REORIENTATION OF PROSTAGLANDIN ENDOPEROXIDE METABOLISM BY A THROMBOXANE SYNTHETASE INHIBITOR: *IN VITRO* AND CLINICAL OBSERVATIONS

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- 1 Work with dazoxiben *in vitro* and *in vivo* suggests that a diminished capacity of platelets to synthesize thromboxane A₂ results in a reorientation of the metabolism of cyclic endoperoxides.
- 2 Platelets of patients with congenital thromboxane synthetase deficiency show the same phenomenon.
- 3 In the presence of endothelium or leukocytes which have prostacyclin synthetase capacity, significant amounts of prostacyclin can be generated if thromboxane synthesis is blocked.
- 4 The local generation of aggregation inhibiting prostaglandins in areas of vascular damage may be an interesting therapeutic concept.
- 5 Pilot clinical studies of thromboxane synthetase inhibitor dazoxiben (UK 37248) in a small number of patients with peripheral arterial disease did not reveal major or consistent haemodynamic changes.
- 6 Attention is directed towards optimizing pharmacologically the generation and efficacy of the inhibitory prostaglandins produced when thromboxane synthetase is inhibited.

A THROMBOXANE synthetase inhibitor should selectively block the transformation of cyclic endoperoxides into thromboxane A₂ (TXA₂), without affecting the generation of these cyclic endoperoxides from arachidonic acid. Inhibition of thromboxane synthesis alone is probably not the most efficient way to impede platelet function, since platelet activation occurs through a number of parallel pathways (Vermynen *et al.*, 1982), of which thromboxane formation is only one. On the other hand, all these pathways are initiated by an increased concentration of ionized calcium in the cytoplasm (Detwiler *et al.*, 1978). Inhibition of this mobilization of Ca²⁺ from the dense tubular system would prevent platelet activation by any agonist. This therefore could be an ideal step at which to intervene pharmacologically.

We now know that there is an active mechanism that reduces cytoplasmic calcium levels. This mechanism is adenosine-3',5'-cyclic monophosphate (cyclic AMP)-dependent (Käser-Glanzmann *et al.*, 1977). An increase in platelet cyclic AMP reduces the effect of whatever platelet activator is used. Prostaglandins D₂ and I₂ (prostacyclin) activate platelet adenylate cyclase, the enzyme responsible for the synthesis of cyclic AMP. If thromboxane synthesis inhibition would reorient the metabolism of cyclic endoperoxides within platelets towards PGD₂ or would allow cyclic endoperoxides to escape from the platelets and be transformed by other cells into prostacyclin, these reactions could constitute additional indirect means of inhibiting platelet function, that

could be as important as the direct prevention of thromboxane synthesis itself. We review here the evidence we have that such reorientation may occur.

Our interest in thromboxane synthetase inhibition arose in part as a result of a detailed study of a family with congenital partial platelet thromboxane synthetase deficiency (Defreyn *et al.*, 1981). This family presents a moderate bleeding tendency, a strikingly prolonged bleeding time, absent platelet aggregation with arachidonic acid, reversible aggregation with adenosine-5'-diphosphate, moderately reduced malondialdehyde formation after platelet stimulation with N-ethylmaleimide and decreased serum thromboxane B₂ (TXB₂) levels. When their washed platelets are incubated with [³H]-arachidonic acid, the final metabolite of the lipoxygenase pathway (12-L-hydroxytetraenoic acid, HETE) is produced in normal amounts but the production of TX B₂ and HHT (12-L-hydroxy-5,8,10-heptadecatrienoic acid) is decreased, whereas prostaglandins F_{2α}, E₂ and probably D₂ are increased.

Evidence for enhanced production of PGD₂ is also provided by the rise in the patient's platelet cyclic AMP levels following stimulation with arachidonic acid in plasma. The patient's washed platelets strongly stimulate the production of 6-ketoprostaglandin F_{1α} (the stable degradation product of prostacyclin) by aspirin-pretreated cultured bovine endothelial cells. The decreased production of TXB₂, HHT and malondialdehyde and increased formation of prostaglandins F_{2α}, E₂, D₂ and of 6-ketoprostaglandin F_{1α}

(in the presence of endothelial cells) are compatible with a partial platelet thromboxane synthetase deficiency and reorientation of cyclic endoperoxide metabolism towards other prostaglandins. The markedly prolonged bleeding time would result not only from reduced formation of TXA_2 but also from increased production of the aggregation inhibiting prostaglandins I_2 and D_2 .

