Effect of Chronic Low Dose Aspirin on Platelet and Vascular Eicosanoid Metabolism in Nonhuman Primates (Macaca fascicularis)

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It has been suggested that inhibition of platelet cyclooxygenase by chronic low dose aspirin may spare vascular prostacyclin production. Conventional doses of aspirin (>5 mg/kg) have been shown to inhibit the generation of both thromboxane A2 and prostacyclin. Low dose aspirin inhibits prostacyclin production by excised human venous tissue, thus questioning the selectivity of such regimens. However, many clinical and surgical conditions requiring platelet inhibition involve the arterial system. We have studied the effects of various aspirin regimens on platelet, venous, and arterial cyclooxygenase activity in a nonhuman primate (Macaca fascicularis). We determined the lowest chronic dose of oral aspirin required to effectively inhibit platelet cyclooxygenase and aggregation to be 1 mg/kg. After 14 days of 0, 1, or 2 mg/kg aspirin, intact veins and arteries were surgically removed and perfused, and luminal prostacyclin (6-keto-PGF1a) generation was assessed. Levels of 6-keto-PGF1a in venous perfusates were reduced by 89% and 86% (p<0.05) after 1 and 2 mg/kg, respectively. Arterial 6-keto-PGF1a levels were unchanged by 1 mg/kg aspirin, but after 2 mg/kg were reduced by 66% (p<0.05). Preferential inhibition of platelet over arterial cyclooxygenase is thus achievable, but only over a narrow dose range. (Arteriosclerosis 7:599–604, November/December 1987)

The ability of aspirin to acetylate and thereby inactivate the enzyme cyclooxygenase underlies the suggestion that aspirin may be an effective therapeutic agent for limiting platelet activity. Such modulation may be beneficial in the treatment of those obstructive vascular diseases, such as ischemic heart disease and stroke, in which platelets are thought to play a pathologic role. Conceptually, an ideal aspirin regimen would be one that resulted in profound inhibition of platelet thromboxane A2 production while sparing vascular prostacyclin production. Since conventional doses of aspirin appear to inhibit both platelet and vascular cyclooxygenase,1–4 an alternative approach possibly employing lower doses of aspirin may be advantageous. One such approach was originally suggested by the observations of Burch et al.5 that repeated small doses of aspirin had a cumulative inhibitory effect on platelet cyclooxygenase activity. A single low dose resulted in submaximal inhibition of platelet cyclooxygenase. However, when the same dose was given repeatedly at daily intervals a cumulative inhibitory effect was demonstrated. These observations have subsequently been confirmed and extended by several other groups.6–9

The effect of such low dose regimens on vascular prostacyclin production is unclear. Patrignani et al.7 and Fitzgerald et al.10 reported that chronic low dose aspirin had little effect on total urinary excretion of prostacyclin metabolites, although it is not clear what portion of this reflects vascular prostacyclin production. In contrast, Preston et al.11 reported that similar aspirin regimens reduced prostacyclin production by excised veins of the human forearm by greater than 80%. Because of the limited availability of human arterial tissue, it is not clear what effect chronic low dose aspirin has on the ability of arterial endothelium to produce prostacyclin.

In view of the conflicting results and limited information, we have studied the effect of chronic low dose aspirin on cyclooxygenase of platelets and venous and arterial endothelium in a nonhuman primate (Macaca fascicularis). This model was chosen since it corresponds more closely to man than do the more commonly used animal models. In addition, nonhuman primates are frequently used as models of dietary, hypertensive, and surgical arteriosclerosis often including studies of aspirin intervention.12,13

Methods

Animals

Male, 4 to 5 kg cynomolgus monkeys (Macaca fascicularis) were housed in accordance with National Institutes of Health guidelines and fed Purina monkey chow, but fasted overnight prior to surgery. Aspirin was given in the morning at 24 hour intervals, 1 to 2 hours before feeding. Buffered aspirin (Alka Seltzer) was dissolved to a final concentration of 10 mg/ml aspirin in water. Upon complete dissolution (10 minutes with stirring) the volume corresponding to the
desired aspirin dose was mixed with an equal volume of orange juice. This procedure resulted in a preparation that the animals, after a brief period of familiarization, would eagerly accept from a 1 ml syringe. This method allowed administration of precise aspirin doses without submitting the animals to stress. Except where noted, all platelet and vascular studies were conducted immediately before and after 14 days of aspirin or vehicle administration. A 14 day aspirin-free recovery period was allowed before restudy of aspirin-treated animals. The recovery of platelet aggregation and thromboxane A₂ production confirmed that this was an appropriate period. All invasive procedures were performed under ketamine anesthesia (initial: 15 mg/kg intramuscularly; maintenance: 7.5 mg/kg intramuscularly, as required). The animal studies described had prior institutional review board approval.

