Endogenous Biosynthesis of Prostacyclin and Thromboxane and Platelet Function during Chronic Administration of Aspirin in Man

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ABSTRACT To assess the pharmacologic effects of aspirin on endogenous prostacyclin and thromboxane biosynthesis, 2,3-dinor-6-keto $PGF_{1\alpha}$ (PGI-M) and 2,3-dinor-thromboxane B_2 (Tx-M) were measured in urine by mass spectrometry during continuing administration of aspirin. To define the relationship of aspirin intake to endogenous prostacyclin biosynthesis, sequential urines were initially collected in individuals prior to, during, and subsequent to administration of aspirin. Despite inter- and intra-individual variations, PGI-M excretion was significantly reduced by aspirin. However, full mass spectral identification confirmed continuing prostacyclin biosynthesis during aspirin therapy. Recovery of prostacyclin biosynthesis was incomplete 5 d after drug administration was discontinued. To relate aspirin intake to indices of thromboxane biosynthesis and platelet function, volunteers received 20 mg aspirin daily followed by 2,600 mg aspirin daily, each dose for 7 d in sequential weeks. Increasing aspirin dosage inhibited Tx-M excretion from 70 to 98% of pretreatment control values; platelet TxB_2 formation from 4.9 to 0.5% and further inhibited platelet function.

An extended study was performed to relate aspirin intake to both thromboxane and prostacyclin generation over a wide range of doses. Aspirin, in the range of 20 to 325 mg/d, resulted in a dose-dependent decline in both Tx-M and PGI-M excretion. At doses of 325–2,600 mg/d Tx-M excretion ranged from 5 to 3% of control values while PGI-M remained at 37–23% of control. 3 d after the last dose of aspirin (2,600 mg/d) mean Tx-M excretion had returned to 85% of control, whereas mean PGI-M remained at 40% of predosing values. Although the platelet aggregation response (T_{max}) to ADP ex vivo was inhibited during administration of the lower doses of aspirin the aggregation response returned to control values during the final two weeks of aspirin administration (1,300 and 2,600 mg aspirin/d) despite continued inhibition of thromboxane biosynthesis.

These results suggest that although chronic administration of aspirin results in inhibition of endogenous thromboxane and prostacyclin biosynthesis over a wide dose range, inhibition of thromboxane biosynthesis is more selective at 20 than at 2,600 mg aspirin/d. However, despite this, inhibition of platelet function is not maximal at the lower aspirin dosage. Doses of aspirin in excess of 80 mg/d resulted in substantial inhibition of endogenous prostacyclin biosynthesis. Thus, it is unlikely that any dose of aspirin can maximally inhibit thromboxane generation without also reducing endogenous prostacyclin biosynthesis. These results also indicate that recovery of endogenous prostacyclin biosynthesis is delayed following aspirin administration and that the usual effects of aspirin on platelet function ex vivo may be obscured during chronic aspirin administration in man.

INTRODUCTION

The use of aspirin as an analgesic, antiinflammatory, and antipyretic agent has evolved empirically in ther-

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apeutic practice. The more recent interest in aspirin as a platelet-inhibiting agent has derived from the observation that aspirin prolonged the bleeding time and inhibited platelet aggregation ex vivo (1). It is now appreciated that aspirin has several actions potentially relevant to platelet function and that these may be mutually antagonistic. Furthermore, the relative prominence of these actions may be highly dependent on the dose of aspirin administered.

Inhibition of human platelet aggregation ex vivo by aspirin is seen after doses as low as 20 mg daily (2). These low doses inhibit the cyclooxygenase enzyme of the platelet and it is likely that this explains the aggregation defect. Aspirin irreversibly acetylates this enzyme and thereby inhibits the capacity of the platelet to synthesize thromboxane A_2, which can induce irreversible platelet aggregation (3). This is generally regarded as a beneficial action of aspirin. However, the cyclooxygenase pathway in other tissues leads to the formation of prostaglandins that may themselves inhibit platelet activation. Recent attention has focussed on prostacyclin, a vasodilator and potent inhibitor of platelet aggregation formed in vascular endothelium (4). It has been suggested that tissue sensitivity to cyclooxygenase inhibition by aspirin is highly variable and thus aspirin may selectively alter the capacity to synthesize endogenous prostaglandins (5, 6).

Although the enzyme remains inhibited for the lifetime of exposed platelets, enzyme turnover permits functional recovery in the vascular endothelium in vitro (5). In addition, platelet cyclooxygenase may be more sensitive than the vascular enzyme to aspirin inhibition in vitro (6), suggesting that it might be possible to achieve a dose-dependent, selective inhibition of thromboxane biosynthesis by aspirin in platelets, while leaving prostacyclin synthesis by vascular endothelium relatively intact. We have sought to establish the comparative effects of aspirin on endogenous thromboxane and prostacyclin synthesis in normal individuals.

METHODS

Study design

Three investigations are described. Initially, serial 24-h urine collections were performed by two male volunteers (aged 31 and 32 yr, wt 74 and 76 kg) for analysis of urinary 2,3-dinor-6-keto-$PGF_{1\alpha}$ (PGI-M).[1] This was done before, during, and subsequent to ingestion of aspirin capsules 650 mg four times per day. Full mass spectral analysis was performed on pooled urines obtained before and during aspirin administration to confirm continuing prostacyclin synthesis during aspirin therapy.

In another investigation five different male volunteers (aged 28–33 yr; wt 78±7 kg) received aspirin in daily doses of 20 and 2,600 mg (650 mg four times daily), each dose for 7 d, in successive weeks. Urine was obtained for determination of 2,3-dinor-thromboxane B_2 (Tx-M) and blood drawn for platelet function studies on the day prior to dosing and on the final day of each dosing period. Blood samples were obtained following an overnight (12-h) fast and were drawn 1 h after the morning doses during the treatment period.

In the final study, five different male volunteers received aspirin capsules in oral doses of 20, 40, 80, 160, 325, 650, 1,300, and 2,600 mg daily. Each dose was given for 7 d and ascending doses were administered in consecutive weeks. Aspirin was administered as a single daily dose except the two highest doses, which were given as 650 mg twice per day and 650 mg four times per day. Highly motivated research workers were selected as volunteers to enhance compliance. Additionally, compliance was assessed by capsule count. 24 h collections of urine for prostacyclin and thromboxane metabolite determination were performed on the day prior to the first dose of aspirin, on the final day of each dosage period and for the 3 d after the last dose of aspirin. Prostaglandin metabolites were adjusted to creatinine excretion to reduce the possibility that incomplete collection of urine might have confounded the results. Urinary values of creatinine did not significantly alter during the course of any of the studies described.