On the basis of this study of patients with platelet thromboxane synthetase deficiency we suggested that an effective thromboxane synthetase inhibitor may be a more ideal suppressor of platelet activity than a cyclo-oxygenase inhibitor, which inhibits formation of all prostaglandins. A thromboxane synthetase inhibitor on the other hand would be expected not only to diminish the production of aggregating TXA_2 but also to enhance formation of the aggregation inhibiting compounds prostacyclin and PGD_2 . We therefore were very interested in studying dazoxiben (Tyler *et al.*, 1981).

By means of *in vitro* experiments we confirmed that dazoxiben reduces formation of TXB_2 and HHT by washed platelets and found that this was compensated for by an increased production of prostaglandins E_2 and $\text{F}_{2\alpha}$; arachidonic acid challenged platelets pretreated with dazoxiben strongly stimulated the production of prostacyclin by aspirin pretreated cultured endothelial cells. This confirmed that blockage of thromboxane synthetase increases release of platelet cyclic endoperoxides, which then become available for transformation into prostacyclin by the endothelial cells. In a double-blind placebo controlled study to examine the *in vivo* properties of dazoxiben, human volunteers ingested 200 mg of the compound. Their serum TXB_2 levels dropped and their plasma 6-ketoprostaglandin $\text{F}_{1\alpha}$ values rose. Arachidonic acid-induced platelet aggregation was inhibited whereas that elicited by adenosine-5'-diphosphate was unaffected (Vermylen *et al.*, 1981).

Further *in vitro* studies showed that the cyclic AMP level did not consistently change when dazoxiben pretreated platelets were challenged with arachidonic acid in plasma. This may indicate that with the drug smaller (or more variable) quantities of PGD_2 are formed than in the family with thromboxane synthetase deficiency, when the metabolism of the cyclic endoperoxides is reoriented. Unfortunately quantitative assays of PGD_2 in plasma are not readily available. The involvement of PGD_2 in the inhibition of platelet aggregation by dazoxiben is discussed by others during this symposium. We found that dazoxiben does not activate platelet cyclic AMP degrading phosphodiesterases.

We realized that platelet-rich plasma (PRP) is not suited for the study of possible reorientation of cyclic endoperoxides towards prostacyclin by a

thromboxane synthetase inhibitor, as it does not contain a prostacyclin synthetase. We therefore decided to study the effect of dazoxiben on prostaglandin generation in citrated whole blood (Defreyn *et al.*, 1982). Immediately after addition of collagen to citrated whole blood, the levels of 6-ketoprostaglandin $\text{F}_{1\alpha}$ and PGE_2 rise markedly, particularly when thromboxane synthetase is blocked. There is a further gradual rise of 6-ketoprostaglandin $\text{F}_{1\alpha}$ as a function of time. Further analysis revealed that the initial "jump" of 6-ketoprostaglandin $\text{F}_{1\alpha}$ results from transfer of cyclic endoperoxides from platelets to leukocytes possessing prostacyclin synthetase, and that the further gradual increase is due to prostacyclin synthesis from endogenous substrate by the activated leukocytes. A thromboxane synthetase inhibitor enhances both the amount of endoperoxides escaping from the platelet and serving as substrate for the prostacyclin synthetase and the amount of prostacyclin produced from endogenous substrate.

These *in vitro* findings may offer new therapeutic perspectives. In the area of a vascular lesion where blood would be in contact with collagenous sub-endothelial structures, a thromboxane synthetase inhibitor would reduce local formation of thromboxane and enhance local production of prostacyclin: the increased formation of vasodilating prostacyclin at the site of a vascular lesion may improve local perfusion. If so, thromboxane synthetase inhibitors may form an entirely new class of vasodilators, for the first time acting selectively at the level of the diseased blood vessels only.