**Platelet Studies**

Platelet studies were performed before initiating the various aspirin regimens and 2 hours after the last aspirin dose on Day 14 unless otherwise indicated. Aspirin or vehicle administration was designed such that on any one study day platelets from aspirin-treated and control animals were examined simultaneously.

Blood (approximately 10 ml) was drawn from a superficial posterior leg vein (anatomically equivalent to the lesser saphenous vein in man) via a 21 gauge butterfly cannula and added to a one-sixth volume acid citrate dextrose (ACD) or a one-tenth volume 3.8% trisodium citrate. In separate experiments duplicate 1 ml blood samples were allowed to clot in glass tubes at 37°C for 1 hour, and serum was prepared. Platelet-rich plasma (PRP) was prepared from ACD and citrated blood by centrifugation at 1500 g for 5.5 minutes. The platelet count in citrated PRP was adjusted to 2.5 x 10⁹/ml with autologous citrated platelet-poor plasma (PPP) prepared by high speed centrifugation (13,000 g for 5 minutes) of the blood remaining after aspiration of PRP. Platelet aggregation in citrated PRP in response to the sodium salt of arachidonic acid was measured in a Chronolog aggregometer by using autologous citrated PPP as the reference.

³H-arachidonic acid-labelled gel-filtered platelets were prepared from ACD PRP as described. The platelet count was adjusted to 2.5 x 10⁹/ml with gel-filtration buffer, and Ca²⁺ and bovine serum albumin (essentially fatty acid free, Sigma) were added to final concentrations of 3 mM and 0.05%, respectively. Platelet suspensions were stimulated with human α-thrombin (Fentom J, New York Department of Health) for 5 minutes at 37°C in an oscillating water bath. The incubation was terminated by the addition of a one-tenth volume of 10% DMSO in 200 mM EDTA, cooling to 4°C, and immediate centrifugation (13,000 g for 5 minutes). The supernatant medium was aspirated and stored at -20°C prior to high performance liquid chromatography (HPLC) and radioimmunoassay.

**Vascular Studies**

Surgery was performed immediately after blood collection, and segments of the superficial venous system (cephalic vein) and arterial system (femoral and iliac artery) were obtained in the following manner. With a sterile surgical technique, approximately 4 cm of the vessel was exposed, dissected free of connective tissue, and any side branches were tied off. These procedures were performed without direct manipulation of the vessel wall. The proximal end of the vessel (relative to the direction of blood flow) was cannulated in situ, tied onto a Teflon cannula (Quick-Cath, Travenol Laboratories, Deerfield, Illinois), and flushed free of blood with sterile tissue culture medium 199 (M.A. Bioproducts, Walkersville, Maryland). The vessel attached to the cannula was then removed after tying and cutting both ends of the parent vessel. The vessel was perfused at 37°C with medium 199 containing 0.1% bovine serum albumin at a flow rate of 0.05 ml/min. After discarding the initial 2 minute sample, the perfusate was collected for 10 minutes and stored at -20°C prior to radioimmunoassay. The vascular segments were then perfused with medium containing arachidonic acid (10 μg/ml) for a further 10 minute period and samples stored at -20°C. Preliminary experiments demonstrated that in the venous system 6-keto-PGF₁α production was stable and maximal during these periods.

**High Performance Liquid Chromatography**

The released metabolites of ³H-arachidonic acid were resolved by HPLC by using the method of Russel and Deykin using a Waters high performance liquid chromatograph equipped with a reverse phase fatty acid column (Millipore Corporation, Bedford, Massachusetts) as the stationary phase. Elution and separation was achieved with a quaternary solvent system containing varying proportions of tetrahydrofuran, acetonitrile, water, and acetic acid (HPLC grade, Fisher Scientific, Medford, Massachusetts). Radioimmunoassay

Determination of thromboxane B₂ (TXB₂) in the medium obtained from gel-filtered platelet incubations and 6-keto-PGF₁α in vascular perfusates was performed by specific radioimmunoassays with ¹²⁵I-labelled ligands (New England Nuclear Dupont, Boston, Massachusetts). The standard curve for each assay contained an equivalent volume of the gel-filtration buffer containing Ca²⁺, bovine serum albumin, EDTA, and DMSO or medium 199 to that being assayed (100 μl). The lower level of sensitivity for each assay was 2 pg/tube. Serum TXB₂ was measured with ³H-TXB₂, as previously described. Antibodies were provided by Lawrence Levine (Brandeis University, Waltham, Massachusetts).

