The biochemical pharmacology of thromboxane synthase inhibition in man

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ABSTRACT Selective inhibitors of thromboxane synthase have two theoretical advantages over inhibitors of the cyclooxygenase enzyme as potential antithrombotic compounds. First, they do not prevent formation of prostacyclin, a platelet-inhibitory, vasodilator compound, coincident with inhibiting thromboxane biosynthesis. Second, the prostaglandin endoperoxide substrate that accumulates in the platelet in the presence of thromboxane synthase inhibition may be donated to endothelial prostacyclin synthase at the site of platelet-vascular interactions (endoperoxide “steal”). Selective inhibition of thromboxane biosynthesis coincident with enhanced prostacyclin formation in vivo has been observed after administration of these compounds to man. Despite these attractive features and the efficacy of these compounds in diverse short-term animal preparations of thrombosis, investigations of their efficacy in human disease have proven disappointing. This may reflect on the importance of thromboxane A₂ in the diseases that have been investigated. Alternatively, the lack of drug efficacy may have resulted from either incomplete suppression of thromboxane biosynthesis and/or substitution for the biological effects of thromboxane A₂ by prostaglandin endoperoxides during long-term dosing studies. Given that selective inhibition of thromboxane formation can be approached with aspirin, the particular value of these compounds is dependent on enhancing prostacyclin formation. Aspirin inhibits thromboxane-dependent platelet activation, but many platelet agonists are likely to act in concert in vivo and prostacyclin inhibits platelet aggregation induced by both thromboxane-dependent and thromboxane-independent mechanisms. To test the hypothesis that thromboxane synthase inhibitors are efficacious in human disease, compounds of longer duration of action are required. Combination with antagonists of the prostaglandin/thromboxane A₂ receptor may be necessary to reveal their full beneficial action.

Circulation 72, No. 6, 1194-1201, 1985.

ASPIRIN irreversibly acetylates platelet cylooxygenase and thereby inhibits production of thromboxane A₂, a potent vasoconstrictor and platelet agonist.¹ ² These observations provided a molecular basis for prospective studies of aspirin in syndromes putatively associated with platelet activation. These trials were initially mounted on the basis of aspirin’s effects on platelet aggregation³ and retrospective data suggesting a reduced cardiovascular mortality in patients with arthritis who consumed aspirin over the long term.⁴ In prospective, double-blind, placebo-controlled studies aspirin has since been shown of benefit in the prevention of transient ischemic attack and stroke,⁵ ⁶ the prevention of death and nonfatal myocardial infarction in patients with unstable angina,⁷ ⁸ the prevention of hemodialysis shunt occlusion⁹ and, with dipyridamole, the prevention of coronary graft occlusion¹⁰ ¹¹ and renal deterioration in patients with membranoproliferative glomerulonephritis.¹²

The efficacy of aspirin alone in a recent study of coronary graft occlusion¹³ and the similar efficacy of aspirin with or without dipyridamole in the prevention of transient ischemic attacks¹⁴ and coronary graft occlusion¹⁵ suggests that dipyridamole may have contributed little benefit in the last three studies cited.¹⁰ ¹¹ ¹² Residual doubts about the efficacy of aspirin remain, however, particularly fostered by a number of negative studies in the secondary prevention of myocardial infarction.¹⁶ ¹⁸ Many factors, including delay from the time of presentation to randomization and inadequate sample size may have contributed to these results. However, it has also been suggested that the dosage of aspirin used may have resulted in coincidental depression of formation of prostacyclin, the major cylooxygenase product of vascular endothelium and a potent inhibitor of platelet aggregation.¹⁹ Consequently, the development of compounds that selectively inhibit thromboxane synthase²⁰ ²¹ prompted considerable interest. Unlike cylooxygenase inhibitors, such agents would prevent formation of thromboxane A₂ with-
out coincident inhibition of prostacyclin biosynthesis. Furthermore, they might actually enhance vascular prostacyclin formation if platelets could “donate” their accumulated prostaglandin endoperoxide substrate to endothelial prostacyclin synthase.22

**Endoperoxide rediversion.** A compound that enhanced vascular prostacyclin formation would be of substantial potential benefit as a platelet inhibitor in vivo. Aspirin-like drugs might be expected to prevent thromboxane-dependent platelet activation. However, many endogenous mediators of platelet activation, such as thrombin and collagen, could stimulate aggregation despite the presence of aspirin. Prostacyclin, by increasing platelet cyclic AMP, prevents platelet aggregation induced by all known agonists and has been shown to disaggregate aggregated platelets in vitro.23 Multiple agonists are likely to act in concert in vivo, so even in a syndrome of platelet-mediated vascular occlusion, inhibition of thromboxane formation alone would not be expected to be completely effective. Enhanced production of prostacyclin, particularly at the site of platelet-vessel wall interaction, as envisaged by the endoperoxide rediversion or “steal” hypothesis,24 might represent an important advantage over aspirin.