We have recently performed a series of as yet unpublished experiments to substantiate *in vivo* this concept derived from *in vitro* experiments. A nylon thread is introduced into a rabbit external jugular vein; the size of the thread is such that an occlusive thrombus does not form; blood that has just passed the thread is sampled at regular intervals for prostaglandin assays via the facial vein. Control samples are taken from the opposite side. The thread results in local production of TXA_2 , that continues throughout the experiment (3–4 h). Local prostacyclin generation initially is high, but decreases markedly with time. Infusion of the thromboxane synthetase inhibitor dazoxiben through the femoral vein results in a prompt drop of local TXA_2 production; on the contrary, local prostacyclin formation, instead of falling, tends to rise. This finding confirms that *in vivo* dazoxiben can also reorient the metabolism of cyclic endoperoxides away from TXA_2 and towards prostacyclin.

A word of caution should be introduced here. Although indeed collagen stimulation of citrated whole blood in the presence of dazoxiben reorients some cyclic endoperoxides towards prostacyclin, much

Table 1 Plasma and serum levels of 6-ketoprostaglandin $F_{1\alpha}$ and serum levels of TXB_2 before and during treatment with dazoxiben

	Plasma (pg/ml)	6-keto-PGF _{1α} Serum (ng/ml)	TXB ₂ serum (ng/ml)
Normal values	44±29	2.6±1.9	290±50
Patient A			
Pretreatment	51	—	162
Dazoxiben 400 mg daily for 1 week	123	28	84
Placebo for 1 week	14	5	132
Dazoxiben 800 mg daily for 3 d	218	31	39
Patient B			
Pretreatment	0	0	58
Dazoxiben 400 mg daily for 1 week	187	4	12
Dazoxiben 800 mg daily for 3 d	418	5	6
Patient C			
Pretreatment	7	1.1	128
Dazoxiben 800 mg daily for 4 d	17	1.6	1.4
Patient D			
Pretreatment	8	—	—
Dazoxiben 400 mg daily for 1 week	101	—	—
Dazoxiben 400 mg daily for 1 week	121	1.5	1.8
Dazoxiben 800 mg daily for 4 d	122	2.9	0.5

more cyclic endoperoxides are transformed into PGE_2 . Prostaglandins E_2 and I_2 share a common receptor on platelet adenylate cyclase (Miller & Gormans, 1979). In that way, PGE_2 could antagonize prostacyclin binding and thus diminish prostacyclin-mediated inhibition of platelet aggregation. In other words, although dazoxiben indirectly stimulates prostacyclin production, the latter may be a less effective inhibitor as a result of simultaneously formed PGE_2 . On the other hand, the affinity of the receptor for these two prostaglandins could be different. Furthermore, the extrapolation of these findings to inhibition or not of platelet aggregation *in vivo* is complicated by the different rates at which the prostaglandins are metabolized. PGD_2 has a distinct receptor. At least from a theoretical point of view, association to dazoxiben of a compound, that reorients cyclic endoperoxides away from PGE_2 towards its isomer PGD_2 , would be worth studying.

The second half of this presentation concerns initial observations in patients. The purpose of this pilot study with dazoxiben in peripheral arterial disease was to investigate whether dazoxiben produces acute haemodynamic effects. Additionally it was aimed to further document the effect of dazoxiben on platelet aggregation and prostaglandin metabolism. In view of the limited number of patients studied until now, the emphasis here is on the second aim, although the clinical observations are presented briefly. This pilot study received the approval of the ethical committee of our university. Patient A, a 44-year-old male, developed acute ischaemia of the left leg 2 months before admission.

A fortnight before admission a 2.5×1.5 cm ischaemic ulcer had developed on the back of his left foot. Arteriography revealed occlusions of the superficial femoral, popliteal, anterior tibial, posterior tibial and fibular arteries. He was no candidate for reconstructive surgery and had already undergone lumbar sympathectomy. The patient was treated with dazoxiben 100 mg four times daily for 1 week; the following week he received matched placebo tablets; biochemical results (Table 1) indicated that thromboxane generation had only partially been suppressed by the 400 mg daily dose. For that reason, the patient was given dazoxiben 200 mg four times daily for 3 days. At that moment it became apparent that the ulcer had perforated between the metatarsal bones and that amputation had become unavoidable.