**Results**

The effect of aspirin on thrombin-induced platelet thromboxane A₂ production measured after 14 days of various aspirin doses is shown in Figure 1. Control platelet suspensions released 75.3 ± 24.1 ng TXB₂/10⁹ platelets in response to 2 U/ml thrombin for 5 minutes at 37°C. After 14 days of 0.5, 1.0, and 2.0 mg/kg aspirin, identical stimulation of the platelets resulted in the release of 16.2 ± 4.6, 5.2 ± 4.9, and 2.8 ± 4.0 ng TXB₂/10⁹ platelets, respectively. These levels were all significantly different from control...
values ($p<0.01$) and represent 79%, 93%, and 96% inhibition of thromboxane A2 production. These results indicated that the lowest chronic dose of aspirin required to inhibit thromboxane A2 production by > 90% was 1 mg/kg. The response of platelets in citrated plasma to arachidonic acid after 14 days of various aspirin regimens is illustrated in Figure 2. Animals tested before aspirin and those receiving vehicle alone consistently showed full platelet aggregation in response to 2 mM arachidonic acid (the majority also responded to 1 mM arachidonic acid, results not shown). After 14 days of 0.5 mg/kg aspirin, platelet aggregation was only partial and spontaneously reversible; after 14 days of 1 mg/kg aspirin, platelet aggregation was completely inhibited in each animal. Figure 3 illustrates the time course of platelet cyclooxygenase inhibition as reflected in serum TXB2 levels. The data indicate that the maximum extent of inhibition evident after this chronic low dose aspirin regimen was achieved in a cumulative fashion. Serum TXB2 measurements confirmed the effectiveness of 1 mg/kg aspirin with the mean control value of 444 ± 102 ng/ml falling to 14.7 ± 4.0 ng/ml after 8 days (mean ± SD, n = 5, $p<0.001$).

Table 1 details the effects of 14 days of 1 mg/kg aspirin on thrombin induced release and further metabolism of $^3$H-arachidonic acid by prelabelled, gel-filtered monkey platelets. The total release of $^3$H-radioactivity was not significantly changed after 14 days of aspirin treatment. Of the $^3$H-radioactivity released, 37.3% ± 9.4% was found in cyclooxygenase-derived products. After aspirin this value was reduced by 90% to 3.7% ± 1.5% ($p<0.01$). The proportion of $^3$H-radioactivity present as lipoxygenase products was 21.6% ± 4.7% prior to aspirin and increased significantly ($p<0.05$) to 32.2% ± 8.3% after aspirin. Before aspirin 29.8% ± 8.0% of the total radioactivity released was arachidonic acid. After aspirin this value increased to 54.0% ± 6.6% ($p<0.02$).

Figure 4 (VEIN) shows the effects of chronic aspirin administration on prostacyclin release (measured as 6-keto-PGF1α) from venous endothelium. It can be seen that there was a profound reduction in the release of 6-keto-PGF1α after 14 days of aspirin administration. Unstimulated control levels (13.0 ± 6.5 pg/mg) were reduced to 1.44 ± 0.9 (p < 0.05) and 1.76 pg/mg (p < 0.05) after 1 and 2 mg/kg aspirin, respectively; these values represent 89% and 86% inhibition. Arachidonic acid stimulated levels showed a similar response to 14 days of 1 and 2 mg/kg aspirin. The levels of 6-keto-PGF1α in the perfusates of arterial endothelium are also illustrated in Figure 4 (ARTERY). The data indicate that, in contrast to its effect on venous tissue, 1 mg/kg aspirin had no significant effect on the release of 6-keto-PGF1α (10.8 ± 4.8 vs. 8.1 ± 1.3 pg/mg tissue, $p > 0.05$) by arterial endothelium. At the higher dose of 2 mg/kg aspirin the difference was statistically significant ($p < 0.05$), and the level of 3.7 ± 0.7 pg/mg represents 66% inhibition of 6-keto-PGF1α release. Similar effects were noted when the tissues were perfused with 10 μg/ml arachidonic acid. Because of the small diameter of the veins it was most convenient to express the 6-keto-PGF1α data on a wet weight basis. Expression of the data in this manner may underestimate the relative capacity of the arterial endothelium to generate prostacyclin ex vivo.
Table 1. Thrombin Induced Metabolism of 3H-Arachidonic Acid by Platelets from Control and Aspirin-Treated Monkeys

