In Vivo Measurement of Thromboxane B₂ and 6-Keto-Prostaglandin F₁α in Humans in Response to a Standardized Vascular Injury and the Influence of Aspirin

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The effects of smoking, aspirin ingestion, and sex differences on bleeding times and bleeding time thromboxane B₂ and 6-keto-prostaglandin (PG)F₁α production were examined. Nonsmoking men produced more thromboxane B₂ (3.99±0.76 ng/ml) than nonsmoking women (2.13±0.24 ng/ml). Female smokers produced more thromboxane B₂ (5.01±0.97 ng/ml) than nonsmoking women. Twenty-four hours after a single dose of 600 mg aspirin, in vitro production of thromboxane B₂ in response to collagen fell by 95%, whereas in vivo production of thromboxane B₂ and 6-keto-PGF₁α in bleeding time blood fell by 87% and 66%, respectively. Subjects with the lowest absolute levels of thromboxane B₂ 24 hours after aspirin were also those with the longest postaspirin bleeding times. Recovery of 6-keto-PGF₁α production was faster than recovery of thromboxane B₂ production, but 6-keto-PGF₁α production for most subjects was still below basal 72 hours after aspirin. The influence of two different doses of long-term aspirin (80 mg every other day and 325 mg daily) on the in vivo production of thromboxane B₂ and 6-keto-PGF₁α was studied in normals and diabetics. After 14 days of 80 mg aspirin every other day, thromboxane B₂ and 6-keto-PGF₁α production were both substantially inhibited (93% and 78%, respectively). After 14 days of 325 mg aspirin daily, thromboxane B₂ production was similarly substantially inhibited (93%), whereas 6-keto-PGF₁α was significantly less affected (only 45% inhibition). Study of a second group of five normal subjects confirmed that 6-keto-PGF₁α production was significantly inhibited 24 hours after the first dose of 325 mg aspirin but was not significantly less than basal after 14 days of 325 mg aspirin. The results suggest that 325 mg aspirin daily is more antithrombotic compared with 80 mg every other day due to the superior preservation of prostacyclin production. (Circulation 1989;79:29–38)

Thromboxane A₂, a potent vasoconstrictor and platelet-aggregating agent produced by platelets, and prostacyclin, a potent vasodilator and platelet inhibitor produced by vascular tissue, are believed to exert critical modulatory roles in vivo.¹,² Variations in the production of these compounds may be involved in the genesis of hemostatic and thrombotic disorders.²,³ Because of the short-lived nature of these compounds and the difficulties encountered in measuring them in vivo, much of the evidence to date has relied on measurements made in vitro or ex vivo.⁴ The assessment of the role of these compounds in vivo, and their contribution to disease requires improved assessment of their production in vivo.

It is in the nature of prostaglandins and thromboxanes that they are generally produced in response to specific stimuli. For platelet thromboxane and vascular prostacyclin, vessel wall injury is believed to be a major initiator. Measurement of plasma or urinary levels of these compounds, or rather their metabolites, is likely to reflect not only the response to a specific injury but also the number and extent

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of injury sites where interaction between platelets and the subendothelium occurs. For example, individuals with atherosclerosis have been found to have increased urinary prostacyclin and thromboxane metabolites, and this has been suggested to result from increased vascular injury in such patients. Because the measurement of the production of these compounds in response to a single vascular injury is more likely to be useful in the diagnosis and treatment of bleeding and clotting disorders, we have assessed the production of thromboxane and prostacyclin in response to a specific and standardized vessel wall injury—the template bleeding time. We have done this by measuring their immediate metabolites—thromboxane B$_2$ and 6-keto-prostaglandin (PG)F$_{1\alpha}$, respectively. The present study extends the work of others suggesting this may be a useful approach and evaluates the influence of sex, smoking, and aspirin on such production.

Despite much investigation, there is still debate as to the extent to which prostacyclin production is inhibited on varying long-term doses of aspirin and which dose is optimum. In this respect, studies with long-term aspirin should look not only at normal individuals but also at specific populations for whom a beneficial effect of antiplatelet therapy has been advocated (e.g., diabetics). Such individuals are significantly different from normal individuals in that they have an increased platelet turnover and decreased platelet life span, which may have important effects on the ability of the platelets and the vessel to recover from the effects of aspirin. Therefore, in this study, we have looked at two separate long-term doses of aspirin and evaluated normal subjects and matched diabetic controls. Because the results of the study show the remarkable finding that vascular prostacyclin was less inhibited on the daily higher dose aspirin, an additional group of controls was evaluated.