Initial attempts to demonstrate endoperoxide rediversion were unsuccessful.25 However, Marcus et al.26 demonstrated that in a perturbed mixture of endothelial cells and platelets, approximately half of the prostacyclin produced by the endothelial cells originated from the platelet endoperoxides. This phenomenon was amplified by thromboxane synthase inhibitors. These observations, which were subsequently confirmed by others,27 provide important experimental support for the concept of endoperoxide transfer. However, the model lacks important components such as flow, shear stress, and albumin (which avidly binds eicosanoids28), which operate in vivo. Early reports that were purported to demonstrate an increase in plasma 6-keto-prostaglandin (PGF)\(_{1\alpha}\) after administration of a thromboxane synthase inhibitor to human volunteers29 were uninterpretable due to methodologic shortcomings.30 More commonly, inhibitors were screened for evidence of endoperoxide rediversion by measurement of immunoreactive thromboxane B\(_2\) and 6-keto-PGF\(_{1\alpha}\) formation in serum in vitro or ex vivo.31,32 However, although 6-keto-PGF\(_{1\alpha}\) is formed in small amounts in whole blood,33 immunoreactive 6-keto-PGF\(_{1\alpha}\) may greatly overestimate actual 6-keto-PGF\(_{1\alpha}\) formation in serum in the presence of a thromboxane synthase inhibitor.34 PGE\(_2\), PGF\(_{2\alpha}\), and PGD\(_2\) are formed in much greater amounts than 6-keto-PGF\(_{1\alpha}\) in serum in the presence of a synthase inhibitor.33 Thus, although cross reactivity of a 6-keto-PGF\(_{1\alpha}\) antibody with these compounds may be small in percentage terms, increased PGE\(_2\), PGF\(_{2\alpha}\), or PGD\(_2\) production would be likely to introduce a substantial quantitative artifact into estimates of 6-keto-PGF\(_{1\alpha}\) production in the presence of a synthase inhibitor. Confirmation of this hypothesis was obtained by measurement of both thromboxane B\(_2\) and 6-keto-PGF\(_{1\alpha}\) by radioimmunoassay and gas chromatography-mass spectrometry in serum with and without addition of the thromboxane synthase inhibitor dazoxiben.34

An alternative approach to the detection of enhanced prostacyclin formation after the administration of these compounds has been the measurement of 2,3-dinor-6-keto-PGF\(_{1\alpha}\) (PGI-M), a major urinary prostacyclin metabolite in man.35 We have conducted three double-blind, short-term studies with structurally distinct thromboxane synthase inhibitors.36-38 PGI-M excretion increased approximately two- to threefold after two of these compounds, dazoxiben and CGS 13080, while the increase after dazmargel was less marked. Assuming that the “basal” level of 6-keto-PGF\(_{1\alpha}\) is 2 to 3 pg/ml or less,39,40 a threefold increment would result in levels that are still below the limits of sensitivity of most assay systems. This is consistent with the marginal increase in plasma 6-keto-PGF\(_{1\alpha}\) reported by Patrignani et al.41 after administration of dazoxiben to volunteers. Obviously, such an increment would be insufficient to result in circulating concentrations of prostacyclin of biological significance. However, it might reflect production at sites of platelet-vessel wall interaction that could be relevant to local thromboreistance. It is also of interest that in patients with severe peripheral vascular disease, prostacyclin metabolite excretion is increased three- to fourfold.42 Finally, although these data are consistent with the vascular steal hypothesis, it is important to remember that one cannot definitively attribute a tissue of origin to metabolites measured in urine. It is possible that the increase in PGI-M reflects its production by a tissue other than vascular endothelium.

In summary, information has been provided in constrained circumstances in vitro that demonstrates enhanced transfer of platelet endoperoxides to vascular prostacyclin synthase in the presence of thromboxane synthase inhibitors. Data compatible with this mechanism has been obtained after short-term administration of thromboxane synthase inhibitors in vivo.