Biochemical analyses

Quantification of Tx-M. Both Tx-M and PGI-M were measured by stable isotope dilution assay using gas chromatography-mass spectrometry. Briefly, the assay for Tx-M required the addition of 100 ng of a combined tritium and deuterium labeled internal standard to a 10% aliquot of a 24-h collection of urine. The urine was acidified to pH 3.2 and absorbed onto a column of Amberlite XAD-2 and the metabolite eluted with ethanol. After solvent evaporation the residue was dissolved in pH 3.2 buffer and partitioned into ethyl acetate. The ethyl acetate extract was then applied to an open column of silicic acid and the metabolite eluted with ethyl acetate. The sample was then subjected to reversed phase high-pressure liquid chromatography (HPLC) on a fatty acid analysis column (Waters Associates, Milford, MA) under isocratic conditions with the solvent water/acetonitrile/benzene/acetic acid (400:100:1:0.5) (vol/vol/vol/vol), flow rate 2 ml/min, 2-ml fractions. The metabolite was then converted to a methyl ester by treatment with excess ethereal diazomethane and to a tert-butyldimethylsilyl ether derivative by treatment with tert-butyldimethylchlorsilane/imidazole in dimethylformamide (Applied Science Laboratories, Waltham, MA) for 12 h at 45°C.

Quantification of PGI-M. The assay for PGI-M follows similar principles and has been described in detail elsewhere (7). Briefly, the urine was initially acidified to pH 3.2 and the metabolite extracted with dichloromethane using Clin-Elute. The organic phase was then back-extracted with pH 8.0 buffer. The organic phase residue obtained after solvent evaporation was dissolved in pH 8.0 buffer and extracted with ethyl acetate. The ethyl acetate extract was discarded and the aqueous phase acidified to pH 3.2 and extracted with dichloromethane. The dichloromethane extract residue

[1] *Abbreviations used in this paper*: GC-MS, gas chromatography-mass spectrometry; HPLC, high pressure liquid chromatography; PGI-M, 2,3-dinor-6-keto-$PGF_{1\alpha}$; PPP, platelet-poor plasma; PRP, platelet-rich plasma; T_{max}, platelet aggregation response, Tx-M, 2,3-dinor-thromboxane B_2.

obtained after solvent evaporation was then treated with pyridine/water/triethylamine (10:10:10) (vol/vol/vol) and after solvent evaporation the metabolite is converted to a methyl ester by treatment with excess ethereal diazomethane for 5 min, converted to a methyloxime derivative by treatment with 2% methoxyamine HCL in pyridine for 2 h at 70°C, and a trimethylsilyl ether derivative by treatment with *N,O*-bis(trimethylsilyl)-trifluoroacetamide for 30 min at 70°C.

Quantitation by gas chromatography-mass spectrometry. These were performed on a Hewlett-Packard Corp., Palo Alto, CA (HP) 5981A instrument interfaced with a HP 5710A gas chromatograph. The injection port temperature was 250°C. Chromatography was on a 2-mm i.d. glass column with a length of 2 M packed with 3% SP-2100 on 100/130 mesh supelcoport (Supelco, Inc., Bellefonte, PA). The mass spectrometry was controlled by a high pressure 5947A multiple ion detector, which allowed up to four ions to be sequentially monitored. Electron impact ionization (70 eV) took place at 200°C source temperature.

The ion peak ratio in the Tx-M assay was calculated from the M + −57 ion m/z 641 for endogenous Tx-M and m/z 644 for the deuterium-labeled internal standard. The ion peak ratio for the PGI-M assay was calculated from the M + −31 ions, m/z 570 for endogenous PGI-M and m/z 574 for the deuterium-labeled internal standard.

Identification of PGI-M in urine. We wished to confirm that what was measured as PGI-M by selected ion monitoring of urine obtained from patients receiving the highest dose of aspirin corresponded to continuing biosynthesis of prostacyclin and not an assay blank. Consequently, batches of four 24-h urine collections obtained before and four collections obtained during dosage of two subjects with aspirin 2,600 mg/d were pooled and spiked with 100 ng $^{2}H_4$ PGI-M per 500 ml urine. The total volume of each pooled sample varied between 4 and 5 liters. After equilibration of the deuterated internal standard with the endogenous metabolite and a short period of treatment with alkali (pH 9 for 15 min), the urine was acidified to pH 3 with concentrated HCl and saturated with sodium chloride. The sample was then extracted twice with one-half volume of dichloromethane. The organic phases were combined and washed three times with an equal volume of 50 mM sodium borate pH 8. The aqueous phases were discarded and the dichloromethane was taken to dryness on a rotary evaporator. The residue was dissolved in 5 ml pyridine and then treated with 100 ml 50 mM sodium borate pH 8 for 15 min. The aqueous phase was subsequently washed twice with 500 ml ethyl acetate (which was discarded) and then acidified to pH 3 with HCl. The metabolite was extracted with dichloromethane (three times 5 vol) and the pooled organic phases were washed once with 30 ml distilled water and then taken to dryness on a rotary evaporator. At this stage the sample was sufficiently pure that it could be dissolved in 0.5 ml of water/acetonitrile/acetic acid 80:20:0.1 (vol/vol/vol). The specimen was purified by high-pressure liquid chromatography (HPLC) using a Waters C_{18} μBondapak analytical column and the aforementioned water/acetonitrile mixture as the eluting solvent. The retention volume of the metabolite was established on two consecutive prior runs using authentic radiolabeled PGI-M. The column and injection port were then carefully rinsed and the column effluent appearing at the appropriate elution volume (63–93 ml) was collected. The metabolite was extracted into ethyl acetate and subsequently further purified by thin-layer chromatography (5 × 20 cm plate of silica gel 60 (Merck, Inc., Rahway, NJ), in a solvent system of ethyl acetate with 0.5% glacial acetic acid, retardation factor (R_f) authentic PGI-M = 0.32). One of the four samples was converted to the methoxime derivative and purified again by reversed-phase HPLC under the same conditions as before (elution volume of the methoxime derivative 28-32 ml) but this procedure was omitted with the other three samples in order to avoid the 50% loss of material associated with each chromatography step. The methyl ester methoxime trimethylsilyl ether derivative of the purified urinary extract was prepared and subjected to analysis by GC-MS using a capillary column of OV-101 as described below.

GC was performed on a 23-m vitreous silica WCOT capillary column (0.2 mm i.d.) coated with OV-101, (Scientific Glass Engineering, Inc., Houston, TX). The oven temperature was 240°C and the helium flow rate was ~1 ml min^{-1}. The column extended through the interface directly into the MS ion source, and 25,000 theoretical plates were calculated for this compound. The injection system was a Ross type sliding needle (all glass) (Charles Ross & Son Co., Hauppauge, NY); vaporization chamber temperature was 260°C.

Mass spectra were acquired on a Ribermag 10-10B quadrupole MS interfaced with a Girdel model 31 GC. The system was controlled by an RDS 1000 data system (all from Nermag, Inc., Santa Clara, CA).

Ionization was by electron impact at 70 eV. Spectra were continuously acquired, scanning from amu 100 to 650 with 7 ms integration times per amu.