**Subjects and Methods**

**Drugs**

Thromboxane B$_2$ and 6-keto-PGF$_{1\alpha}$ were obtained from the Upjohn Company (Kalamazoo, Michigan). Sep-Pak C$_{18}$ cartridges were obtained from Waters Associates (Milford, Massachusetts). 6-(5,8,9,11,12,14,15-H')-Keto-PGF$_{1\alpha}$ 160 Ci/mmol was purchased from New England Nuclear (Boston, Massachusetts).

**Methods**

Enzyme-linked immunoabsorbent assays (ELISAs) for 6-keto-PGF$_{1\alpha}$ and thromboxane B$_2$ were used to measure levels of these substances in bleeding time blood. The assay for 6-keto-PGF$_{1\alpha}$ used micro-ELISA plates coated with 6-keto-PGF$_{1\alpha}$-bovine serum albumin (BSA) conjugate prepared by reaction with carbodiimide. The remaining protein binding sites on the plates were saturated by coating with fatty acid free bovine serum albumin (BSA), and the plates were thoroughly washed. The competitive ELISA was then performed by addition of 6-keto-PGF$_{1\alpha}$ standard in phosphate-buffered saline containing 10 mg/ml BSA (PBS-BSA) or unknown samples in the same buffer plus 100 µl anti-6-keto-PGF$_{1\alpha}$ at a dilution of 1:32,000 in PBS-BSA. After reaction at room temperature for 1 hour and overnight with shaking at 4°C, wells were washed thoroughly; then, 200 µl 1:1,000 diluted goat anti-rabbit IgG alkaline phosphatase conjugate in PBS-Tween was added, and the mixture was incubated for 2.5–3 hours at room temperature on a plate shaker. Again, wells were washed thoroughly; then, 200 µl alkaline phosphatase substrate was added to each well and incubated in an oven at 37°C for 3–4 hours before reading absorbance on an automated microplate reader. A full description of this assay is provided in Docherty and Gerrard. The ELISA method is capable of detecting 0.1 pg/ml 6-keto-PGF$_{1\alpha}$. Cross-reactivity of the antibody with PGF$_{2\alpha}$ is 0.5% of 6-keto-PGF$_{1\alpha}$, with PGF$_{1\alpha}$ equal to 0.01% and less than 0.01% for various other prostaglandins and prostaglandin metabolites.

To investigate the possibility of other plasma constituents interfering with the ELISA, results of the assay were double-checked by passing a portion of a sample of bleeding time blood isolated by the Sep-Pak extraction through a further high-pressure liquid chromatography (HPLC) separation. Part of the plasma sample was assayed directly by the ELISA, and part was further purified by HPLC before assay by the ELISA. Briefly, (3H)-6-keto-PGF$_{1\alpha}$ (0.4 nCi/ml) was added to plasma and was then extracted with Sep-Pak cartridges and resuspended in 26% CH$_3$CN. This sample was separated by HPLC (Beckman) by a fatty acid analysis column and an isocratic solvent system of 26% CH$_3$CN (pH 3.5) with a flow rate of 2.5 ml/min. The perfusate was collected at 30-second intervals. A 250-µl aliquot was dried under nitrogen, resuspended in an equal volume of PBS-BSA, and analyzed by the ELISA. The remainder was quantified by liquid scintillation counting to identify the fraction containing the purified 6-keto-PGF$_{1\alpha}$ and to calculate recovery. Samples passed through the HPLC association procedure gave results that were very similar (105 ± 11%, n = 5) to the aliquots of the same samples assayed directly.