**"Nonresponse" to thromboxane synthase inhibition.** Bertele et al.43 reported interindividual differences in the platelet-inhibitory response to thromboxane synthase inhibitors in vitro. Despite efficient inhibition of
thromboxane formation, some “nonresponders” would fully aggregate in response to collagen or arachidonate. A possible explanation for this phenomenon is substitution for the platelet-aggregatory effects of thromboxane A$_2$ by prostaglandin endoperoxides at their common receptor site during thromboxane synthase inhibition. An alternative explanation would be differential rates of platelet uptake of the inhibitor between individuals. We performed experiments with the use of the lag time to 50% maximal aggregation induced by arachidonic acid as a sensitive index of inhibition of arachidonate-dependent aggregation. Prolongation of the time platelets were preincubated with a thromboxane synthase inhibitor, dazoxiben, progressively prolonged the lag time and ultimately inhibited aggregation (figure 1). In further experiments, we found that prolongation of the lag time was reflected by increasing inhibition of platelet thromboxane generation and conversion of “nonresponders” into “responders” (figure 2). In some individuals, however, despite prolongation of the preincubation time to 10 min and comparable inhibition of platelet thromboxane production, full aggregation was preserved. In such true nonresponders, aggregation was completely inhibited by coincubation with both dazoxiben and 5-end0-(6'-caboxyhex-2'Z-etyl-6-exo N-(phenylcarbamoyl) hydrazono methyl-becycl0 2,2,1 heptane (EPO45; a gift of Dr. R. L. Jones), an antagonist of the shared thromboxane A$_2$/prostaglandin endoperoxide receptor (figure 3). Responder status may also be altered by adenylate cyclase inhibitors or influenced by the proaggregatory properties of PGE$_2$ and the antiaggregatory properties of PGD$_2$. However, nonresponders and responders do not differ with respect to generation of PGE$_2$ and PGD$_2$ and complete inhibition of platelet aggregation by thromboxane A$_2$/endoperoxide antagonists suggests that endoperoxides play the predominant role.

In summary, experiments in vitro suggest that prostaglandin endoperoxides may substitute for the proaggregatory effects of thromboxane A$_2$ during efficient synthase inhibition. Even responders demonstrate less marked inhibition of platelet aggregation ex vivo than would be anticipated on the basis of comparable inhibition of thromboxane formation by aspirin.

**Clinical trials with thromboxane synthase inhibitors.** In contrast to their efficacy in certain short-term models of platelet activation, the experience with thromboxane synthase inhibitors in clinical trials has been unencouraging. Although the greatest experience has been gained with dazoxiben, the first of these compounds to be studied in man, the human pharmacology of the subsequently developed inhibitors suggests that their effects would be similar.

Dazoxiben has been studied in human syndromes putatively associated with platelet activation. For example, Belch et al. administered a daily dose of 400 mg for 6 weeks to patients with Raynaud’s phenomenon. Subjective clinical improvement occurred in some patients treated with dazoxiben, but there was no improvement in the objective parameters (hand temperature, plasma viscosity, and red cell deformability). Furthermore, two double-blind placebo-controlled studies in patients with Raynaud’s phenomenon have failed to detect any benefit from dazoxiben. Luderer et al. found no improvement in total digital blood flow, capillary flow, or forearm blood flow at 28° or 20° C in 21 patients receiving 100 mg dazoxiben four times a day for 2 weeks. Ettinger et al. reported that the subjective evaluation of severity of disease and episode rate were unaltered by dazoxiben. Recently, we have failed to demonstrate efficacy in a double-

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**FIGURE 1.** Preincubation of platelets for 2.5 min with a thromboxane synthase inhibitor, dazoxiben (2μg/ml), prolonged the lag time to 50% of full aggregation induced by arachidonic acid (1.33 mM). Preincubation of the platelets with dazoxiben for a longer period (5 min) permitted complete inhibition of platelet aggregation.
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APPENDIX

PLATELETS AND VASCULAR OCCLUSION

blind placebo-controlled study of another thromboxane synthase inhibitor (CGS 13080 100 mg qid and 200 mg qid) in patients with severe peripheral vascular disease.59 Zipser et al.60 have recently reported that the imidazol analog dazmagrel has no effect on renal or hepatic function in patients with the hepatorenal syndrome. A reported benefit of dazoxiben therapy in patients with diabetic nephropathy based on a reduction in microalbuminuria61 has not been confirmed by a subsequent, larger study. *