Platelet function studies

Platelet aggregation studies. These were done according to the method of Born (8) using a Payton Dual Channel Aggregometer (Payton Associates, Buffalo, NY). The platelet count in platelet rich plasma was adjusted to 300,000/min^3 prior to the aggregation studies using platelet-poor plasma prepared from the autologous blood sample spun down at 8,000 *g* for 2 min. The aggregometer base line (10% light transmission) was set using platelet rich plasma and buffer added in concentration equivalent to the test system. Full transmission (100%) was set using platelet-free plasma. Aggregation was measured using percent maximum transmission attained at 6 min after addition of aggregating agent. The slope value (9), which represented the change along a line tangent to the sharpest increase in light transmission, was also measured. Aggregating materials used were ADP (Sigma Chemical Co., St. Louis, MO), collagen (Biodata, Hatboro, PA) and epinephrine (Parke-Davis, Inc., Detroit, MI). They were diluted in 0.01 M phosphate-buffered saline, pH 7.4, and freshly prepared each study day. Blood samples were drawn at the same time on each study day from subjects who were fasting. Blood was drawn by the two-syringe method into a second syringe that contained 0.11 M citrate buffer, pH 5.0, to make a final blood to buffer ratio of 9:1. The blood was processed as described previously (10).

Release of [^{3}H]serotonin from platelets. This was measured as described (10). Briefly, platelet-rich plasma was incubated at room temperature with 0.05 μCi/ml of [^{3}H]serotonin (5-hydroxy G-[^{3}H] tryptamine creatinine sulfate, Amersham Corp., Arlington, IL) for 4 min. Incorporation of [^{3}H]serotonin into the platelets ranged from 82 to 90%. The amount of released serotonin was determined after incubation in an aggregometer cuvette for 10 min with constant stirring at 37°C and in the presence of imipramine (Ciba-Geigy Corp., Summit, NJ) at a concentration of 0.15 μg/ml to prevent reuptake of [^{3}H]serotonin. Samples of platelet-rich plasma (PRP) were taken for total radioactivity counts, the incubation mixture containing PRP with agonist

or buffer were centrifuged at 8,000 *g* for 2 min and the platelet-poor plasma (PPP) assayed for released radioactivity. The release of [^{3}H]serotonin was calculated according to the formula:

% release

= CPM in PPP with agonist-CPM in PPP with buffer/

CPM in PRP × 100.

Thromboxane B_2 radioimmunoassay

Thromboxane B_2 was measured in PRP ex vivo using a highly specific antibody generously supplied by Dr. Fitzpatrick of the Upjohn Company (11) and a procedure previously described (12).

Bleeding time

The forearm template bleeding time (Simplate General Diagnostics, Morris Plains, NJ) was measured before and 1 h following administration of aspirin.

Statistical analysis

Data were analyzed by nonparametric methods (13, 14) thereby avoiding assumptions as to the distributions of the variables involved. The data in the 8- and 2-wk studies were subjected to one-way analysis of variance by the method of Kruskall and Wallis and subsequent pairwise comparison with control values by the Lord U test. The unpaired data in Fig. 1 were analyzed by the Mann-Whitney U test. The paired samples illustrated in Fig. 6 were compared by the Wilcoxon signed-ranks test. Two-tailed probabilities were used throughout the analysis.

RESULTS

Inhibition of prostacyclin synthesis. PGI-M excretion was quantitated in sequential 24-h periods in two subjects who received 2,600 mg aspirin/d (650 mg four times daily) for 1 wk (Fig. 1). Besides interindividual variation, PGI-M excretion varied within both individuals (coefficients of variation 13 and 23%) before dosing with aspirin. However, a significant reduction in PGI-M excretion occurred in both cases during aspirin administration. Collections were not performed by subject 1 in the early dosing period, but the degree of inhibition of PGI_2 biosynthesis appeared to remain constant in subject 2 during aspirin administration. In both subjects recovery of PGI_2 biosynthesis was delayed.

To determine whether the selected ion monitoring traces obtained during the aspirin dosing period were

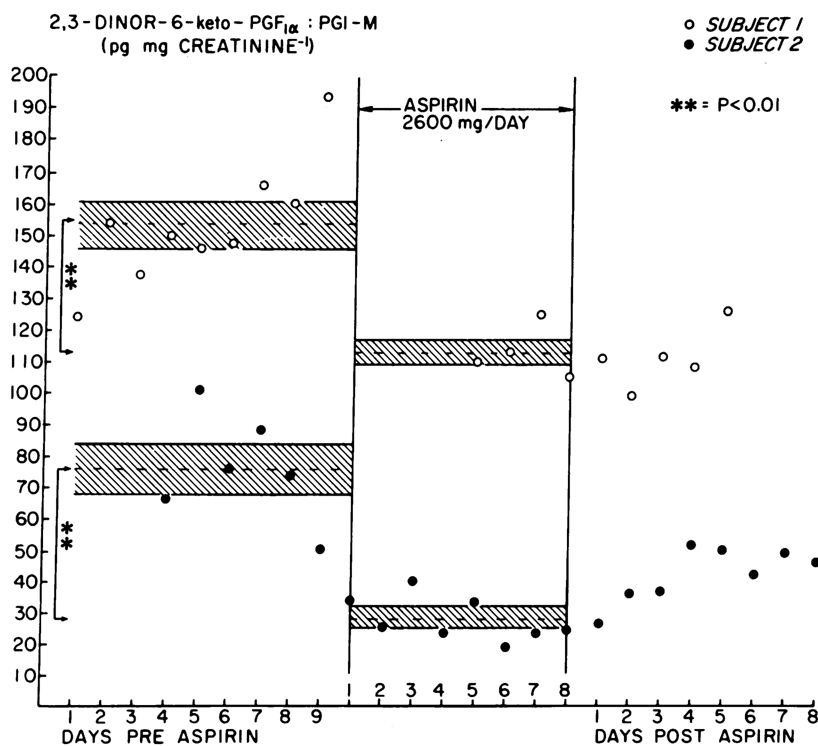


FIGURE 1 Urinary PGI-M in two subjects before, during, and subsequent to aspirin (2,600 mg/d × 8) administration. Predosing values of PGI-M were significantly reduced by aspirin in both subjects ($P < 0.01$). Urine collections by subject 1 was not performed in the first 4 d of dosing with aspirin. Mass spectra derived from subject 1 are illustrated in Fig. 2.