The ELISA for thromboxane B$_2$ was performed according to a similar procedure except plates coated with thromboxane B$_2$-BSA conjugate and a highly specific rabbit antibody to thromboxane B$_2$ were used. The sensitivity of this ELISA for thromboxane B$_2$ is similar to the one for 6-keto-PGF$_{1\alpha}$. The antibody used showed cross-reactivity with PGF$_{2\alpha}$ of 0.14% of thromboxane B$_2$, whereas cross-reactivity with PGA$_2$, PGB$_2$, PGF$_{2\alpha}$, 6-keto-PGF$_{1\alpha}$, 13,14-dihydro-15-keto-PGF$_{2\alpha}$, and 13,14-dihydro-15-keto-PGF$_{1\alpha}$ was uniformly less than 0.01%.
Studies With Aspirin In Vivo

Part 1: Single aspirin dose: Influence of sex and smoking. Twenty-three normal individuals (nine men and 14 women; nine smokers and 14 nonsmokers), 22–49 years old, each took a single dose of 600 mg aspirin. Bleeding times were performed with a blood pressure cuff inflated to 40 mm Hg before aspirin and at 2, 24, 48, and 72 hours and at 2 weeks after aspirin (normals for the laboratory range from 3.0 to 9.0 minutes; mean, 6.0). A Simplate II (General Diagnostics, Organon-Teknika, Scarborough, Ontario) bleeding time device was used to produce a longitudinal incision on the volar surface of the forearm. Blood oozing from the 5-mm bleeding time incision was collected into heparinized capillary tubes containing indomethacin every 30 seconds until bleeding stopped and processed in aliquots corresponding to 5-minute time intervals. The plasma was obtained after centrifugation in a microhematocrit centrifuge, extracted by Sep-Pak cartridges,21 and analyzed for 6-keto-PGF_{1α} and thromboxane B_{2}. Results reported are the levels in the blood collected over the first 5 minutes of the bleeding time.

At the same time intervals, blood was also collected by venipuncture to give platelet-rich plasma.22 Platelet aggregation in response to 1.8 μg/ml collagen or 1.6 mM arachidonic acid was monitored by a Payton Dual Channel aggregometer, and thromboxane B_{2} production assessed by the ELISA described above. Percent platelet aggregation was recorded as the maximum extent of aggregation achieved within 3 minutes of addition of the collagen.

To allow comparison of baseline (i.e., preaspirin) and of levels of 6-keto-PGF_{1α} and thromboxane B_{2} in smokers compared with that in nonsmokers and in men compared with that in women, additional subjects were recruited to achieve the numbers reported in “Results.” Statistical analysis used SAS,23 except where specifically indicated. Comparisons between groups were done with a non-paired t test.

Part 2: Influence of long-term aspirin therapy in normal individuals and diabetics. Eight patients (19–31 years old) with type 1 diabetes from the endocrinology outpatient clinic at St. Boniface Hospital volunteered for the study. They fell into two subgroups: those with diabetes for less than 10 years having no complications of diabetes (n = 5) and those with diabetes for more than 10 years having proliferative retinopathy (n = 3). All were being treated with insulin, had ingested no aspirin or other prostaglandin-inhibitory drug for 2 weeks before the study, and were notsmoten. Random blood glucose levels (Hexokinase—Beckman Astra, Beckman Incorporated, Brea, California) ranged from 13.8 to 18.7 mmol/l. HbAlc levels

74 ranged from 9.6% to 11.8%.

Seven normal subjects (age, 21–33 years) matched for age (±5 years), sex, and smoking history were recruited as controls. They also had no history of hypertension and had not taken aspirin or any other drug known to inhibit prostaglandin or thromboxane synthesis in the previous 2 weeks. Random blood glucose measurements ranged from 4.6 to 6.8 mmol/l; HbAlc ranged from 5.0% to 6.5%. All subjects gave informed consent.

All 15 subjects were given aspirin (Bayer) according to a crossover design that consisted of two different dosage schedules—either 325 mg/day or 80 mg every 2nd day for 2 weeks. Subjects were randomly assigned schedules. The second schedule was started at least 2 weeks after the first was completed. Each diabetic subject was matched with and studied concurrently with a normal subject (except for an eighth control who withdrew) to allow paired comparisons of the results. One of the controls and two of the diabetics completed only one aspirin regimen. Bleeding time and venipuncture were performed before aspirin and on three later occasions (days 14, 15, and 16) for each of the two doses.

An additional five controls (age, 24–35 years) were studied after the initial trial had been completed. These subjects were placed on 2 weeks of single dose 325 mg aspirin/day and were studied before the start of aspirin therapy, 24 hours after the first aspirin dose, and 4 and 24 hours after the day-14 aspirin dose. For this study, any 6-keto-PGF_{1α} detected by the ELISA in plasma was subtracted from the value obtained from the bleeding time determination to ensure that the measurement represented de novo production. Bleeding time values refer to blood collected in the first 4 minutes after the injury. Comparisons between groups were made with a paired t test. Unless otherwise noted, all results are expressed as the mean ± SEM.

