Increased thromboxane biosynthesis in a human preparation of platelet activation: biochemical and functional consequences of selective inhibition of thromboxane synthase

IRENE A. G. REILLY, M.B., CH.B.,* JOHNIENE B. DORAN, B.A., BRUCE SMITH, M.D., AND GARRET A. FITZGERALD, M.D.

ABSTRACT Although thromboxane A₂ is a potent platelet agonist and vasoconstrictor in vitro, our knowledge of its pathophysiologic importance in human disease is limited. To facilitate the elucidation of its role in vivo, we sought to define a human syndrome in which pharmacologic interventions designed to inhibit the biosynthesis or biologic actions of thromboxane A₂ might be appropriately assessed. Patients with severe peripheral vascular disease were selected on the basis of elevated plasma β-thromboglobulin and circulating platelet aggregates and compared with healthy, age-matched control subjects. In addition to the platelet indexes, their bleeding time was shorter and excretion of 2,3-dinor-thromboxane B₂, a noninvasive index of thromboxane formation in vivo, and 2,3-dinor-6-keto-prostaglandin F₃α, the major urinary metabolite of prostacyclin, was markedly increased. A selective inhibitor of thromboxane synthase, imidazo (1,5-2) pyridine-5-hexanoic acid, was administered to these patients under randomized, double-blind, controlled conditions. Platelet aggregation ex vivo, the circulating platelet aggregate ratio, and the bleeding time were all unaltered, despite almost maximal inhibition of platelet thromboxane formation 1 hr after dosing. By contrast, pronounced inhibition of aggregation was observed when platelet cyclooxygenase was inhibited by aspirin. During long-term dosing with the synthase inhibitor, inhibition of thromboxane biosynthesis was incomplete, which would permit continued thromboxane-dependent platelet aggregation to occur. However, the failure of enzyme blockade to influence platelet function at the time of maximal drug action, despite efficient inhibition of serum thromboxane B₂, suggests that accumulation of proaggregatory endoperoxides is also likely to have contributed to the persistence of platelet activation. We have characterized a human preparation in which platelet activation coexists with increased thromboxane biosynthesis. In this setting, platelet activation persists despite long-term administration of a thromboxane synthase inhibitor in a dosing regimen representative of that employed in clinical trials. Prolongation of drug action and combination with antagonists of the shared endoperoxide/thromboxane A₂ receptor may be necessary to assess the potential of selective inhibition of thromboxane synthase as a therapeutic strategy in man.

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IN VIEW OF the potential importance of the proaggregatory platelet eicosanoid thromboxane A₂ in the mediation of vascular occlusive events in vivo, there has been considerable interest in the effects of selective thromboxane synthase inhibitors in man.¹ Despite apparent efficacy in animal preparations of acute thrombosis,²-⁴ endotoxic shock,⁵,⁶ ventricular fibrillation,⁷,⁸ and in an acute preparation of immune complex glomerulonephritis,⁹ clinical trials of these compounds have failed to demonstrate significant benefit.¹⁰-¹⁴

Several factors may have contributed to this discrepancy. First, in the clinical conditions that have been studied, the pathogenic importance of thromboxane A₂ has not been clearly defined. In addition, while the animal studies reflect the short-term effects of thromboxane synthase inhibitors (those over a period during which maximal inhibition of thromboxane formation...
can be maintained), the clinical investigations involved long-term drug administration. Thromboxane synthase inhibitors, unlike aspirin, are reversible enzyme inhibitors. Moreover, the brevity of their plasma drug half-lives suggests that recovery of thromboxane biosynthesis may have occurred during the dosing intervals commonly employed in clinical trials. Finally, in the presence of thromboxane synthase inhibition, whether partial or complete, accumulated prostaglandin endoperoxides may occupy the shared endoperoxide/thromboxane A2 receptor and substitute for the proaggregatory and vasoconstrictor effects of thromboxane A2.

The aims of this study were twofold. We sought to identify a patient group in which elevated thromboxane biosynthesis coexisted with evidence of platelet activation in vivo and in this manner establish an appropriate human preparation for the assessment of compounds designed to inhibit thromboxane-dependent platelet activation in vivo. Our second objective was to test the hypothesis that platelet activation persists during long-term administration of a thromboxane synthase inhibitor, imidazo (1,5-2) pyidine-5-hexanoic acid (CGS 13080), to such patients and to explore the possible underlying mechanisms of such an observation. Thus, we anticipated that inhibition of thromboxane biosynthesis might be incomplete during long-term doses with these compounds, which, together with the proaggregatory effects of prostaglandin endoperoxides, would provide an explanation for the apparent lack of clinical efficacy of thromboxane synthase inhibitors in man.