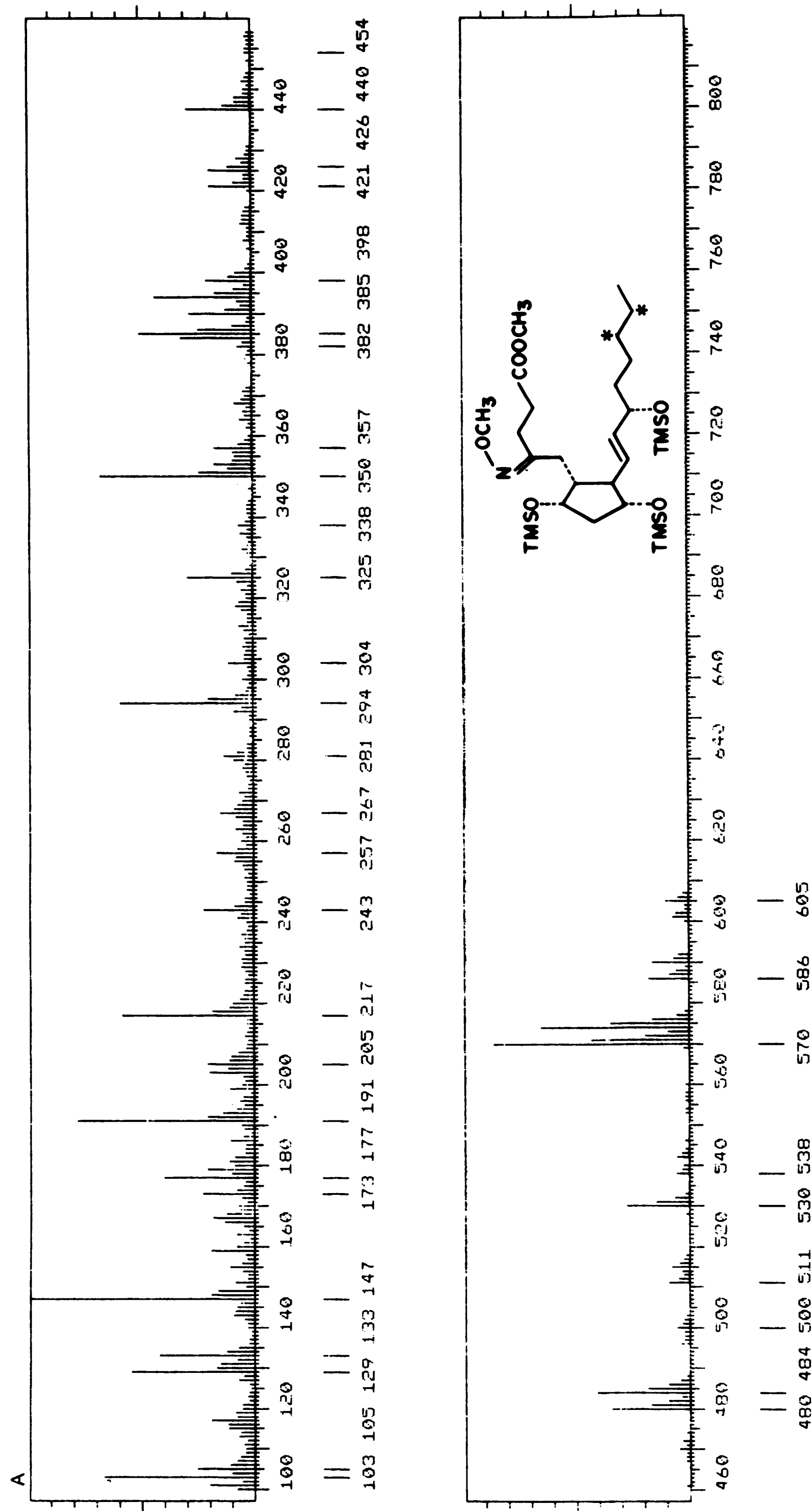


FIGURE 2A Mass spectrum of a mixture of authentic unlabeled and deuterium-labeled PGI-M derivatized as the methyl ester, methoxime, trimethylsilyl ether.

indicative of continuing prostacyclin biosynthesis rather than an assay blank, pooled urines were obtained from two individuals who received aspirin, 2,600 mg/d for 8 d. A known amount of deuterated PGI-M was added to each sample and then the metabolite was extracted, purified, and the mass spectrum recorded. Fig. 2A shows the mass spectrum of a mixture of authentic unlabeled and deuterated dinor-6-keto-$PGF_{1\alpha}$. Fig. 2B shows the spectrum recorded on the endogenous dinor-6-keto-$PGF_{1\alpha}$ and the deuterated internal standard after purification from pooled 24-h urines collected from one of the subjects during daily administration of 2,600 mg aspirin. This spectrum is essentially identical to that shown in Fig. 2A.

Inhibition of thromboxane synthesis and platelet function. To examine the effects of high and low doses of aspirin on indices of thromboxane generation and platelet function, five volunteers received aspirin 20 and 2,600 mg, each dose daily for 7 d in successive weeks. Whereas mean Tx-M excretion fell to 33±9% of control values (273±65 pg mg creatinine^{-1}) on 20 mg/d aspirin, mean immunoreactive thromboxane B_2 formation by platelets stimulated with thrombin (5 U) fell to 4.9±2% of control (44.5±26 ng ml^{-1}). Both Tx-M (3±1% of control) and platelet thromboxane B_2 (0.5±0.02% of control) were essentially maximally inhibited by the higher dose of aspirin. Despite substantial inhibition of thrombin-stimulated platelet thromboxane B_2 formation by the lower dose of aspirin both the platelet aggregation response (T_{max}) and serotonin release response to a variety of agonists (Fig. 3A,B) were further inhibited when the dose of aspirin was increased to 2,600 mg/d. Bleeding time increased slightly from predosing values (5.2±0.7 min) in four of the five subjects after 7 d administration of aspirin 20 mg/d but this prolongation only attained statistical significance ($P < 0.01$) on the 7th d of aspirin 2,600 mg/d (8.2±1.6 min).

Multiple dose study. The effects of aspirin doses intermediate between 20 and 2,600 mg/d on PGI-M and Tx-M excretion were assessed in five volunteers. Before dosing the volunteers excreted 328±96 pg mg creatinine^{-1} of PGI-M and 246±52 pg mg creatinine^{-1} of Tx-M. Individual values of urinary metabolite excretion during the study are expressed as a percentage of control values in Figs. 4 and 5. Considerable interindividual variability was noted in both PGI-M excretion (50–570 pg mg creatinine^{-1}) and Tx-M excretion (90–368 pg mg creatinine^{-1}) in these studies. Expression of results as a percentage of individual control values tends to minimize this factor. One subject was excluded in the 4th wk of the study following the development of an intercurrent illness. Mean (±SEM) data for the remaining four subjects are included in both figures.