Results

Thromboxane Production

It was important to assess whether calculating thromboxane production as picograms per minute or as nanograms per milliliter was the more appropriate. A review of the data obtained showed that the results in most patients were similar regardless of which way the results were calculated. In three subjects, all high thromboxane producers, an increase in the volume of blood oozing from the bleeding time incision 2 hours after aspirin associated with significant, but incomplete, inhibition of thromboxane synthesis (measured as ng/ml or in vitro in response to collagen or arachidonic acid) gave an apparent and artifactual increase in post-aspirin thromboxane production, if this measure was calculated as picograms per minute. We conclude from this observation that thromboxane is formed in the flowing blood when platelets encounter released soluble mediators such as adenosine diphosphate, as well as the injured vessel surface, and that
thromboxane B₂ production is more meaningful if reported as nanograms per milliliter. A significant correlation between the length of the bleeding time and the level of thromboxane B₂ measured as nanograms per milliliter (see below) but not as picograms per minute further supports the contention that nanograms per milliliter is the more relevant physiological parameter.

**Baseline Levels of 6-Keto-PGF₁α and Thromboxane B₂**

*Influence of sex.* To evaluate a possible influence of sex on bleeding time thromboxane B₂ and 6-keto-PGF₁α levels, we compared nonsmoking men (n = 11) and nonsmoking women (n = 11). Men had bleeding time thromboxane B₂ levels of 3.99 ± 0.76 ng/ml, whereas women produced only 2.13 ± 0.24 ng/ml (p < 0.04). Men also had significantly shorter bleeding times (5.5 ± 0.4 min) than women (6.89 ± 0.38 min) (p < 0.03). The volume of bleeding time blood was slightly, but not significantly, lower in men. There was no significant difference in 6-keto-PGF₁α production between men (5.82 ± 1.35 pg/min) and women (6.10 ± 1.23 pg/min).

*Influence of smoking.* To assess a possible influence of smoking on bleeding time thromboxane B₂ levels, nonsmoking women (n = 11) were compared with smoking women (n = 12). Thromboxane B₂ levels were 5.01 ± 0.97 ng/ml in smokers and 2.13 ± 0.24 ng/ml in nonsmokers (p < 0.02). Bleeding times were also shorter in smokers (5.64 ± 0.39 min) than in nonsmokers (6.89 ± 0.38 min) (p < 0.04) as was the volume of bleeding time blood, but the latter did not quite reach statistical significance. There were no significant differences between 6-keto-PGF₁α production in smokers (5.51 ± 0.82 pg/min) and in nonsmokers (6.10 ± 1.23 pg/min).

*Relation to length of bleeding time.* For all subjects entered in the single-dose aspirin study, no correlation was observed between the length of the bleeding time and the production of 6-keto-PGF₁α (r = 0.19). There was, however, a significant correlation between the length of the bleeding time and the production of thromboxane B₂ (r = -0.68, p < 0.002) (Figure 1). There was also a correlation between the length of the bleeding time and the volume of bleeding time blood (r = 0.57, p < 0.0005), but there was no association between the level of thromboxane B₂ and the volume of bleeding time blood (r = -0.21, p < 0.3).

**Influence of Aspirin on Production of 6-Keto-PGF₁α and Thromboxane B₂**

The ingestion of 600 mg aspirin was associated with an increase in the bleeding time, an increase in the volume of blood oozing from the bleeding time incision (Figure 2, top left panel), and a decrease in vitro production of thromboxane B₂ in response to collagen (Figure 2, top right panel) and arachidonic acid (data not shown). Significant decreases also occurred in both 6-keto-PGF₁α and thromboxane B₂ production in bleeding time blood (Figure 2, bottom panel). The timing of the nadir varied, with the lowest level of thromboxane B₂ recorded occurring in the 2-hour sample for 11 subjects (occurred for 11 for 6-keto-PGF₁α), in the 24-hour sample for nine subjects (10 for 6-keto-PGF₁α), and in the 48-hour sample for three subjects (two for 6-keto-PGF₁α). Smokers tended to have an earlier nadir (six of nine smokers showed the lowest level of thromboxane B₂ at 2 hours after aspirin compared with only four of 14 nonsmokers, p = 0.08). The percent decrease in thromboxane B₂ levels was proportionately greater (86.3 ± 2.8% and 86.9 ± 2.5% at 2 and 24 hours, respectively) than the decrease in 6-keto-PGF₁α levels (64.5 ± 5.5% and 65.8 ± 5.2% at 2 and 24 hours, respectively; p < 0.0001).

