Effect of Aspirin and Salicylate on Platelet-Vessel Wall Interactions in Rabbits

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We performed studies to examine the mechanism responsible for the antithrombotic effect of aspirin in experimental animals. We measured the effect of 10 and 100 mg/kg doses of aspirin, salicylate, or a combination of both on the accumulation of radiolabeled platelets onto injured carotid arteries in rabbits. The effects of these agents on thrombogenicity measured as platelet accumulation onto injured carotid arteries, were correlated with the ability to inhibit platelet thromboxane A2 and vessel wall prostacyclin synthesis. We found that a low dose of aspirin significantly inhibited platelet accumulation onto the injured vessels, while a high dose reversed this effect. Thromboxane A2 and prostacyclin production, however, were maximally inhibited in rabbits given either aspirin dose. The reversal of the antithrombotic effect of the low dose of aspirin by a high dose of aspirin was simulated by administering a combination dose of high-dose salicylate and low-dose aspirin. We concluded that the reversal of the antithrombotic effect of a 10 mg/kg dose of aspirin by a higher dose of aspirin could not be explained solely by the inhibition of PGI2 synthesis and was affected by the salicylate moiety of aspirin. (Arteriosclerosis 4:403–406, July/August 1984)

We reported that aspirin in doses of 1 to 10 mg/kg inhibits thrombus formation, whereas higher doses enhance thrombus formation and accelerate hemostasis in a number of experimental animal models.1–4 The thrombogenic effect of aspirin was attributed to the inhibition of PGI synthesis. However, we noted that aspirin retained its antithrombotic effect even at doses that inhibited PGI synthesis, and that much higher doses were required to produce a thrombogenic effect. This disparity between the antithrombotic effect of aspirin and the dose required to inhibit PGI2 synthesis prompted us to investigate again the mechanisms by which aspirin is antithrombotic, and in particular, to test the possibility that the loss of this effect is through mechanisms other than the inhibition of PGI2 production.

Methods

Arterial Injury Model

Accumulation of 51Cr-labeled platelets onto injured rabbit carotid arteries was determined using a modification of our injury model previously described.2 Briefly, rabbits were injected with homologous 51Cr-labeled platelets. After 24 hours, each animal was anesthetized with sodium pentobarbital, and both carotid arteries were isolated. The blood was emptied from the vessel, and two clamps were applied 2 cm apart onto each artery. After 10 minutes, the clamps were removed, and blood flow was restored. Then 1 hour later, a 5-ml citrated blood sample was collected, and each rabbit was 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 the glass tube containing 1.5 ml of saline. The vessel wall radioactivity was determined in a gamma counter. The vessel was removed from the tube, slit longitudinally, and laid flat with the endothelial side down on a transparent film of acetate, and was photocopied. The surface area of the vessel wall was determined by cutting out the photocopied, weighing it, and comparing it with the weight of an adjacent 1 cm2 cut from the same piece of paper.

In the interim, platelet-rich plasma was prepared from the citrated blood collected before removing the injured vessel walls and both platelet count and platelet specific activity were determined. The radioactivity of the vessel wall was then expressed as platelets/mm2 of vessel wall surface.

In other experiments, both uninjured and injured carotid artery segments were prepared from the same animals for scanning electron microscopic examination with standard techniques.2 Each vessel segment was fixed in 2% glutaraldehyde, postfixed in 1% aqueous osmium tetroxide, and dehydrated in a series of graded ethanol (50% to 95%). After dehydration, the vessels were critical-point dried.
with CO₂, coated with gold/platinum (200Å), and viewed in a Phillips scanning electron microscope (PSEM Model 501B).

**Experimental Design**

We injected 42 rabbits (male and female New Zealand White, 2.1 kg to 3.6 kg) via the marginal ear vein with 0 (suspending vehicle), 10 or 100 mg/kg of aspirin, 10 mg/kg of aspirin followed 1 hour later with 100 mg/kg sodium salicylate (salicylate), or 100 mg/kg of salicylate alone. All treatments were administered in 1 ml/kg equivalents. After 2 hours, both carotid arteries were injured as described above. Then, 1 hour later, the injured carotid artery segments were removed and the number of platelets adherent to the injured surface was determined. At the same time, citrated blood samples were collected to determine thromboxane A₂ (TxA₂) production.