Chronic dosing with aspirin in the range of 20 to

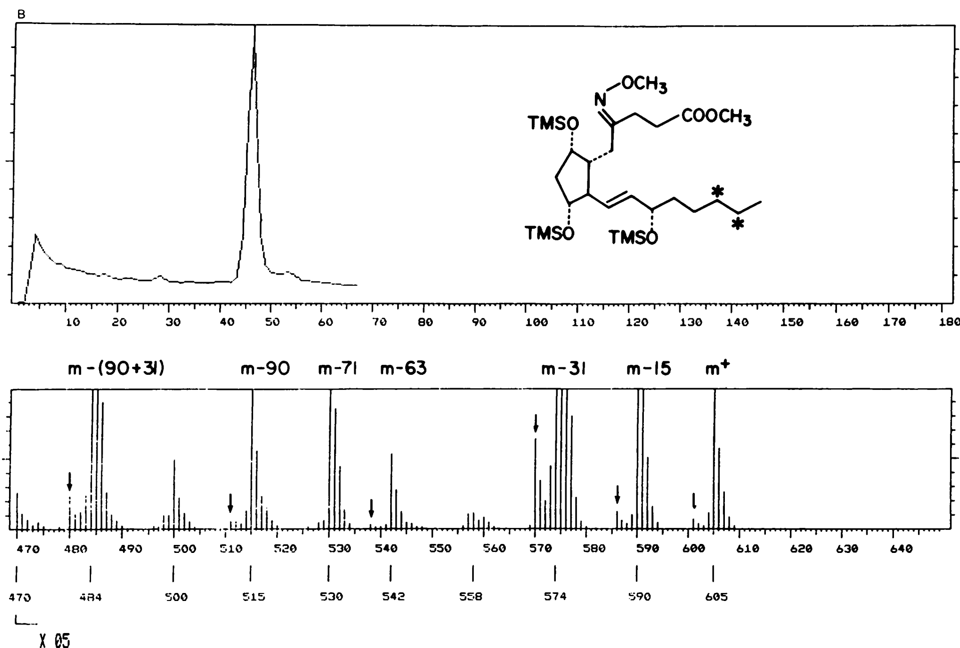
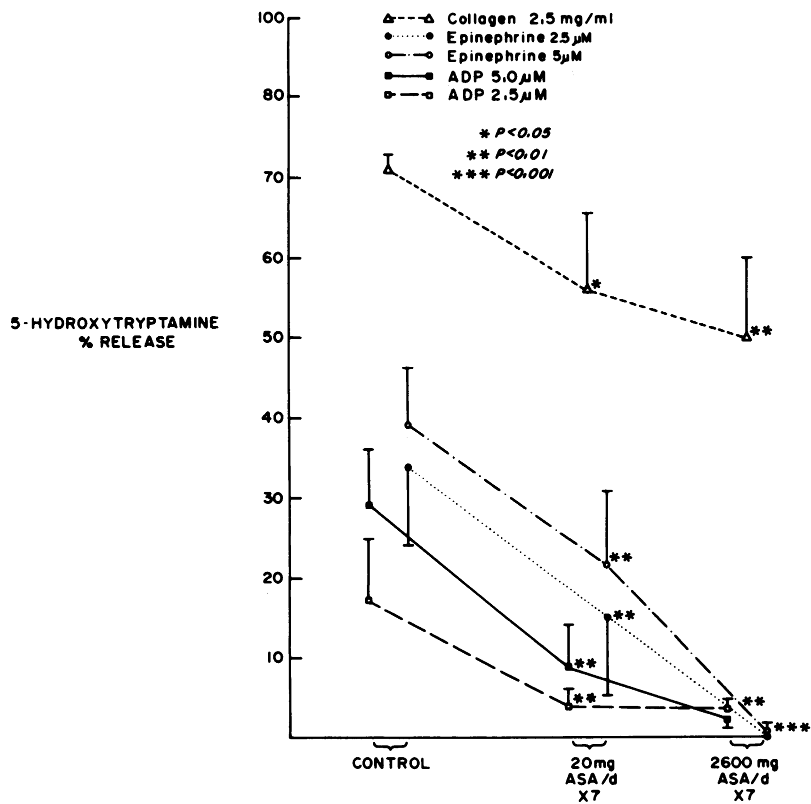


FIGURE 2B Mass spectrum of material isolated from the urine of subject 1 (Fig. 1) receiving 2,600 mg aspirin daily. Deuterium-labeled PGI-M was added to the urine that was purified as described under Methods. The total ion current is illustrated in the upper panel of the figure.



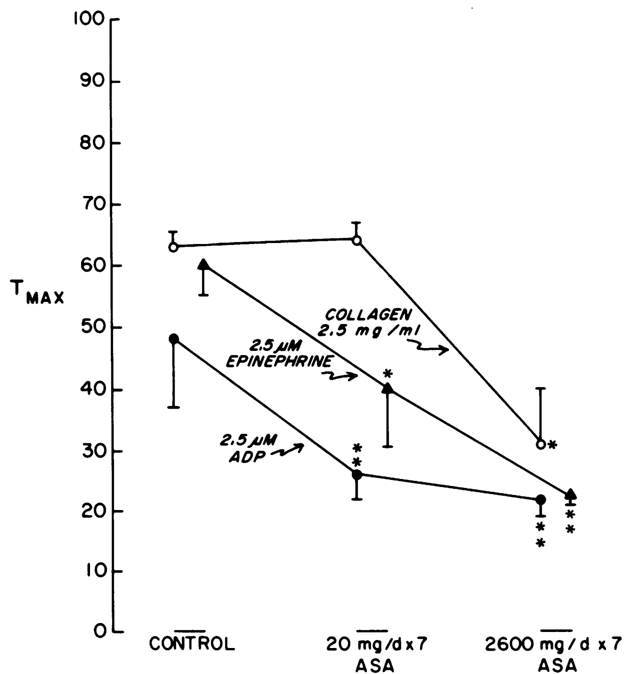


FIGURE 3 A. Platelet aggregation response to collagen (2.5 mg ml^{-1}) epinephrine (2.5 μM) and ADP (2.5 μM) ex vivo in normal volunteers prior to and on the 7th d of treatment with aspirin 20 mg d^{-1} and 2,600 mg d^{-1}. Differences are expressed from predosing control values. B. Platelet aggregation response. Percentage serotonin (5-HT) released from PRP after exposure to collagen (2.5 mg ml^{-1}), epinephrine (2.5 and 5.0 μM), and ADP (2.5 and 5.0 μM) in volunteers before aspirin and on the 7th d of dosage with aspirin 20 mg d^{-1} and 2,600 mg d^{-1}. Differences are expressed from predosing control values. $^{*}P < 0.05$, $^{**}P < 0.01$.