Long-term dosing studies with thromboxane synthase inhibitors have not been reported in patients with primary cardiac disease. McGibney et al.62 have reported that predosing with dazoxiben (100 mg) failed to influence the time to exercise-induced angina despite the inhibition of thromboxane formation in serum ex vivo by 80% or more. Reuben et al.63 performed a double-blind randomized crossover study of 200 mg dazoxiben four times daily for 7 days in patients with stable angina who had had a positive stress test. Serum thromboxane B2 was reduced by approximately 80% at the time of exercise testing during dazoxiben therapy. No change was observed in the duration of exercise to angina, the cumulative ST depression, the angina attack rate or in glyceryl trinitrate consumption. Kiff et al.64 reported that pretreatment with 200 mg dazoxiben had no effect on the hemodynamic response to atrial pacing in patients with chronic stable angina. Lactate extraction and the percentage oxygen uptake were also unaltered. By contrast, Thaulow et al.65 reported that pretreatment with dazoxiben (200 mg) reduced lactate accumulation and ST depression during pacing-induced angina. Myocardial extraction of free fatty acids and the arteriovenous oxygen difference were unaltered by dazoxiben. However, the changes in lactate accumulation in this study were marginal, and the study did not include a placebo group.

Let us consider several factors that might explain the discrepancy between the efficacy of thromboxane synthase inhibitors in short-term animal preparations of thrombosis and their lack of effect in clinical studies.

The role of thromboxane A2 in the syndromes studied. Although the clinical conditions that have been studied are putatively associated with platelet activation, evidence of increased thromboxane biosynthesis has generally not been provided. However, despite documentation of increased thromboxane formation in vivo in patients with the hepatorenal syndrome60 and severe peripheral vascular disease59 by acceptable methodolo-

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*Tyler H: Personal communication.

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FIGURE 3. Aggregation of platelet-rich plasma to arachidonic acid (1.33 mM) resulted in formation of 216 ng/ml thromboxane (Tx) B_2 6 min after addition of the agonist. Preincubation with the thromboxane synthase inhibitor dazoxiben did not alter the aggregatory response in this nonresponder, despite inhibition of thromboxane formation. Coincubation with dazoxiben and the antagonist of the prostaglandin endoperoxide/thromboxane A_2 receptor EPO45 completely inhibited aggregation.

...h, in controlled studies with synthase inhibitors no effect was observed. Similarly, although increased thromboxane formation was not documented in the studies of patients with Raynaud's phenomenon, we have recently found that such patients do indeed excrete excessive quantities of the urinary thromboxane metabolite 2,3-dinor-thromboxane B_2. Thus, in these long-term dosing studies, one assumes that the synthase inhibitors were ineffective either because thromboxane formation was an epiphenomenon of little relevance to the clinical syndrome or that it was of importance but that the compounds studied inadequately blocked its biological action. Although synthase inhibitors fail to alter the response to exercise and pacing in patients with chronic stable angina in short-term dosing studies, the only investigations that have suggested that thromboxane formation is enhanced in these settings have been based on plasma immunoreactive thromboxane B_2. These reports are likely to have been confounded by platelet thromboxane formation ex vivo and/or problems of analytic methodology.

In summary, despite enhanced in vivo thromboxane synthesis in patients with Raynaud's phenomenon, severe peripheral vascular disease, and the hepatorenal syndrome, conventional long-term dosage schedules for thromboxane synthase inhibitors have proven ineffective. The effects of short-term administration of synthase inhibitors in pacing- and exercise-induced angina have also been unconvincing. However, whether thromboxane formation is increased in these settings remains to be determined. Two reports suggest that short-term administration of thromboxane synthase inhibitors may reduce the incidence of ventricular fibrillation after coronary artery occlusion in the dog. Whether thromboxane formation is enhanced in this setting has yet to be established and the antiarrhythmic properties of thromboxane synthase inhibition remain to be explored in man.