The level of thromboxane B₂ in the bleeding time blood at 2 and 24 hours showed a correlation with the length of the bleeding time; subjects with the lowest levels of thromboxane B₂ in their bleeding time blood 24 hours after aspirin ingestion had the longest postaspirin bleeding times (Figure 1). Four subjects (two smokers and two nonsmokers) had more than 1.0 ng/ml thromboxane B₂ in their bleeding time blood 24 hours after aspirin inges-

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**Figure 1.** Plot of bleeding time and thromboxane B₂ levels in bleeding time blood before (●) and 24 hours after (○) 600 mg aspirin.
tion. These four individuals had been high thromboxane producers before aspirin and did show a mean postaspirin decrease in bleeding time blood thromboxane production of 64% and an average decrease in thromboxane production in vitro in response to arachidonic acid and collagen of 81% and 82%, respectively. The postaspirin bleeding times of all four of these individuals were within the normal range.

Recovery of 6-Keto-PGF$_{1\alpha}$ and Thromboxane B$_2$ Production After Aspirin

To assess the rate of recovery of thromboxane B$_2$ and 6-keto-PGF$_{1\alpha}$ after aspirin and to evaluate the influence of sex and smoking, we evaluated those subjects who had the largest decreases in production after aspirin. For this analysis, we included only those subjects who had a more than 90% decrease in thromboxane B$_2$ levels at some point after aspirin (this removed one smoker and two nonsmokers); for 6-keto-PGF$_{1\alpha}$ production, we included those who had a 70% or more decrease in 6-keto-PGF$_{1\alpha}$ production at some point after aspirin (this removed three smokers and five nonsmokers). There was no difference between men and women in recovery from aspirin. However, smokers showed a greater percent of basal thromboxane B$_2$ production 24–72 hours after aspirin than did nonsmokers ($p<0.01$) (Figure 3). There were no significant differences in the rate or extent of recovery between any of the 6-keto-PGF$_{1\alpha}$ groups. Overall, in smokers, there was no significant difference between the percent recovery of 6-keto-PGF$_{1\alpha}$ and thromboxane B$_2$, whereas in nonsmokers, 72 hours after aspirin ingestion, the relative recovery of 6-keto-PGF$_{1\alpha}$ (69±8.7% of basal) was significantly greater than the recovery of thromboxane B$_2$ production (33.9 ±9.4% of basal) ($p<0.05$). When smokers and nonsmokers were grouped together (Figure 4, left panel), recovery of 6-keto-PGF$_{1\alpha}$ occurred more quickly than that of thromboxane B$_2$ ($p<0.05$). When all subjects were included, the level of 6-keto-PGF$_{1\alpha}$ at 72 hours was still significantly lower than basal ($p<0.01$, Wilcoxon's matched pairs test).
Influence of Long-term Aspirin Therapy

There was no significant difference in the length of the bleeding time or the production of thromboxane B2 or 6-keto-PGF1α when diabetics were compared with controls. A small subgroup of diabetics with significant retinopathy did show an increased thromboxane B2 production compared with the other diabetics (3.18±0.35 ng/ml for those with proliferative retinopathy compared with 1.60±0.25 ng/ml for those without, \( p < 0.01 \)). Because the differences between controls and diabetics were not significant (with the exception of thromboxane B2 production 24 hours after 325 mg aspirin where diabetics produced more [0.23±0.06 ng/ml] than controls [0.03±0.004 ng/ml]), we have combined results on controls and diabetics to assess the influence of long-term aspirin therapy.