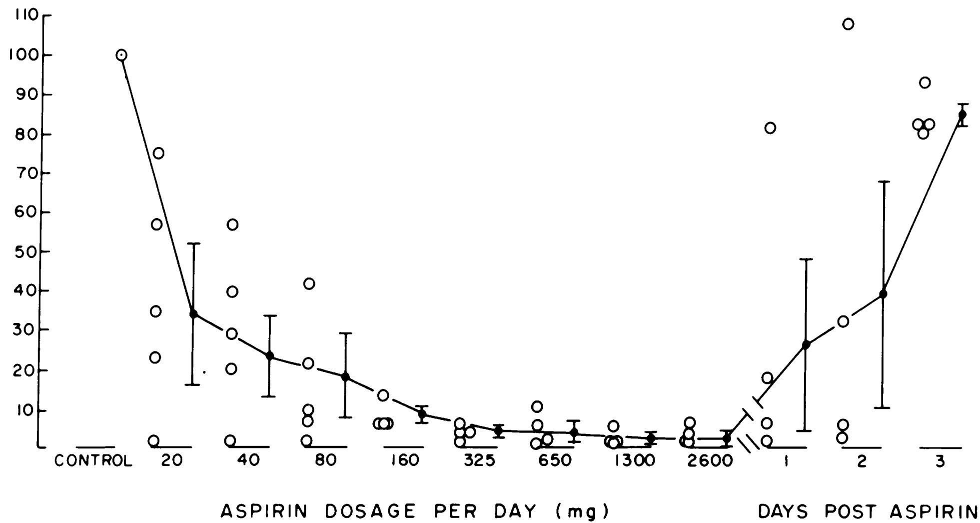


FIGURE 4 Urinary Tx-M expressed as a percentage of control values, during aspirin administration. The values corresponding to each dosage was obtained on the 7th d of each dosage period. Doses were administered in successive weeks. One subject completed only 3 wk of the study (see text). Mean±SEM data are included for the remaining subjects.

325 mg/d resulted in a dose-related decrease in excretion of both PGI-M and Tx-M. At 20 mg/d, a dose of aspirin that significantly inhibits the platelet release reaction (see above) PGI-M excretion had fallen to 70.6±12.5% and Tx-M excretion to 33.1±15.7% of control values. The variance of both parameters declined as aspirin dosage was increased. At doses of 325–2,600 mg/d aspirin, mean Tx-M excretion ranged from 4.7 to 3.3% of control. Mean PGI-M ranged from 23 to 37% of control over the corresponding dose range (Figs. 4 and 5). When the data in these four subjects during aspirin therapy are compared with that before dosing without adjustment to individual control values, the fall in PGI-M attained statistical significance ($P < 0.05$) at doses of aspirin of 160 mg/d and greater. Tx-M fell significantly ($P < 0.05$) from control values

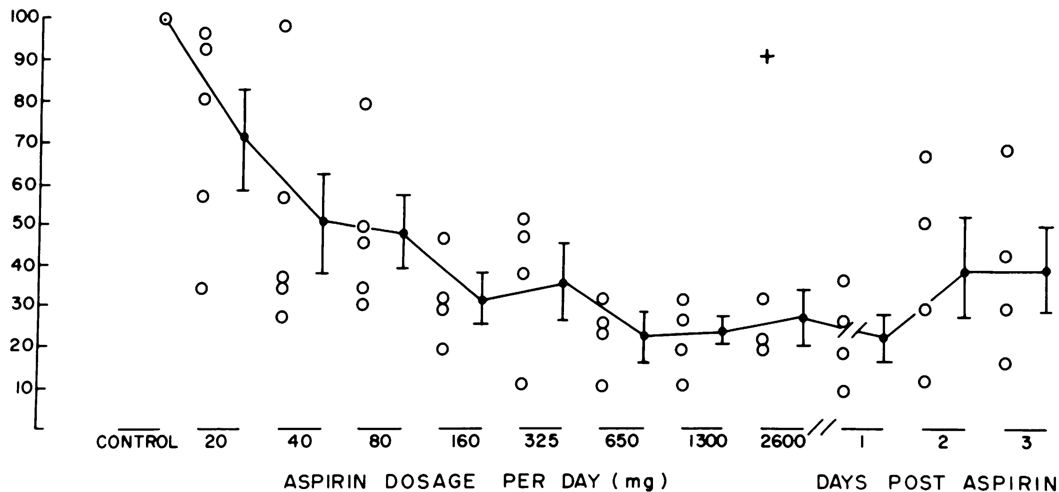


FIGURE 5 Urinary PGI-M, expressed as a percentage of control values, during aspirin administration. The values corresponding to each dosage were obtained on the 7th d of each dosage period. Doses were administered in successive weeks. One subject completed only 3 wk of the study (see text). Mean±SEM data are included for the remaining subjects. The unexpectedly high result indicated by the + symbol, appeared to have been due to an interfering substance. Reanalysis of the sample did not provide ion traces of sufficient technical quality to resolve this question.

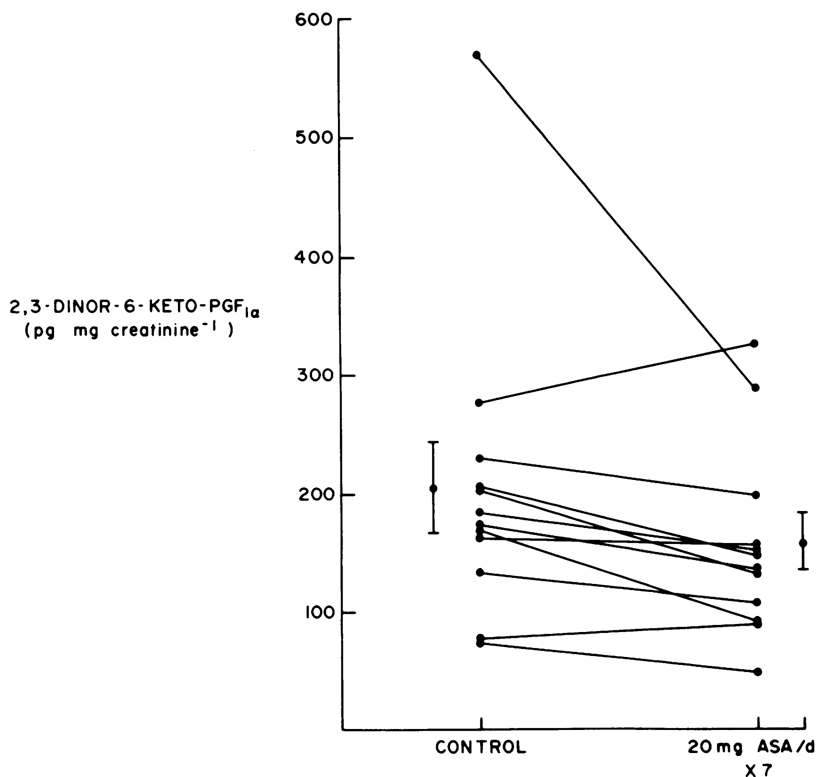


FIGURE 6 Urinary PGI-M before dosing (control) and on the 7th d of dosage with aspirin 20 mg/d in 12 volunteers. PGI-M excretion on aspirin (159 ± 25 pg mg creatinine^{-1}) was not significantly ($P > 0.05$) reduced compared with control values (204 ± 39 pg mg creatinine^{-1}).

at doses of 80 mg/d and above. Thus, although chronic aspirin therapy led to a reduction in excretion of both metabolites, inhibition of thromboxane biosynthesis was more marked than that of prostacyclin at all doses tested. Urinary prostaglandin metabolite excretion was also measured in the 3 d after chronic aspirin therapy. Although the rate of recovery was variable, Tx-M excretion had attained 85±3% of control values 3 d after dosing (Fig. 4). By contrast, PGI-M excretion remained depressed at 40±11% of control values (Fig. 5).