Methods

Clinical methods

Patient selection. All patients had severe, angiographically confirmed, obstructive arterial disease of the lower limb with pain at rest and/or ischemic ulcers (grades III or IV) and met preset criteria consistent with the presence of platelet activation in vivo: plasma β-thromboglobulin of greater than 20 ng/ml and a circulating platelet aggregate (CPA) ratio of greater than 1.3. Concomitant measurement of plasma platelet factor 4 allowed us to minimize artifacts caused by spurious platelet activation ex vivo by excluding samples with a platelet factor 4 of greater than 25 ng/ml. Five of the nine patients studied were insulin-dependent diabetics, seven had electrocardiographic evidence of chronic myocardial ischemia, although only one was taking antianginal medication (occasional sublingual nitroglycerine), and seven of nine were chronic cigarette smokers. Four had ischemic foot ulcers at the commencement of the study and one further patient developed an ulcer during the course of the study.

Study design. Nine patients (eight men and one woman) who were 49 to 75 years old participated in the study, which was performed in the Elliott V. Neumann Clinical Research Center of Vanderbilt University and which was approved by the Committee for the Protection of Human Subjects of Vanderbilt University Medical Center. Patients received placebo, 100 mg CGS 13080, or 200 mg CGS 13080 (kindly provided by Dr. N. Feliciano of the Ciba-Geigy Corporation) as a single dose followed 24 hr later by the beginning of a regimen of one dose four times daily (at 8 A.M., 1 P.M., 6 P.M., and midnight) for 6 days. The order of the three treatment regimens was randomized according to a double-blind, crossover design, the treatments being separated by 7 day drug-free intervals. None of the patients had taken aspirin or other nonsteroidal anti-inflammatory drugs for at least 10 days before or throughout the study.

After an overnight fast and cessation of cigarette smoking for at least 30 min, blood was drawn from a fresh venipuncture for determination of plasma β-thromboglobulin, platelet factor 4, and the CPA ratio before and 1, 4, 6, 8, and 24 hr after the single dose and after the final dose of the 6 day regimen. Blood was collected for measurement of plasma drug levels at the same times and for complete blood counts and a routine automated chemistry screen before dosing on each of the study days of the three treatment periods. The bleeding time was estimated before and 1 hr after the first and last dose of each treatment. Twenty-four hour urine collections for measurement of the major urinary metabolites of thromboxane, 2,3-dinor-thromboxane B2 (Tx-M), and prostacyclin, 2,3-dinor-6-keto-PGF1α (PGI-M), were made before the single dose and the final dose of the long-term dosing period. In addition, three aliquots of urine were collected over the 24 hr (0 to 6, 6 to 12, and 12 to 24 hr samples) after the first and final doses of each treatment regimen.

On a separate occasion four of the patients were administered 325 mg aspirin as a single dose. Blood was drawn for measurement of serum thromboxane B2 and the aggregation response of platelets to arachidonic acid, ADP, and collagen before and 1 hr after dosing.

Clinical evaluation. Before the study and at the end of each treatment period, the patients were assessed clinically by physical examination and objectively by Doppler flow measurement of ankle systolic pressure, by analysis of the waveform of arterial flow, and by calculation of the ankle/brachial systolic pressure index. Measurements were made in patients at rest with a unidirectional Doppler ultrasound velocity detector (Parks Electronics, Aloha, OR) and a mercury sphygmomanometer. A visual analog scale was used to monitor pain at rest. This consisted of a 10 cm line labeled “no pain” and “the worst pain I have ever experienced” at opposite poles. Blood pressure, measured indirectly by a mercury sphygmomanometer, and heart rate were monitored in patients in the supine position before and ½, 1, 2, 4, 6, 8, and 24 hr after dosing on each study day and every 6 hr during the long-term dosing period.

Analytic methods

Platelet α-granule constituents. Blood for measurement of β-thromboglobulin and platelet factor 4 was collected without stasis immediately into an ice-cold acid dextrose anticoagulant mixture containing indomethacin (10 μM) and prostaglandin E1 (1 μM). The samples were kept on ice and spun within 30 min at 80,000 g for 20 min and the plasma was stored at −20°C for later analysis. β-Thromboglobulin and platelet factor 4 were quantitated by radioimmunoassay with commercially available kits (Amersham Corporation, Arlington Heights, IL, and Abbott Laboratories, Chicago, respectively). 