Both 325 mg aspirin daily and 80 mg aspirin every other day significantly prolonged the bleeding time (from 4.9±0.2 to 8.1±1.2 min with 80 mg every other day and from 5.2±0.2 to 9.6±1.6 min with 325 mg every other day). Similarly, both doses were associated with marked inhibition of thromboxane B2 production after 2 weeks, measured at 24 hours after the last dose (Figure 5). However, with 80 mg aspirin every other day, 6-keto-PGF1α production was more inhibited (82%) than after the daily 325 mg dose (45%) (Figure 5) \(( p = 0.02 \), paired \( t \) test). Though the mean bleeding time after the daily 325 mg dose was correspondingly longer than after 80 mg every other day (see above), this was not statistically significant. Recovery of prostacyclin and thromboxane production after 80 mg aspirin every other day (Figure 4, right panel) was very similar to the recovery after a single dose of 600 mg aspirin (Figure 4, left panel).

The production of 6-keto-PGF1α was statistically higher at 24 hours after the higher daily dose of aspirin than after the lower alternate day aspirin dose. However, the percent of basal production in individuals on the long-term 325 mg daily aspirin was somewhat variable. It was, therefore, critical to repeat this study of 325 mg aspirin daily for 2 weeks to confirm these results and to probe whether there was a real difference between a single dose and a long-term daily dose of 325 mg aspirin. This study (Figure 6) showed no difference between single dose and long-term aspirin for thromboxane B2 with a substantial and highly significant \( p < 0.01 \) decrease in both compared with basal. In contrast with 6-keto-PGF1α, although there was a significant decrease in production 24 hours after the first aspirin dose \(( p < 0.05 \), there was recovery to baseline 24 hours after the 14th daily aspirin dose. Four hours after the 14th aspirin dose, 6-keto-PGF1α production was higher then, but not significantly different from, 24 hours after the 14th aspirin dose (data not shown).

Evaluation of the relation between bleeding time and thromboxane B2 production in bleeding time blood before and after long-term aspirin therapy showed a significant association \(( p = 0.005 \), although the regression line was less steep (Figure 7) than after a single dose of 600 mg aspirin (Figure 2).

Discussion

In measuring the production of thromboxane and prostacyclin in bleeding time blood, we first had to

Figure 4. Plots of recovery of thromboxane \( B_2 \) (■) and 6-keto-prostaglandin (PG)F\(_{1α}\) (●) production in bleeding time blood after a single dose of 600 mg aspirin (left panel) and after 14 days of 80 mg aspirin every other day (right panel).

Figure 5. Bar charts of production of thromboxane \( B_2 \) (Panel A) and 6-keto-prostaglandin (PG)F\(_{1α}\) (Panel B) before aspirin (ASA) and 24 hours after 14 days of daily aspirin therapy in eight diabetics and seven controls. Stippled bars show results with 80 mg aspirin every other day. Hatched bars show results with 325 mg aspirin daily.
decide the best way to express production of these substances. 6-Keto-PGF$_{1a}$ is produced on reasonable evidence from the blood vessel. Because the extent to which vessels are injured by the bleeding time incision is standardized and consistent as far as possible from sample to sample, expression of the level as total production per time interval or picograms per minute is logical. For 6-keto-PGF$_{1a}$ to express the level as nanograms per milliliter of blood is misleading in that equal production from two injured vessel surfaces would appear to be less when blood flow is heavier due to dilution of the 6-keto-PGF$_{1a}$ in the larger volume of blood.

A substantial amount of evidence suggests that thromboxane B$_2$ in bleeding time blood is derived largely from platelets (Reference 6 and Docherty and Gerrard, unpublished data). Because platelets are constituents of the blood, the production of thromboxane may well be proportional to the number of platelets and to the amount of blood flowing past the site of injury. If so, thromboxane production should be expressed as nanograms per milliliter of bleeding time blood. On the other hand, if platelets only produce thromboxane when they have adhered to the site of injury, then it may be only those platelets that become attached to the side of the injured vessel that produce thromboxane. If so, thromboxane production would be better expressed as picograms per minute. Thromboxane production was calculated both ways; in fact, in most patients, the results from the two methods gave reasonably comparable results. There were, however, three patients who were clearly exceptions in that the thromboxane production when expressed at picograms per minute gave a very erratic pattern after aspirin, and in some samples, production calculated as picograms per minute appeared to increase instead of decreasing after aspirin. The reason for the results was traced to a considerable postaspirin increase in blood flow associated with a decreased, but still significant, thromboxane production because these subjects were high thromboxane producers to begin with. When thromboxane production was expressed as nanograms per milliliter, the results were consistent in showing a similar pattern of inhibition after aspirin as the other subjects. Thus, while 6-keto-PGF$_{1a}$ production is logically expressed as picograms per minute, thromboxane B$_2$ appears more appropriately expressed as nanograms per milliliter.