<table>
<thead>
<tr>
<th>Study period</th>
<th>% Total 3H release (mean ± SD)</th>
<th>% Distribution of released 3H-radioactivity (mean ± SD)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Before aspirin</td>
<td>12.0 ± 2.0</td>
<td>37.3 ± 9.4</td>
</tr>
<tr>
<td>After aspirin</td>
<td>10.3 ± 6.5</td>
<td>3.7 ± 1.5</td>
</tr>
<tr>
<td>Significance</td>
<td>NS</td>
<td>p&lt;0.01</td>
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Platelets were labelled with 3H-arachidonic acid, gel-filtered, and stimulated with 2 U/ml thrombin for 5 minutes at 37°C. The major classes of released 3H-radioactivity were resolved by high performance liquid chromatography. Studies were performed before and after 14 days of 1 mg/kg aspirin, n = 5; NS = not significantly different (p > 0.05).

Discussion

By using a nonhuman primate model, which in the parameters that we have examined corresponds closely to man, we have determined the effect of various chronic (14 day) doses of aspirin on platelet, venous, and arterial endothelial eicosanoid metabolism. With the use of thrombin induced thromboxane A2 production and serum TXB2 levels as quantitative biochemical indicators we have demonstrated that chronic aspirin administration to macaque monkeys results in a dose-dependent inhibition of platelet cyclooxygenase activity. The minimum effective dose, that is, that required to inhibit platelet thromboxane A2 production by >90%, was 1 mg/kg. That this dose was indeed a functionally effective dose was independently confirmed by the absence of ex vivo arachidonic acid-induced platelet aggregation. The temporal studies indicate that this level of inhibition was achieved in a cumulative manner, a phenomenon that has been well characterized in man.5-8 These data show that while the lowest serum TXB2 values were evident at Day 8, the bulk of the effect was present after Day 3. This time course is similar to that found in man during chronic ingestion of low dose aspirin.5-8 In addition, we examined the effects of 1 mg/kg aspirin on the qualitative aspects of arachidonic acid metabolism by examining the distribution of 3H-arachidonic acid metabolites released from prelabelled platelets. The data showed good agreement with our quantitative measurements of TXB2 by radioimmunoassay and confirmed a 90% decrease in cyclooxygenase activity after 14 days of 1 mg/kg aspirin administration. Further, the data also demonstrate that the in vitro release of both the lipooxygenase product, 12-hydroxyeicosatetraenoic acid (12-HETE), and free arachidonic acid is significantly increased after aspirin treatment.

The minimum inhibitory dose of aspirin needed to give the required degree of biochemical and functional inhibition of platelet cyclooxygenase activity was not greatly different in this nonhuman primate model from those reported in human studies (assuming a body weight of 70 kg): 0.45 mg/kg, Patrignani et al.7; 0.57 mg/kg, Preston et al.11; Hoogendijk and ten Cate6; 0.3 mg/kg, Fitzgerald et al.10

With the use of perfused vascular segments to assess the cyclooxygenase status of the vascular endothelium, we found that there was profound (89%) inhibition of venous prostacyclin production with a chronic dose of 1 mg/kg aspirin. These results are in close accord with those of Preston et al.,11 who found that production of 6-keto-PGF1α by vascular rings was reduced by 81% to 100% when an equivalent human tissue (subcutaneous forearm vein) was studied. Weksler et al.16 also demonstrated that human saphenous vein endothelium was substantially inhibited 3 hours after low dose aspirin.