CPAs: The presence of CPAs was determined by the method of Wu and Hoak, with the calculated platelet aggregate ratio expressed as the inverse such that an abnormal value would be elevated.

Platelet aggregation. Platelet aggregation in platelet-rich plasma was measured with the light transmission method of Born with the use of a dual-channel aggregometer (Payton Associates, Buffalo, NY). Platelet-rich plasma was prepared as previously described and the platelet count was adjusted to
300 × 10^3 liter with autologous platelet-poor plasma. Platelet aggregation was measured in response to increasing doses of arachidonic acid (0.33 to 1.33 mM; Sigma Chemical Co., St. Louis), epinephrine (1 to 10 μM; Sigma Chemical Co.), and collagen (9.5 to 95 μg/ml; Biodata Corporation, Horsham, PA). Platelet aggregation was quantitated with the lag time to 50% of maximal alteration in light transmission, expressed as the LT50, and as the percentage of maximum light transmission achieved 6 min after addition of the agonist.

**Bleeding time.** The bleeding time was measured on the forearm in a blinded fashion by the template method (Simplate, General Diagnostics, Morris Plains, NJ). All determinations were performed by the same investigator.

**PGI-M.** Urinary excretion of PGI-M was measured with a stable isotope dilution assay with the use of gas chromatography/mass spectrometry modified from the original method as previously described. Briefly, 5 ng of a deuterated internal standard was added to 5 ml of urine, which was then subjected to extraction and backextraction under alkaline and acidic conditions. The sample was derivatized as the methoxime, pentafluorobenzyl ester, and purified by thin-layer chromatography and the derivatization was completed by formation of the trimethylsilyl ether derivative. Quantitation was accomplished with a Hewlett-Packard 5980 instrument operating in the negative ion mode, monitoring m/z 586.5 for endogenous PGI-M and m/z 590.5 for the deuterated internal standard.

**Tx-M.** Urinary excretion of Tx-M was measured by a newly developed stable isotope dilution assay in which gas chromatography/mass spectrometry is used, as described in detail elsewhere. Briefly, methoxymine hydrochloride (0.5 ng in 1.5 ml 6M acetate buffer) was added to a 5 ml aliquot of urine previously spiked with 12.5 ng of a deuterated internal standard. The urine was then applied to a phenylboronic acid column (Analytichem International, Harbour City, CA), rinsed with 0.05M HCl followed by methanol, and eluted from the column with 4 ml methanol/0.1N NaOH. The sample was then diluted to 15 ml with distilled water, acidified to pH 3.5 with 10% formic acid, and applied to a reverse-phase Sep-Pak (Waters Associates, Milford, MA). After washing with 5 ml distilled water followed by 5 ml methanol/water (1:3 by volume), the sample was eluted with ethyl acetate and evaporated to dryness under nitrogen. Further purification was carried out by thin-layer chromatography in a solvent system of ethyl acetate/acetic acid/hexane (54:12:25 vol/vol/vol). The sample was extracted into ethyl acetate and further derivatized as the pentafluorobenzyl ester by addition of 12.5% pentafluorobenzyl bromide and N,N-dimethylformamide and heating to 40°C for 30 min. This reaction mixture was subjected to thin-layer chromatography with a solvent system of ethyl acetate/isooctane (85:15 vol/vol) and extracted as above and final derivatization was completed by formation of the trimethylsilyl ether by treatment with bis(trimethylsilyl)trifluoroacetamide and pyridine for a minimum of 15 min at 20°C. Quantitation was established with a Nermag 10-10C instrument operating in the negative ion mode monitoring m/z 586.5 for endogenous Tx-M and m/z 590.5 for the tetradeuterated internal standard.

**Thromboxane B2.** Urinary excretion of thromboxane B2 was measured by a stable isotope dilution assay reported in detail elsewhere. A 5 ml aliquot of urine was spiked with 12.5 ng of a deuterated internal standard of thromboxane B2. Purification and derivatization of the sample was carried out as described above for Tx-M with the exception that, since the relative migration rates for thromboxane B2 and Tx-M (approximately 0.33 and 0.21, respectively) are different, the appropriate areas of the plates were scraped at each step of the thin-layer chromatography procedure. Quantitation was established with the Nermag 10-10C instrument operating in the negative ion mode monitoring m/z 614.5 for endogenous thromboxane B2 and m/z 618.5 for the deuterated internal standard.