Another group of rabbits were given similar treatments; 2 hours later the carotid arteries were removed and their PGI₂ activity was determined.

**Determination of 6-Keto-PGF₁α**

PGI₂ activity was determined by measuring⁵ a stable endproduct, 6-keto-PGF₁α. Purified 6-keto-PGF₁α was purchased from Upjohn Company (Kalamazoo, Michigan), and the specific antibody to 6-keto-PGF₁α was raised in male rabbits using a 6-keto-PGF₁α-albumin conjugate.⁵ Briefly, 6-keto-PGF₁α was coupled to bovine serum albumin using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The conjugate was purified and dissolved in Freund’s complete adjuvant. Male rabbits were then immunized with the conjugate and given a series of booster shots at 6-week intervals for 6 months. The antisera produced by these animals was comparable in sensitivity and cross-reactivity with a commercial grade antisera.⁵

For the radioimmunoassay itself, 6-keto-PGF₁α was dissolved in the presence of EDC. An aliquot of the conjugate was iodinated (¹²⁵I) using the chloramine T-procedure. Equal volumes of tracer, standard, or test samples and the antisera were incubated for 2 hours at 22°C. The bound and free moieties were separated using a double-antibody technique and the bound fraction was counted in a gamma counter. The 6-keto-PGF₁α concentrations were calculated by a computer-optimized Scatchard analysis.⁵ The assay sensitivity was 30 pg/ml with an operating range of 30–2000 pg/ml.

**Thromboxane B₂ Assay**

Purified thromboxane (TxB₂) was purchased from the Upjohn Company; the specific antisera was from Seragen Company, Boston, Massachusetts. The assay for TxB₂ was essentially the same as that described for the 6-keto-PGF₁α except that the TxB₂ was dissolved in ethanol before being coupled with EDC. The assay sensitivity was 25 pg/ml with an operating range of 25–2000 pg/ml.⁵

**Assessment of Vessel Wall PGI₂ Activity**

Endothelial cells were stripped in toto from the rest of the vessel wall with electroperborahetic cellulose acetate as previously described.⁶ This stripping technique provided a standardized mechanical stimulus for PGI₂ synthesis by both endothelial cells and the remaining vessel wall segment. Both the cellulose acetate-endothelial cell preparation and the remaining denuded vessel wall (smooth muscle cells) were immediately placed together in a cuvette containing 1 ml of modified Eagle’s medium (Gibco, Grand Island, New York) and 4% albumin, 5 mM Hepes buffer (pH 7.5) and incubated for 5 minutes at 37°C. The tissues were then removed from the cuvette and the medium was maintained at 37°C for an additional 60 minutes to permit total degradation of PGI₂ to 6-keto-PGF₁α.

**Data Analysis**

All data were analyzed using analysis of variance and Dunnett’s multiple comparison tests.⁶,⁷

**Results**

**Scanning Electron Microscopy**

The luminal surface of injured carotid arteries was markedly abnormal 2 hours after the stasis- and anoxia-induced injury. The surface was partially denuded, and in several places the endothelial cells were lifted off the basement membrane (Figure 1 A). Platelets and platelet aggregates adhered both to the partially detached endothelial cells and to the underlying basement membrane. Figure 1 B shows a control vessel for comparison.

**Effect of Aspirin and Salicylate on Platelet Accumulation**

Approximately 2.44 × 10⁶ platelets adhered to a 10-mm² area of the vessel wall of the injured carotid arteries in untreated animals. This represented the thrombogenicity of the injured vessels (Figure 2 A). A dose of 10 mg/kg of aspirin significantly inhibited platelet accumulation (p < 0.01), whereas the higher aspirin dose of 100 mg/kg reversed this effect. Similarly, treatment with a combination of salicylate and low-dose aspirin reversed the inhibitory effect on platelet accumulation achieved with the low dose of aspirin alone. Salicylate alone had no effect.