12 patients had PGI-M excretion measured before and on the 7th d of administration of aspirin 20 mg/d (Fig. 6). Although PGI-M fell in 10 of the 12 subjects, the variance was substantial and the decline from pretreatment values (204±39 pg mg creatinine^{-1}) was not statistically significant during administration of this dose of aspirin (159±25 pg ng creatinine^{-1}; $P > 0.05$).

T_{max} to ADP ex vivo was reduced in the early weeks of the multiple dose study. However, in the final 2 wk of the study (doses of 1,300 and 2,600 mg aspirin/d) the aggregation response returned despite continuing inhibition of thromboxane biosynthesis (Fig. 7). This effect was evident at all three doses of ADP (2.5, 5, and 10 μM) used and in all subjects studied. No alteration in the slope of the primary wave of the aggregation response to ADP was observed during aspirin administration (Fig. 8). The platelet response to other agonists was not investigated in this section of the study.

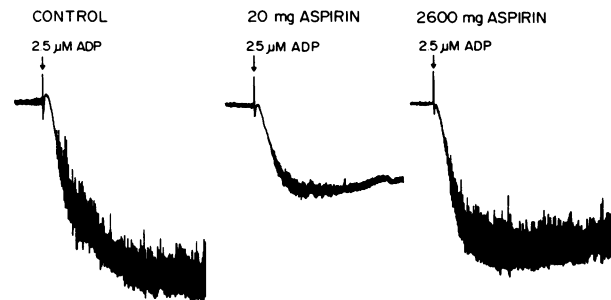


FIGURE 7 Platelet aggregation response to ADP (2.5 μM) of a volunteer in the 8-wk study. Although the change in optical density (T_{max}) was reduced by the end of the first week of aspirin administration (20 mg d^{-1}) it had returned to predosing values by the final day of dosing (2,600 mg d^{-1}).

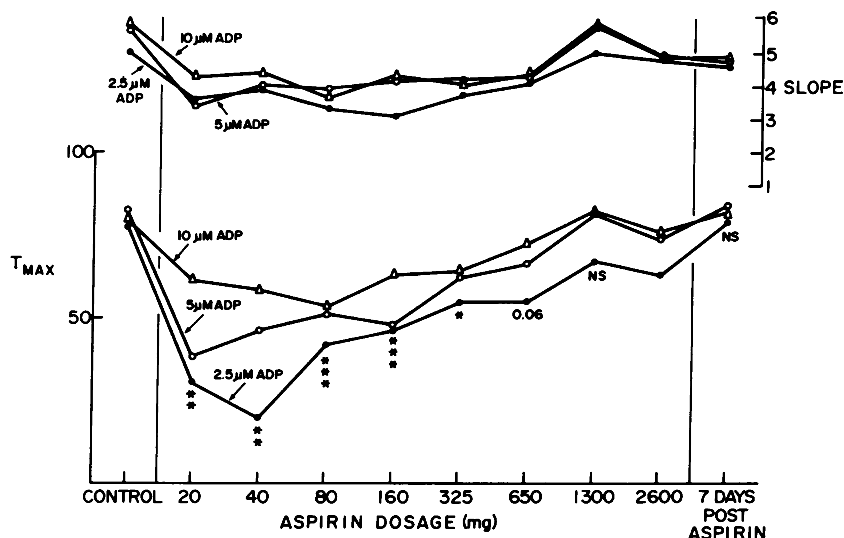


FIGURE 8 The change in optical density (T_{max}) and slope of the primary phase of the platelet aggregation response induced by ADP (2.5, 5.0, and 10.0 μM) ex vivo in five volunteers before receiving aspirin (control) on the 7th d of each dosage period and 7 d postdosage. Doses of 20, 40, 80, 160, 325, 650, 1,300, and 2,600 mg were administered, each dose for 7 d in sequential weeks. Differences are expressed from predosing control values. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ for the lowest concentration of ADP (2.5 μM).

DISCUSSION

Blood levels of prostaglandin and thromboxane products are very low and generally assays in plasma are insufficiently sensitive to detect suppression of endogenous biosynthesis of these compounds. To obtain an estimate of biosynthesis in vivo we have measured major urinary metabolites of prostacyclin (15) and thromboxane (16). Use of this noninvasive methodology avoids problems of artifactual increases in prostaglandin biosynthesis associated with blood sample withdrawal or tissue manipulation ex vivo (17). This approach also establishes whether endogenous prostacyclin and thromboxane synthesis does indeed occur and to what extent it is modulated by antiplatelet drugs in man. This is distinct from evidence provided by methods which measure the capacity of isolated tissues to synthesize eicosanoids ex vivo. The measurement of urinary prostaglandin metabolites has been successfully applied to estimate endogenous prostanoid biosynthesis by several groups of workers (18–20). For example, we have previously demonstrated an association between elevations of urinary PGE-M and indomethacin-sensitive hypercalcaemia (21) and that alterations in Tx-M parallel (22, 23) changes in platelet count.

The results of the present study are in accord with previous observations that thromboxane generation is highly susceptible to aspirin inhibition (24–26). In addition, we have shown that inhibition of prostacyclin biosynthesis appeared less marked than that of thromboxane over the dose range tested. In the multiple dose study, depression of Tx-M attained statistical significance ($P < 0.05$) at doses of aspirin 80 mg/d and above. The fall in PGI-M excretion attained significance at doses in excess of aspirin 160 mg/d. A depression at the lower doses may have been obscured to some extent by day to day variation in metabolite excretion. However, it is apparent from Fig. 6 that the effect of continued administration of aspirin 20 mg/d has a very marginal effect on PGI-M excretion. Due to both the cumulative nature of drug administration in our studies and the possibility that aspirin may result in increasing prostaglandin synthesis inhibition during chronic dosing (26), these results may well differ from those obtained after single dose administration.

Patrignani et al. (26) have recently reported that a low dose of aspirin (0.45 mg/kg/d) significantly reduced thromboxane B_2 formation in serum. Urinary 6-keto-$PGF_{1\alpha}$ (the stable hydrolysis product of prostacyclin) fell by 20% during the first 4 d of treatment but this failed to attain statistical significance. This is in accord with our findings of a rather subtle depression of urinary PGI-M excretion at the lower doses of aspirin. It is noteworthy that 2,3-dinor-6-keto-$PGF_{1\alpha}$ is the major metabolite of intravenously administered radiolabeled prostacyclin in man (15) and may thus largely reflect extrarenal endogenous prostacyclin bio-

synthesis (27). Although 6-keto-$PGF_{1\alpha}$ is recovered in urine after systemic administration of prostacyclin (15) it may largely reflect renal prostacyclin synthesis under physiological conditions (26), although this has been disputed (28). The close correspondence between our data and that of Patrignani et al. (26) provide indirect evidence that renal and extrarenal (i.e., largely vascular) sites of prostacyclin synthesis do not differ substantially in their response to aspirin inhibition in man.