**Serum thromboxane B2.** Formation of thrombin-stimulated thromboxane B2 ex vivo was determined by the method of Patrone et al. Venous blood was allowed to clot in a warmed glass tube, which was then incubated at 37°C for 1 hr. The blood was spun at 2000 g for 15 min and the serum was stored at −20°C for later analysis by radioimmunoassay.

**Plasma drug levels.** Plasma concentrations of CGS 13080 were determined by Dr. Chan of the Ciba-Geigy Corporation using high-pressure liquid chromatography. After purification with an Amberlite CG-50 cation exchange column, the samples were applied to a C18 µ-Bondpak column (Waters Associates, Milford, MA) with a mobile phase of 0.01M sodium 1-pentane-sulfonate, pH 3, containing 27% acetonitrile. Levels of CGS 13080 and the internal standard CGS 13067 were measured by fluorescence at an excitation wavelength of 280 nm. The limit of detection of the assay was 10 ng/ml.

**Statistical analysis.** Data were analyzed by nonparametric methods, thereby avoiding assumptions as to the distribution of the variables involved. Two-way analysis of variance was by the method of Friedman, with subsequent pairwise analysis by the Wilcoxon rank-sum test and unpaired analysis by the Mann-Whitney U test.

**Results.** Evidence for platelet activation in vivo. All patients fulfilled the preset criteria consistent with the presence of platelet activation in vivo. Plasma β-thromboglobulin (113 ± 22 vs 13 ± 3 ng/ml, mean ± SEM; p < .001) and the CPA ratio (1.4 ± 0.01 vs 1.0 ± 0.02, p < .05) were both significantly elevated compared with those in healthy age-matched control subjects (figure 1), while the levels of platelet factor 4 measured in the same samples (11.7 ± 0.5 vs 11.0 ± 2 ng/ml) were within the normal range. A small number of samples (12 of 162) from different subjects were excluded from analysis because elevated levels of β-thromboglobulin were accompanied by platelet factor 4 levels of greater than 25 ng/ml. In addition, the atherosclerotic patients were characterized by a significantly shorter bleeding time (172 ± 12 vs 300 ± 24 sec; p < .001) and by markedly enhanced excretion of Tx-M (912 ± 99 vs 223 ± 22 pg/mg creatinine; p < .001) in comparison with the age-matched control subjects (figure 1). Basal PGI-M excretion was significantly greater in the patients with severe atherosclerosis (323 ± 89 vs 197 ± 21, pg/mg creatinine, p < .05) than in healthy age-matched control subjects, providing further indirect evidence of platelet activation in vivo in our preparation.

**Effects of drug on thromboxane synthesis.** Tx-M excretion was unaltered after administration of placebo and did not differ significantly on the control days that preceded each treatment period. After administration of a single dose, CGS 13080 caused a significant, reversible fall in Tx-M (maximum 66.0 ± 4% from control values) 6 to 12 hr after dosing (figure 2).
Persisted long-term dosing, significant elevations of plasma \( \beta \)-thromboglobulin (\( \beta \)TG) (\( p < .001 \)), the CPA ratio (\( p < .05 \)), and Tx-M excretion (\( p < .001 \)) were observed in the patients. The template bleeding time was shorter (\( p < .001 \)) in the patients than in the healthy controls.

Although Tx-M excretion remained depressed during long-term dosing, significant biosynthesis of thromboxane persisted and no further decrement in Tx-M occurred with administration of the final dose. There was no significant difference between the 100 and 200 mg dose with respect to the effects observed on Tx-M excretion after short- and long-term dosing. In the four patients in whom we measured the urinary excretion of both thromboxane B\(_2\) and Tx-M, a similar degree of depression of both metabolites was noted after administration of 200 mg CGS 13080 (table 1). In addition, basal levels of urinary thromboxane B\(_2\) were markedly elevated compared with those observed in healthy subjects (247 ± 86 vs 46 ± 14 pg/mg creatinine).

Administration of placebo did not significantly alter serum thromboxane B\(_2\) levels from predosing values. After short-term administration of CGS 13080, serum thromboxane B\(_2\) generation ex vivo was reversibly inhibited, with peak inhibition measured 1 hr after dosing (figure 3). Recovery of serum thromboxane was linear and gave a calculated "biological" half-life (50% recovery to predosing values) of between 6 and 8 hr. The level of serum thromboxane B\(_2\) immediately before the last dose (140 ± 54 ng/ml), which was measured 8 hr after the previous dose, was not significantly different from that measured 8 hr after the first dose (115 ± 38 ng/ml). Thus, no cumulative inhibition of serum thromboxane B\(_2\) occurred during long-term dosing. As observed with Tx-M excretion, there was no difference in the effects of the two doses of CGS 13080 on serum thromboxane B\(_2\) formation, suggesting their close proximity on the dose-response curve.

