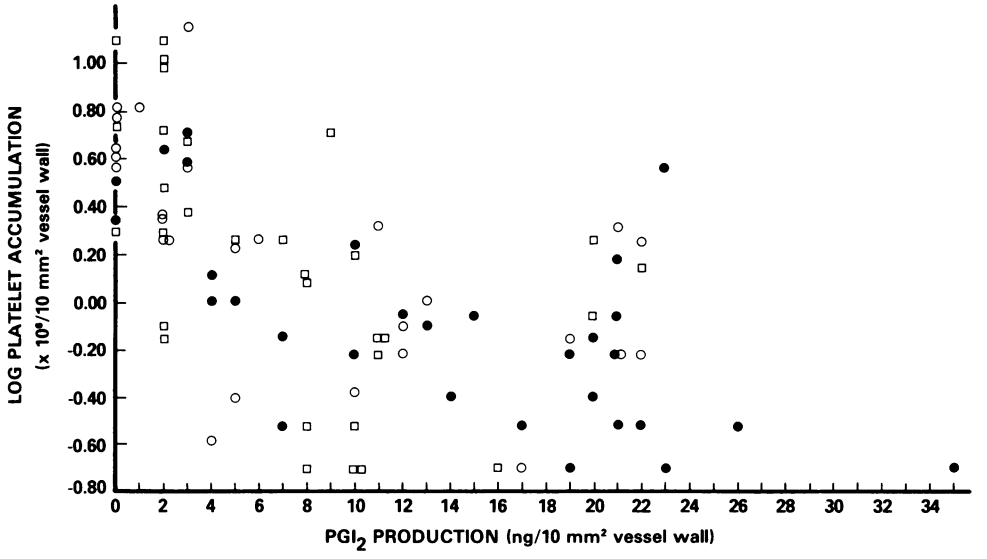


FIGURE 3 Log platelet accumulation onto injured vessel wall plotted against $PGI_2$ production by vessel wall in the same material. Platelet accumulation was measured on one injured carotid artery and $PGI_2$ production was measured in the other uninjured carotid artery in the same animal. Vessels were obtained from control animals (●) and from animals given 5 (○) or 10 mg/kg ASA (□) either 3 or 20 h beforehand. There was a significant negative correlation between $PGI_2$ 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).

synthesis in human fibroblasts and arterial smooth muscle cells. These and other investigators have also demonstrated that the effect of ASA on prostaglandin synthesis lasts for the life span of the platelet (3). In contrast, Jaffe and Weksler (6) reported that prostaglandin synthesis by cultured endothelial cells is inhibited by concentrations of ASA similar to those that inhibit platelet prostaglandin synthesis, but that the effect on vascular wall cells is rapidly reversed after ASA is removed. Kelton et al. (9) reported that very high doses of ASA are required to enhance $^{125}$I-fibrin accretion onto injured rabbit jugular veins and that this thrombogenic effect of ASA is lost within 2½ h of ASA treatment (9), supporting the suggestion that, compared with its effect on platelets, the inhibitory effect of ASA on vessel wall $PGI_2$ synthesis is short lived.

We have previously reported that the inhibitory effect of ASA on $PGI_2$ synthesis is more prolonged in arteries than in veins (7). We now report that ASA, in a dose of 10 mg/kg i.v., inhibits $PGI_2$ 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 $PGI_2$ 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 $PGI_2$ 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 $PGI_2$ does not influence platelet adhesion onto vascular wall cells (18–20). Curwen et al. (19) reported that the adhesion of platelets to cultured endothelial cells in vitro was not increased when $PGI_2$ production was inhibited by ASA (19). Dejana et al. (20) reported that ASA treatment did not influence the adherence of $^{51}$Cr-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 $PGI_2$ production (21), and that treatment with ASA enhanced platelet adhesion to the fibrin mesh by inhibiting $PGI_2$ production. These findings suggest that $PGI_2$ 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 $PGI_2$ synthesis.

Although recommendations about the therapeutic

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 $TxA_2$ production (4, 17).

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