**Effect of Aspirin and Salicylate on Platelet TxB₂ and Vessel Wall 6-Keto-PGF₁α Production**

Platelet TxB₂ production, which was 67.6 ± 10.2 pg/10⁶ platelets (mean ± sem) in control rabbits, was maximally inhibited in all rabbits in the three aspirin-treated groups (Table 1). TxB₂ production in salicylate-treated rabbits was not significantly different from controls.
Vessel wall 6-keto-PGF$_{1\alpha}$ production was also maximally inhibited in all three aspirin-treated groups of rabbits (Figure 2 B). In contrast, the 6-keto-PGF$_{1\alpha}$ production was elevated 40% in rabbits given salicylate alone ($p < 0.05$).

**Discussion**

We demonstrated that aspirin in a dose that inhibits both TxB$_2$ and PGI$_2$ synthesis is antithrombotic in an experimentally injured vessel wall model and that this antithrombotic effect is reversed either by increasing the dose of aspirin or by adding a high dose of salicylate to the antithrombotic aspirin dose. These observations suggest that the reversal of the antithrombotic effect of a low dose of aspirin by increasing the aspirin dose is not solely due to the inhibition of PGI$_2$, and that this reversal is contributed to by an effect of the salicylate moiety on platelet-vessel wall interactions.

**Figure 1.** Scanning electron micrograph of the luminal surface of an injured (A) and an uninjured (B) carotid artery. BM = basement membrane; P = platelets; EC = endothelial cells. × 1000.

**Figure 2.** Effect of aspirin and salicylate on (A) $^{51}$Cr-platelet accumulation onto injured carotid arteries and (B) 6-keto-PGF$_{1\alpha}$ production in rabbits given 0 (none), 10 mg/kg aspirin (LO aspirin), 100 mg/kg aspirin (HI aspirin), 10 mg/kg aspirin plus 100 mg/kg salicylate (ASA/SAL), or 100 mg/kg salicylate alone (HI salicylate). Data are expressed as means ± SEM; n = 8.
The mechanism by which salicylate influences the effect of aspirin on platelet-vessel wall interactions is unknown, but there are a number of possibilities. Cerletti et al.9 and Dejana et al.8 have reported that salicylate inhibits the acetylation of platelet cyclooxygenase by aspirin and therefore interferes with the inhibition of TxA2 production. However, this does not explain the loss of antithrombotic effect in the rabbits treated with a low dose of aspirin and a high dose of salicylate. Therefore, it is more likely that salicylate in high doses acts on the vessel wall through an unknown mechanism independent of the cyclooxygenase pathway and the inhibition of PGI2 synthesis. Lipoxygenase metabolites have reportedly10 affects platelet function, but this effect is to inhibit platelet aggregation, so this would not explain the loss of antithrombotic effect in the rabbits treated with a low dose of aspirin and a high dose of salicylate. Therefore, it is more likely that salicylate in high doses acts on the vessel wall through an unknown mechanism independent of the cyclooxygenase pathway and the inhibition of PGI2 synthesis. Lipoxygenase metabolites have reportedly10 been produced by rabbit vessel wall segments, and salicylate in high concentrations has been shown12 to inhibit the production of lipoxygenase metabolites in platelets.

More recently, we reported13 that human endothelial cells in culture produce a lipoxygenase metabolite that inhibits platelet-endothelial cell interactions; salicylate inhibits the production of this metabolite by endothelial cells and reverses its effect on platelet-endothelial cell interactions. It is therefore possible that the observed effect of salicylate on reversing the antithrombotic effect of aspirin is caused by this mechanism. Observations of an increase in 6-keto-PGF1α levels in animals treated with salicylate alone is consistent with an inhibitory effect of salicylate on the lipoxygenase pathway and the shunting of excess arachidonic acid to the cyclooxygenase pathway. A similar shunting mechanism from the lipooxygenase to the cyclooxygenase pathway has been reported in platelets.14,15 These observations do not, however, exclude the possibility that salicylate has other effects independent of an effect on arachidonic acid metabolism, although this possibility seems unlikely.

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