**TABLE 1**

Urinary excretion of Tx-M and thromboxane B\(_2\) (TxB\(_2\)) in four atherosclerotic patients after long-term administration of 200 mg CGS 13080

<table>
<thead>
<tr>
<th>Time</th>
<th>Tx-M</th>
<th>TxB(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before last dose</td>
<td>84.7 ± 5</td>
<td>89.4 ± 1</td>
</tr>
<tr>
<td>After dose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–6 hr</td>
<td>78 ± 6</td>
<td>88.2 ± 7</td>
</tr>
<tr>
<td>6–12 hr</td>
<td>71.6 ± 7</td>
<td>72.7 ± 4</td>
</tr>
<tr>
<td>12–24 hr</td>
<td>52.1 ± 12</td>
<td>52.3 ± 7</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of determinations in four patients. Depression of Tx-M and TxB\(_2\) excretion was not significantly different by analysis of variance (\( p > .10 \)).
Effect of drug on platelet function. Plasma β-thromboglobulin and platelet factor 4 were unchanged by placebo. There was no significant depression of β-thromboglobulin after short-term administration of either dose of CGS 13080 (figure 4). During long-term dosing, however, β-thromboglobulin levels fell significantly, although they remained more than three times normal: 61 ± 21 ng/ml (200 mg) and 45 ± 15 ng/ml (100 mg) vs 13 ± 3 ng/ml (age-matched control subjects). Platelet factor 4 levels were within the normal range and were unaltered by CGS 13080 (figure 4).

The CPA ratio and bleeding time (figure 5) were not significantly different before and after administration of CGS 13080 or placebo. Platelet aggregation in platelet-rich plasma in response to a wide range of doses of arachidonic acid, epinephrine, and collagen was also unchanged by CGS 13080 or placebo, whether assessed as the LT50 or as the maximum extent of increase in light transmission at 5 min after addition of the agonist (figure 5). By contrast, administration of aspirin significantly inhibited platelet function in these patients (table 2).

Effect of drug on prostacyclin formation. There was no change in PGI-M excretion with placebo. Coincident with the fall in Tx-M after 200 mg of CGS 13080, PGI-M excretion increased significantly (p < .05) from 291 ± 54 to 538 ± 150 and 442 ± 150 pg/mg creatinine in the 0 to 6 and 6 to 12 hr aliquots. Similar increments in PGI-M were obtained after the 100 mg dose. PGI-M remained significantly elevated during long-term dosing but no further increase occurred after the final dose of CGS 13080 (figure 2).

**TABLE 2**

<table>
<thead>
<tr>
<th>Platelet aggregation (%) after:</th>
<th>Before aspirin</th>
<th>After aspirin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet agonist AA 0.3 mM</td>
<td>87 ± 0.9</td>
<td>No agg.</td>
</tr>
<tr>
<td>0.66 mM</td>
<td>87 ± 20</td>
<td>No agg.</td>
</tr>
<tr>
<td>1.33 mM</td>
<td>87 ± 1.5</td>
<td>No agg.</td>
</tr>
<tr>
<td>Collagen 9.5 µg/ml</td>
<td>61 ± 20</td>
<td>No agg.</td>
</tr>
<tr>
<td>95 µg/ml</td>
<td>92 ± 2</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>Epinephrine 5 µM</td>
<td>89 ± 0.8</td>
<td>No agg.</td>
</tr>
<tr>
<td>10 µM</td>
<td>88 ± 2.5</td>
<td>No agg.</td>
</tr>
<tr>
<td>Serum TxB2 (ng/ml)</td>
<td>332 ± 62</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>Bleeding time (sec)</td>
<td>218 ± 39</td>
<td>308 ± 50</td>
</tr>
</tbody>
</table>

Aggregation was inhibited in all individuals at all doses of agonist except high-dose collagen. Serum TxB2 was depressed and bleeding time prolonged in all patients after aspirin.