Unlike many previous studies of chronic low dose aspirin in humans the use of a nonhuman primate in this study allowed examination of the ability of intact arterial endothelium...
lium to release prostacyclin after chronic low dose aspirin. We found that, in contrast to its effect on venous tissue, 1 mg/kg aspirin had no significant effect on the ability of arterial endothelium to release prostacyclin. It is of course possible that a larger number of animals had been studied and that the small decrease in arterial prostacyclin release that was observed may have become statistically significant. At the higher dose of aspirin studied (2 mg/kg) there was a more marked and significant reduction in arterial prostacyclin production. Thus, although arterial cyclooxygenase does appear to exhibit a relative insensitivity to aspirin over platelet cyclooxygenase, this differential sensitivity is lost at high doses of aspirin.

The combined use of $^{3}H$-arachidonic acid-labelled platelets and HPLC resolution of the radiolabelled metabolites released from the monkey platelets has demonstrated that, by inhibiting the ability of cyclooxygenase to metabolize arachidonic acid, there is an increased release of 12-HETE and free arachidonic acid. This shunting phenomenon has also been reported for human platelets. The local release of increased amounts of free arachidonic acid in the vicinity of arterial endothelial cells, which in this study we have shown to have intact cyclooxygenase activity (at 1 mg/kg aspirin), may fuel the production of prostacyclin. It has been established that endothelial cells are able to utilize exogenous arachidonic acid to augment prostacyclin production, both in vivo and in culture, in a variety of species. However, the concomitant increased release of platelet and neutrophil lipoxigenase products, which may modulate prostacyclin synthesis, must also be considered. Clearly platelet-vesSEL wall interaction and concomitant eicosanoid generation by endothelial cells during the use of chronic low dose aspirin warrants further study.

Although our studies provide no explanation for the differential sensitivities of venous and arterial tissue to the inhibitory effects of aspirin, the results described here are in accordance with a previous report. Villa et al. demonstrated that rat platelets and venous tissue are more sensitive to the inhibitory effects of aspirin than is arterial tissue. In a more recent study, however, the same group failed to confirm their earlier observations. Burch et al. by using a microsomal preparation, reported that arterial cyclooxygenase is less sensitive to aspirin than is platelet cyclooxygenase, while Jaffe and Weksler reported that cultured venous endothelial cell cyclooxygenase has a sensitivity equal to aspirin, as does platelet enzyme. In contrast, Baenziger et al. reported that cyclooxygenase in cultured venous endothelium is far less sensitive to aspirin than is platelet cyclooxygenase.

An alternative explanation to differential sensitivities of venous and arterial cyclooxygenase to aspirin is differential recoveries. Indeed, Kelton et al. have shown in a rabbit model that the ability of the carotid artery to inhibit thrombus formation returns as soon as 2.5 hours after aspirin administration. This time frame is similar to the time period between aspirin administration and vascular perfusion (approximately 3 hours) in our studies. Subramanian et al. reached similar conclusions regarding recovery of arterial cyclooxygenase in a comparative study of human vascular tissues. Weksler et al. demonstrated that prostacyclin production by the endothelium of human saphenous veins was substantially depressed 3 hours after low dose aspirin but had fully recovered by 24 hours. By using a rat model, Livio et al. reported that venous prostacyclin-like activity took 24 hours for 50% recovery and 48 hours to return to normal, but arterial prostacyclin-like activity had returned to normal by 24 hours. However, if accelerated recovery by arterial tissue were responsible for the difference in our studies, similar data should have been obtained after the higher aspirin dose of 2 mg/kg. Whatever the mechanism, it is apparent that differential inhibition of platelet cyclooxygenase is apparent 3 hours after the last dose of aspirin, and thus chronic low dose aspirin may be more selective than conventional dose (5 to 10 mg/kg) aspirin regimens.

The results presented here suggest that the measurement of total urinary excretion of prostacyclin metabolites may not accurately reflect the effects of aspirin on cyclooxygenase in all vascular tissues.

In conclusion, we have found that in a nonhuman primate model the lowest chronic dose of aspirin (1 mg/kg/day) that effectively inhibited platelet cyclooxygenase activity had similar effects on the cyclooxygenase activity of venous tissue, but had only a minor effect on arterial prostacyclin production. This differential effect may be achieved only within a narrow dose range. Conventional aspirin doses previously used in clinical trials fall outside the limits of this dose range. The similarities in the effects of aspirin on this animal model and man indicate that this nonhuman primate model is appropriate for further studies of low dose aspirin in occlusive vascular disease.

References


Index Terms: aspirin • primates • blood platelets • arteries • veins • endothelium • prostaglandins • thromboxane A2 • cyclooxygenase

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