There has been considerable debate over the extent of inhibition of thromboxane B$_2$ and 6-keto-PGF$_{1a}$ formation in vivo in response to aspirin.$^{13-16,25-32}$ Previous studies have used the production of these substances in samples of platelets or blood or blood vessels ex vivo, the latter usually removed from the body at surgery. In some instances, measurements of urinary metabolites have been made.$^{14,16,32}$ In the present study, we have shown that a single dose of 600 mg aspirin taken orally in normal volunteers will inhibit production of both of these compounds in vivo in response to a bleeding time injury, though 24 hours after the
aspirin, thromboxane B₂ was inhibited more than 6-keto-PGF₁α (86% vs. 65%). Recovery of 6-keto-PGF₁α was also faster than recovery of thromboxane B₂ production. This is consistent with an ability of the endothelial cells to make new enzyme and thereby recover production faster, whereas recovery of thromboxane B₂ occurs more slowly because platelets, lacking the ability to make new protein, must wait until new platelets are formed in the bone marrow.

Our finding of 65% inhibition of prostacyclin production in bleeding time blood 24 hours after 600 mg aspirin is consistent with previous data of FitzGerald et al., measuring urinary metabolites and only slightly lower than the 75% inhibition found by Weksler et al. in large vessels. Our data are also consistent with studies on human vascular tissue ex vivo but are at variance with results of Heavey et al. and Vesterqvist. Heavey and coworkers gave volunteers an oral dose of 600 mg aspirin and then assessed bradykinin-stimulated PGI₂ production at 30, 90, and 180 minutes and at 6 hours after aspirin. They found that production of prostacyclin had recovered by 6 hours. Vesterqvist measured a urinary prostacyclin metabolite and failed to find, in three subjects, a significant decrease in urinary 2,3-dinor-6-keto-PGF₁α, although the level after aspirin did decrease to a mean of 70% of the basal level. There are several potential explanations for the differences between our findings and those of Heavey and Vesterqvist. These include the potential contribution to total prostacyclin production (as measured in their studies) from nonvascular sources, variations among different vascular beds (the bleeding time will assess microvascular tissue, and large vessels and other vascular beds could have different responses to aspirin), individual variability (given the small number of subjects evaluated by Vesterqvist), and the possibility (in Heavey’s study) that bradykinin might induce synthesis of vascular cyclooxygenase to overcome the aspirin blockade. Certainly, studies in cultured endothelial cells show that the recovery of cyclooxygenase activity in vitro after aspirin is highly dependent on the culture conditions.

The present study confirms that measurement of production of thromboxane and prostacyclin in a standard vascular injury is possible in vivo in humans. We have shown the effect of a single dose of 600 mg aspirin. Furthermore, we have shown that the production of thromboxane B₂ varies depending on the sex and the smoking status of the individual studied and correlates inversely with the length of the bleeding time. The finding of increased thromboxane B₂ production in long-term smokers is consistent with earlier studies suggesting enhanced thromboxane production in individuals who smoke. We did not find any significant difference in 6-keto-PGF₁α in smokers in contrast to earlier evidence from umbilical cords of infants born to women who smoke showing decreased prostacyclin production. Differences between men and women could represent sex differences in one or more of the enzymes involved in thromboxane production. Alternatively, the higher mean hematocrit in men and associated shorter bleeding time may result in enhanced thrombin production or increased adenosine diphosphate availability leading to a secondarily increased thromboxane formation.

Part 2 of the present study represents the first attempt to measure in vivo prostacyclin and thromboxane production in human diabetes in response to a standard vascular injury. In contrast to expectations that thromboxane is increased and prostacyclin decreased in diabetes, the results do not show a difference in thromboxane production between the diabetic subjects and their matched controls. Our results should be interpreted cautiously, given the small sample sizes. Most of the earlier studies showing increased thromboxane production in diabetics were done with labeled or unlabeled arachidonic acid precursor; when serum thromboxane B₂ has been assessed in diabetics, it has been decreased or normal compared with controls. A decrease in the amount of arachidonic acid in the platelet phospholipid pool could explain why studies with exogenous arachidonic acid show high production while evaluation of endogenous production in serum has shown low levels.