AA = arachidonic acid; No agg. = no aggregation response 6 min after addition of agonist.
Plasma drug levels. Plasma concentrations of CGS 13080 are shown in table 3. These confirmed bioavailability of the compound and the relationship of plasma concentration to dose within each of the volunteers. Significant concentrations of CGS 13080 were not detectable in plasma 8 hr after short-term dosing and in eight of nine patients there was no evidence of accumulation during administration of multiple doses. One patient did, however, exhibit marked accumulation of the drug with long-term dosing, particularly at the higher dose, and this correlated with a greater degree of inhibition of serum thromboxane B$_2$, Tx-M excretion, and plasma $\beta$-thromboglobulin (figure 6) than observed in the other patients.

Clinical evaluation. There was no significant alteration in the Doppler echocardiographic measurements of absolute ankle systolic pressure, the ankle/brachial systolic pressure index, or arterial waveform. In addition, supine systolic and diastolic blood pressure and heart rate were unaffected by short- or long-term administration of CGS 13080 or placebo. The scores measured on the visual analog scale used to assess pain at rest were not significantly different with placebo or either dose of CGS 13080. There were no changes in the complete blood counts or results of routine automated biochemistry screens.

Discussion
To explore the discrepancy between the apparent efficacy of thromboxane synthase inhibitors in diverse animal preparations of acute thrombosis and the disappointing results of their use in clinical trials, we defined a novel human preparation in which evidence of
TABLE 3
Plasma concentrations of CGS 13080 (ng/ml)

<table>
<thead>
<tr>
<th>Time after dosing (hr)</th>
<th>After 100 mg dose</th>
<th></th>
<th>After 200 mg dose</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 8</td>
<td>Day 1</td>
<td>Day 8</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>53 ± 26</td>
<td>ND</td>
<td>260 ± 213</td>
</tr>
<tr>
<td>1</td>
<td>2333 ± 379</td>
<td>1704 ± 397</td>
<td>2476 ± 593</td>
<td>3010 ± 590</td>
</tr>
<tr>
<td>4</td>
<td>149 ± 36</td>
<td>128 ± 30</td>
<td>299 ± 122</td>
<td>437 ± 111</td>
</tr>
<tr>
<td>6</td>
<td>31 ± 8</td>
<td>49 ± 19</td>
<td>98 ± 53</td>
<td>102 ± 33</td>
</tr>
<tr>
<td>8</td>
<td>ND</td>
<td>21 ± 9</td>
<td>20 ± 9</td>
<td>64 ± 23</td>
</tr>
<tr>
<td>24</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of determinations in nine patients. ND = None detected (limit of sensitivity 10 ng/ml).

platelet activation in vivo coexists with enhanced thromboxane biosynthesis. We then addressed the hypothesis that inhibition of thromboxane biosynthesis and platelet function is incomplete during long-term administration of a selective inhibitor of thromboxane synthase.

The patients with severe atherosclerosis fulfilled predetermined criteria for the presence of platelet activation in vivo based on three commonly used indexes: plasma levels of the platelet-specific proteins β-thromboglobulin and platelet factor 4 and estimation of the CPA ratio. Plasma β-thromboglobulin and the platelet aggregate ratio were both elevated in the atherosclerotic patients. While the possibility of platelet activation ex vivo cannot be excluded, the absence of a coexistent increase of platelet factor 4 is highly suggestive of the presence of platelet activation in vivo.17 The limitations of these invasive methods27, 28 led us to employ an additional approach to the study of platelet activation in vivo: measurement of urinary Tx-M, a noninvasive index of thromboxane A2 formation in vivo. While under physiologic conditions the rate of secretion of thromboxane has been estimated to be very low — on the order of 0.1 ng/kg/min29 — this is likely to reflect the relative absence of provocative stimuli to its production. In the setting of severe atherosclerosis with evidence consistent with platelet activation in vivo, endogenous thromboxane biosynthesis is increased more than fourfold. Furthermore, we have shown an increased rate of in vivo prostacyclin production in these patients consistent with the hypothesis, previously proposed,30 that this is a consequence of enhanced interactions between activated platelets and the vessel wall, particularly at sites of vascular injury.

Short-term administration of CGS 13080, a selective inhibitor of thromboxane synthase31 resulted in greater than 95% inhibition of serum thromboxane B2 formation ex vivo 1 hr after dosing, with a biological half-life of 6 to 8 hr, which is comparable to that achieved with other thromboxane synthase inhibitors21, 32 and with this compound in healthy volunteers.33, 34 During long-term dosing, however, trough levels of serum thromboxane B2 remained at approximately 60% of predosing values. Furthermore, Tx-M excretion fell to a mean of 34% after a single dose, and, during long-term dosing, thromboxane biosynthesis persisted at a mean 25% of predosing levels. Although platelets are the predominant source of urinary Tx-M, residual metabolite formation may represent formation by minor, nonplatelet sources in addition to recovery of platelet thromboxane formation during the urine collection period.