In the present investigations recovery of prostacyclin generation was delayed after aspirin. 3 d after the 8-wk dosage study, mean PGI-M excretion remained depressed at 40% of predosing values. The recovery of prostacyclin biosynthesis in man following aspirin administration has been addressed in two recent studies. Preston et al. (29) found that 6-keto-$PGF_{1\alpha}$ production by human venous biopsies was substantially depressed 2 h after acute doses of 150 and 300 mg aspirin. Hanley et al. (30) found that 81 mg of aspirin resulted in ~60% depression of release of prostacyclin-like activity from venous biopsies and that suppression was still present 48 h after a 300-mg dose. Complementing these data, Buchanan et al. (31) noted that inhibition of prostacyclin biosynthesis by rabbit carotid arteries persisted at least 20 h after exposure to aspirin. Interestingly, although endothelial cells in culture rapidly resynthesize new cyclooxygenase (5), recent data obtained in cultured rat smooth muscle cells suggests that aspirin may destroy additional components of the prostacyclin synthetic system that can only be replaced by cell division (32).

The pattern of recovery of Tx-M excretion after discontinuation of aspirin administration was more variable than that of PGI-M but had attained 85±3% of control values 3 d after aspirin treatment (Fig. 1). When we consider that aspirin has a more prolonged effect on thromboxane B_2 or malondialdehyde production by platelets ex vivo (2, 24–26) the most obvious interpretation of these data is that extraplatelet sources contribute substantially to Tx-M excretion. Indeed, we noted a second possible example of extraplatelet thromboxane generation contributing to Tx-M excretion. This was an isolated incident involving the volunteer who was withdrawn from the 8-wk study following the development of symptoms of hay fever. Prior to that, on aspirin 80 mg/d, his PGI-M had fallen to 46% and Tx-M to 8% of predosing control values. However, during the period of symptoms while the volunteer continued to take aspirin 160 mg/d, Tx-M excretion rose to 170% and PGI-M to 109% of control values while the platelet response to ADP ex vivo remained depressed. Although measurement of urinary metabolites is likely to reflect prostacyclin and thromboxane production from all sources, generation of these compounds by tissues other than the vessel wall and platelets, respectively, many modify vascular platelet interactions in vivo. Indeed, thromboxane A_2 was first detected as rabbit aorta contracting substance in the effluent from perfused lung (33). It is known that agents applied topically to the external surface of a blood vessel can exacerbate or suppress the development of intraluminal platelet thrombi (34). Therefore, it is reasonable to consider that thromboxane A_2 or prostacyclin from other than their usual tissue sources might influence the activation of platelets in vivo.

There are other factors besides extraplatelet sources of thromboxane that may explain the rapid recovery of Tx-M excretion following aspirin therapy. One possibility is that inefficient acetylation of cyclooxygenase may have occurred in all tissue sources of thromboxane due to competition with aspirin by salicylate (35). However, the salicylate concentrations required are rather high and as plasma concentrations were not determined in this study, this point remains speculative. An alternative possibility is that although prostacyclin is unlikely to act as a circulating antiplatelet agent under physiologic conditions (27) it may inhibit the release of thromboxane from platelets at local sites of interaction with endothelium. A 60–70% inhibition of prostacyclin synthesis by aspirin may permit newly released platelets to be activated more readily. Thus, increased production of thromboxane by newly released platelets may have been reflected by an increase in total thromboxane production that does not directly correlate with turnover of the inhibitory effects of aspirin on platelet cyclooxygenase in vivo. In this instance it is important to appreciate the distinction between the capacity to synthesize thromboxane by stimulated platelets ex vivo and actual endogenous biosynthesis.

When platelet function was assessed in volunteers receiving aspirin 20 mg/d followed by 2,600 mg/d, thromboxane B_2 formation by stimulated platelets ex vivo was substantially inhibited (4.9 vs. 0.5%) by the lower dose of aspirin, albeit submaximally. Both platelet serotonin release and the aggregation response to a variety of agonists was further inhibited when the dose of aspirin was increased to 2,600 mg/d. This may indicate that platelet thromboxane production must be almost completely inhibited to exert maximal effects on platelet function. It is obvious from our data and that of others (26) that this objective might be attained with doses <2,600 mg/d. Urinary Tx-M excretion was maximally inhibited at doses of 325 mg/d and above in the present studies. Complete suppression of serum TxB_2 occurs following acute doses of aspirin slightly >100 mg (26) and is probable at even lower doses during chronic drug administration. A sec-

ond possible explanation of the data would be that the recently proposed thromboxane independent platelet inhibiting properties of aspirin (36) were more pronounced when the volunteers were receiving the higher dose.

During the extended dose-ranging study, the aggregation response to ADP was measured as a marker of the platelet-inhibiting effect of aspirin. To our surprise, the maximal reduction in optical density associated with T_{max} returned to control values during the final 2 wk of the study despite continued inhibition of thromboxane synthesis. This was not a controlled study so these data must be interpreted cautiously. However, this trend was evident at all doses of ADP used and in all volunteers who participated in the study. Furthermore, the subjects progressed through the 9-wk protocol up to 8 wk out of phase with each other, rendering any time-dependent artefact an unlikely explanation of the findings. All samples were drawn and prepared under carefully standardized conditions. Although further studies are required to clarify this phenomenon, a possible mechanism is that acetylation of platelet membrane proteins renders platelets more liable to aggregate during long-term aspirin therapy. Despite the return of T_{max} to predosing values during the final weeks of the study, a second wave of aggregation was not evident, suggesting that this phenomenon was not dependent on the presence of the platelet release reaction. Evidence for inhibition of the release reaction was provided by measurement of ATP release by the firefly luciferase system (37) in two subjects during the final week of dosing. Finally, the enhanced platelet response to ADP during dosing with aspirin 2,600 mg/d contrasts with the response observed when the same dose was administered in the 2-wk study, suggesting that this may be a time-, rather than dose-dependent phenomenon.

In conclusion, we have demonstrated with noninvasive, specific, and sensitive techniques that endogenous biosynthesis of both prostacyclin and thromboxane are depressed during continued administration of aspirin to healthy volunteers. In normal individuals, synthesis of these products occurs at a rate many orders of magnitude below the body's capacity. This may be a reflection of the exceedingly low frequency and intensity of events that stimulate prostacyclin and thromboxane A_2 biosynthesis when there is minimal vascular pathology. Our investigation has demonstrated that in normal volunteers, inhibition of thromboxane generation was greater than that of prostacyclin at all doses of aspirin tested. However, the curves relating dose to inhibition of metabolite excretion were similar for both prostacyclin and thromboxane suggesting that it is unlikely that any dose of aspirin can maximally inhibit thromboxane generation without also reducing prostacyclin biosynthesis. The recovery of prostacyclin biosynthesis following aspirin treatment was delayed. The clinical implications of quantitative inhibition of prostacyclin and thromboxane biosynthesis remain to be established in man.

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