Is thromboxane formation adequately suppressed throughout the dosing interval in long-term dosing studies? Studies of platelet function both in vitro and ex vivo indicate that maximal (~100%) inhibition of platelet cyclooxygenase is necessary to inhibit thromboxane-dependent platelet activation. Thus, clinical benefit may be anticipated only when inhibition of thromboxane is complete. All of the compounds that have been developed thus far are reversible inhibitors of thromboxane synthase. Plasma drug half-lives vary from ~45 to ~90 min. However, more attention has been paid to the "biological" half-lives. These are calculated from the recovery of platelet thromboxane formation ex vivo after inhibition by short-term administration of a synthase inhibitor and have varied from ~3 to 6 hr. This discrepancy has not been explained by the identification of active drug metabolites, but can be readily anticipated from well-characterized plasma drug kinetic-dynamic relationships. This factor appears to have been ignored during the design of long-term dosing studies with these inhibitors, since the dosing interval has been based on the biological half-life. When dosing interval is based on plasma drug half-life, the pharmacokinetic assumption of accumulation to a steady-state effect in three to four half-lives is readily verified. However, no such assumptions can be derived from the employment of biological half-life. Thus, in a study of patients with severe peripheral vascular disease, recovery of thromboxane formation ex vivo and in vivo was just as rapid after administration of the twenty-fifth dose as after the first. Recovery of thromboxane formation would also be consistent with the discrepancy between the efficacy of thromboxane synthase inhibition in certain short-term animal preparations — in which observations are usually made during the period of maximal thromboxane inhibition — and the lack of effect during long-term dosing stud-
ies in human disease. Although little data are available that permit direct testing of this hypothesis in man, it is noteworthy that despite the failure of long-term dosing with inhibitors to ameliorate Raynaud's phenomenon, a condition associated with enhanced thromboxane biosynthesis, Cowley et al. have demonstrated that short-term administration of dazoxiben suppressed cold-induced vasoconstriction when blood flow was measured at the time of maximal inhibition of thromboxane formation.

In summary, these results suggest that substantial recovery of thromboxane formation is likely to have occurred during the dosing interval in published long-term dosing studies of thromboxane synthase inhibitors. Consequently, even if thromboxane-dependent platelet activation were of relevance to the clinical condition studied, benefit would not be anticipated.

Prostaglandin endoperoxides might substitute for the biological action of thromboxane A_2 during inhibition of the synthase enzyme. Experiments in vitro, as outlined above, combining antagonists of the thromboxane A_2/prostaglandin endoperoxide receptor with synthase inhibitors, suggest that endoperoxides may continue to mediate proaggregatory effects on platelets despite inhibition of thromboxane formation. Data supportive of the concept that this may be of importance in vivo are provided by the observation ex vivo of platelet nonresponders to thromboxane synthase inhibitors. Indeed, even responders demonstrate markedly less inhibition of platelets ex vivo than is obtained after comparable inhibition of thromboxane biosynthesis with aspirin. However, direct evidence in support of this hypothesis is unavailable from human experiments. We have recently addressed this issue by comparing the efficacy of a thromboxane/prostaglandin endoperoxide antagonist, a synthesis inhibitor, and their combination in a canine preparation of coronary vascular occlusion. The parameters of this preparation can be set so that occlusion becomes prostaglandin endoperoxide-dependent in the presence of a synthase inhibitor. Under these circumstances, the antagonist, but not the synthesis inhibitor, prolongs the time to occlusion. This supports the concept of endoperoxide-dependent platelet activation in vivo. Furthermore, combination with the synthase inhibitor improved the efficacy of the antagonist in this preparation, as would be anticipated from increased formation of vasodilator platelet-inhibitory eicosanoids at sites of platelet-vascular interaction.

In conclusion, the future place of thromboxane synthase inhibitors in the treatment of vasoocclusive disease in man is dependent on the importance of thromboxane A_2 and prostacyclin as mediators of platelet and vascular homeostasis in vivo. The development of sensitive methods of measuring enzymatic metabolites of thromboxane in urine and plasma and the availability of receptor antagonists are likely to clarify the role of thromboxane A_2 in the coming years. The efficacy of synthase inhibitors is perhaps especially dependent on a role for prostacyclin in the preservation of endothelial thromboresistance. If prostacyclin is important, inhibition of platelet function without depression of vascular prostacyclin formation would be indeed desirable. However, such "selectivity" can be approached with aspirin. More importantly, enhanced formation of prostacyclin at sites of platelet-vascular interaction might be a particular advantage of the use of this class of compounds since prostacyclin inhibits platelet activation by both thromboxane-dependent and thromboxane-independent mechanisms. Finally, even if these assumptions are proven to be facts, modifications of either drug structure or delivery will be necessary to prolong the duration of maximal thromboxane inhibition throughout the dosing interval. Combination of synthesis inhibitors with receptor antagonists may be necessary to realize the full therapeutic potential of these interesting compounds.

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CIRCULATION
The biochemical pharmacology of thromboxane synthase inhibition in man.
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Circulation. 1985;72:1194-1201
doi: 10.1161/01.CIR.72.6.1194

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