The failure to induce complete, cumulative inhibition of thromboxane generation during long-term drug administration is important for several reasons. First, the dosing interval in this investigation, as in previous clinical studies with this class of compounds,10, 12, 13 was based, not on the plasma drug half-life of approximately 45 min,34 but on the much longer biological

![FIGURE 6. Relationship between plasma concentrations of CGS 13080 and the effects on indexes of thromboxane biosynthesis and platelet function. The results are expressed as percentage inhibition from predosing control values and represent the data obtained immediately before administration of the final dose of 200 mg CGS 13080. There was a greater degree of inhibition of serum thromboxane B2, Tx-M, and plasma β-thromboglobulin in the single patient in whom substantial drug accumulation occurred (shaded bars) compared with that in the other patients (open bars, n = 8).](http://circ.ahajournals.org/doi/abs/10.1161/01.CIR.101.5.1306?journalCode=cir)
half-life. While cumulation to a steady-state effect would be expected in three to four half-lives when the dosing interval is based on the plasma drug half-life, this assumption cannot be made for the biological half-life. Indeed, 6 to 8 hr after dosing, plasma levels of CGS 13080 were barely detectable and it is thus not surprising that cumulative inhibition of thromboxane formation did not occur during this study. While such a discrepancy (between the biological and pharmacological half-lives) might be anticipated on the basis of established plasma drug kinetic-dynamic relationships, other factors, such as the presence of active metabolites or slow elimination of the drug from deep compartments, may have contributed to this observation. However, the same discrepancy has been reported with several structurally distinct synthase inhibitors. In no case have biologically active metabolites of these compounds been identified. This suggests that incomplete inhibition of thromboxane biosynthesis during the dosing intervals is likely to have occurred in clinical trials of synthase inhibitors other than CGS 13080.

In addition, using a variety of methods to characterize platelet function ex vivo and in vivo, we have shown that platelet activation persists in this population during long-term administration of a thromboxane synthase inhibitor. There was no change in the bleeding time, in platelet aggregation in response to three different agonists, or in the CPA ratio and, although β-thromboglobulin fell, it still remained more than three times normal. This is in contrast to our studies in healthy volunteers in whom CGS 13080 significantly prolonged the bleeding time and inhibited platelet aggregation ex vivo. The reason for this discrepancy was not directly addressed in the present study. However, the relationship between inhibition of the capacity of platelets to form thromboxane B2 and thromboxane-dependent platelet activation is nonlinear. There is also a marked discrepancy between the capacity to form eicosanoids and actual production rates in vivo. Thus, although capacity was inhibited by approximately 95% 1 hr after dosing in both groups, this would still theoretically permit a substantial augmentation of thromboxane synthesis in vivo in the presence of stimuli to its production. In the patients in whom biosynthesis of the thromboxane A2 was increased, this may have been sufficient to sustain thromboxane-dependent platelet function. The absence of any substantial changes in the measured indexes of platelet function corresponded to the lack of clinical benefit in these patients.

It is also possible that the lack of platelet-inhibitory effects cannot be completely accounted for by inadequate suppression of thromboxane synthesis alone. For example, accumulation of proaggregatory endoperoxides may also be a contributory factor in the observed continuing platelet activation during selective inhibition of thromboxane synthesis. Inhibition of formation of both thromboxane A2 and prostaglandin endoperoxides by aspirin prolonged the bleeding time and inhibited aggregation in these patients. At present, conclusive evidence for the importance of endoperoxides during thromboxane synthase inhibition in man is lacking. However, in a canine preparation, the combination of a prostaglandin endoperoxide/thromboxane receptor antagonist with a synthase inhibitor has been shown to delay the time to coronary artery occlusion despite maximal inhibition of thromboxane synthesis by the synthase inhibitor alone, and preliminary evidence for the efficacy of this combination has also been obtained with human platelets in vitro.