Contrary to expectations, there was a substantial amount of prostacyclin production after 2 weeks of 325 mg aspirin compared with 2 weeks on the low-dose aspirin. Studies of prostacyclin and thromboxane 24 and 48 hours after the low-dose aspirin were very similar to recovery of prostacyclin and thromboxane production after a single dose of 600 mg aspirin. The anomaly in our results is, therefore, the relatively high prostacyclin production shown in individuals after 2 weeks of 325 mg aspirin. An additional study confirmed that 6-keto-PGF₁α production was significantly inhibited after the first dose of 325 mg aspirin and that production was less inhibited 14 days after 325 mg aspirin.

A possible explanation for the increased vascular prostacyclin production on daily 325 mg aspirin compared with 80 mg aspirin every other day is that the former is associated with rapid synthesis of new vascular cyclooxygenase. If so, it must occur by 4 hours after the 325 mg aspirin because 4 hours after the 14th aspirin dose, the production of prostacyclin was not decreased compared with basal production. The result suggests that aspirin, at a dose of 325 mg daily, may fail to inhibit prostacyclin synthesis in microvascular tissue. The residual salicylate in the circulation on long-term therapy might be sufficient to block the inhibitory effect of aspirin, a phenomenon that had been shown to occur in previous studies. Consistent with this, two individuals who had very low levels of prostacyclin 24 hours after the 14th daily dose of 325 mg aspirin informed us after the study had been completed that they had
been erratic in taking the aspirin dose. They may not have had enough salicylate in their circulation to block the effect of aspirin. Also consistent with the present study is evidence of De Gaetano et al.\textsuperscript{48} that aspirin inhibition of vascular cyclooxygenase can be selectively blocked by salicylate under conditions where the platelet cyclooxygenase remains inhibited. It is probable that there is a relatively fine balance between salicylate and aspirin such that sparing of vascular cyclooxygenase may not occur with higher aspirin doses such as the 600 mg daily. This may explain why FitzGerald et al.,\textsuperscript{14} in studies on four subjects, provided a suggestion of less inhibition of prostacyclin production on 325 mg aspirin daily than with either lower or higher doses. Three of four individuals studied at 160 mg aspirin daily and four of four at 650 mg daily but only one of four at 325 mg daily showed more than 65% inhibition of prostacyclin production, measured as the urinary metabolites.

It is of interest that two of the major trials that have shown a positive benefit of aspirin have used a dose of 325 mg daily or 325 mg every other day; some studies with higher doses of aspirin have shown benefit, whereas others have not.\textsuperscript{49–52} It is possible that the results reported here represent some artifact of the way in which we measure 6-keto-PGF\textsubscript{1α}. We believe we have taken the appropriate precautions to make this unlikely, including evaluation of some samples by HPLC separation of 6-keto-PGF\textsubscript{1α} before subjecting them to ELISA assay. But, given the previous history of artifactual results with radioimmunoassays and the similarity of ELISAs to radioimmunoassays, we are unable to completely exclude this possibility. It will, therefore, be important to have these findings confirmed in additional studies because our results appear to differ from those of Weksler,\textsuperscript{53} who measured prostacyclin ex vivo in aorta and saphenous vein from individuals receiving 325 mg aspirin daily.

The correlation between the length of the bleeding time and the production of thromboxane in bleeding time blood was evident after long-term aspirin doses (Figure 7) as it was after a single aspirin dose (Figure 1). However, the regression was significantly flatter. There are several possible reasons for this. The first study used a much higher aspirin dose. The first study used thromboxane-prostacyclin measurements in the first 5 minutes after the bleeding time incision was made, whereas the study of long-term aspirin used production of thromboxane after only the first 4 minutes. Different individuals performed the bleeding times in the two studies, and the results might be explained by a slight difference in technique. It may be that in the diabetics and, coincidentally, the accompanying controls, changes in other factors in the blood or blood vessels can explain the results because clearly there are other factors besides thromboxane that are involved in determining the length of the bleeding time.

In conclusion, we have demonstrated that measurement of prostacyclin and thromboxane production in bleeding time blood can be a useful technique for assessing the production of these substances in vivo. We have demonstrated expected responses to single doses of aspirin that are very consistent with previous findings in the literature. We have also, for the first time, provided evidence that long-term therapy with daily 325 mg aspirin can be associated with a sparing of inhibition of prostacyclin synthesis.

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