An alternative explanation is that despite the increase in biosynthesis of thromboxane A2, platelet activation in these patients was largely reflective of non-eicosanoid-dependent mechanisms. Nevertheless, that incomplete suppression of thromboxane formation throughout the long-term dosing period is likely to have been the major factor in the continuing platelet activation that we observed is supported by data from the single patient in this study in whom significant drug accumulation occurred. In this patient, cumulative inhibition of thromboxane formation in vivo and ex vivo was virtually complete, coincident with a significantly greater fall in β-thromboglobulin.

It has previously been shown, in a rat glomerular preparation in vitro, that the thromboxane synthase inhibitor dazoxiben has a higher concentration for 50% inhibition of glomerular thromboxane synthase than the enzyme in platelets, suggesting that there may be differential tissue sensitivity of thromboxane synthase to selective inhibitors. It has been proposed that urinary thromboxane B2 primarily reflects renal formation under physiologic conditions. By contrast, dinor-thromboxane B2 is the predominant urinary metabolite of systemically administered thromboxane B2 in man. It is therefore interesting that the pattern of inhibition and recovery was similar for both metabolites. While this may be interpreted as evidence against the hypothesis of a differential effect on renal and platelet thromboxane synthase, it may also reflect species differences in the sensitivity of the enzyme to pharmacologic inhibition. The most likely explanation is that while urinary thromboxane B2 is mainly of renal origin in healthy human subjects, in the setting of...
platelet activation in vivo with increased platelet thromboxane formation, urinary thromboxane B₂ may predominantly derive from platelets. Under such circumstances, a similar degree of inhibition of urinary thromboxane B₂ and Tx-M might indeed have been expected. Further evidence in support of this hypothesis is provided by the observation that the basal levels of urinary thromboxane B₂ as well as of dinor-thromboxane B₂ were elevated compared with those measured in healthy subjects.

Endogenous prostacyclin biosynthesis increased approximately twofold in response to a single dose of the thromboxane synthase inhibitor, which is consistent with endoperoxide diversion to a source of prostacyclin synthase. While the percentage increment in prostacyclin biosynthesis is similar to that observed with the same drug in healthy volunteers, the absolute increment in metabolite excretion was at least twice as great in the patients. This raised the possibility that the transfer of prostaglandin endoperoxides may be enhanced in the presence of platelet activation in vivo. Despite elevated basal secretion of prostacyclin in these patients, the magnitude of the increase in prostacyclin after administration of the synthase inhibitor does not represent the maximum capacity of vascular tissue to generate prostacyclin and would not result in circulating levels sufficient to exert systemic effects. Nevertheless, endogenous prostacyclin may contribute to the efficacy of thromboxane synthase inhibitors by its actions at a local level. The initial significant increment in prostacyclin was not maintained during long-term dosing and there was no further increment after the final dose. These changes mirrored the fall in thromboxane formation and indicate that alterations in prostacyclin biosynthesis are dependent on increased provision of endoperoxide substrate after inhibition of thromboxane synthase.

In conclusion, we have characterized a human preparation of platelet activation in vivo. We have demonstrated enhanced biosynthesis of thromboxane in patients with severe atherosclerosis coincident with evidence of platelet activation in vivo as assessed by more conventional indexes. The use of pharmacologic probes in such a preparation is likely to elucidate the pathophysiologic role of thromboxane A₂ in man. In the present study we have demonstrated that administration of a thromboxane synthase inhibitor on a dosage schedule representative of those used in clinical trials causes reversible inhibition of thromboxane formation in such patients. Cumulative, complete inhibition of thromboxane biosynthesis does not occur, however, and platelet activation persists during long-term drug administration. This may reflect incomplete suppression of thromboxane production during the dosing interval. Furthermore, the lack of effect on measured indexes of platelet function 1 hr after dosing, despite substantial inhibition of platelet thromboxane formation, and the response of these indexes to aspirin suggest that substitution for the proaggregatory effects of thromboxane A₂ by accumulated prostaglandin endoperoxides may also play a role during inhibition of thromboxane synthase in vivo.

This study was designed to investigate the functional and biochemical effects of administration of a representative thromboxane synthase inhibitor in a manner typical of that used in clinical trials of these compounds. Although persistent platelet activation in atherosclerotic patients may indicate the importance of nonthromboxane-mediated platelet stimulation, incomplete suppression of thromboxane biosynthesis with or without the proaggregatory effects of prostaglandin endoperoxides would in itself be sufficient to account for the lack of clinical efficacy of thromboxane synthase inhibitors. Thus, realization of the therapeutic potential of this class of compounds is likely to depend on prolongation of drug action and combination with antagonists of the prostaglandin endoperoxide/thromboxane receptor.

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