Pain sensation was recorded twice daily by the patient on an unmarked 10 cm scale. The mean ($\pm$ s.e.m.) scores were 1.7 ± 0.5 ($n=11$) pretreatment, 3.3 ± 0.4 ($n=15$) during dazoxiben 400 mg daily, 1.7 ± 0.3 ($n=13$) during placebo and 4.0 ± 0.7 ($n=6$) during dazoxiben 800 mg daily. The increase in pain during the periods of treatment with dazoxiben is statistically significant ($P < 0.025$). The left foot tended to be warmer during active treatment (temperature difference between right and left foot 3.9°C ($n=4$) without, 2.7°C ($n=4$) during dazoxiben). There was no improvement of systolic blood pressure at ankle level (30 mm Hg) or of the walking distance on a treadmill.

Patient B, a 74-year-old diabetic female, had developed severe ischaemic rest pain in the right forefoot 3 weeks beforehand. She also had a super-

Figure 1 Evolution of plasma 6-ketoprostaglandin F_{1α} before and following addition of collagen to citrated whole blood drawn before and during dazoxiben administration.

ficial painful skin defect between the first and second toes. Arteriography revealed lesions at the level of the superficial femoral, popliteal, anterior and posterior tibial and fibular arteries. Vascular reconstruction was not feasible. The patient was treated with 100 mg dazoxiben four times daily for 1 week; there was no improvement of the pain and the dose was increased to 200 mg four times daily for three and a half days, followed by placebo, 200 mg four times daily for three and a half days. Because of the persisting severe pain, making the patient consider amputation, transcutaneous phenolization of the right lumbar sympathetic ganglia was performed. This was followed by a gradual reduction of the ischaemic rest pain; the skin defect between the first and second right toe slowly healed and the patient could be discharged. She has now completed a six-month follow-up; there is no residual nocturnal rest pain nor are there skin defects.

The right ankle systolic blood pressure rose from 35 mm Hg to 70 mm Hg in association with dazoxiben administration and remained at that level after interruption of therapy. It is difficult to decide whether this rise is related to dazoxiben administration or to gradual development of collateral circulation following an acute occlusion. Maximal pain was recorded during high dose dazoxiben. Skin temperatures and maximal walking distance did not change.

Patient C, a 25-year-old female, with severe primary Raynaud's disease in hands and feet, of two years duration, had previously been treated twice with PGE₁ infusions, with good subjective and objective (thermographic) improvement. We therefore wished to investigate in a short-term trial whether administration of dazoxiben would be as effective. Dazoxiben 200 mg was administered four times daily for 3 days. There was no subjective improvement. The patient had slight dizziness and minor orthostatic hypotension (blood pressure lying 122/84 mm Hg; standing 104/72 mm Hg). Thermography and thermocouple skin temperature measurements showed no change during this short-term trial: strong symmetrical peripheral cooling, compatible with Raynaud's disease.

Patient D, a 70-year-old diabetic female, had had intermittent claudication for several years. The week before admission she had developed painful skin defects on the left big toe and left heel. Arteriography revealed diffuse distal angiopathy. Dazoxiben 100 mg four times daily was administered for 2 weeks. As there was no reduction of the pain, it was decided to administer dazoxiben 200 mg four times daily for 4 days. The rest pain remained unbearable despite objective improvement of the diabetic ulcers, making the patient consider amputation. Because of a suspicion of associated diabetic neuropathy, treatment was started with

carbamazepine. Following this, there was rapid reduction of the rest pain. The diabetic ulcers continued to heal and the patient was discharged in satisfactory condition. Skin temperature and haemodynamic parameters were not clearly modified by dazoxiben administration.

Measurements of 6-ketoprostaglandin F_{1α} in citrated plasma and in serum and of TXB₂ in serum were performed with radioimmunoassays without previous extraction as described elsewhere (Defreyn *et al.*, 1982). Table 1 summarizes measurements performed before and during dazoxiben administration. It is apparent that in three of the four patients plasma 6-ketoprostaglandin F_{1α} levels rise in association with dazoxiben administration, that at least in the first patient a daily dose of dazoxiben 400 mg seemed insufficient to drastically suppress thromboxane generation during clotting, and that, with the higher dose of dazoxiben 800 mg daily, the amount of 6-ketoprostaglandin F_{1α} formed during coagulation rose to levels similar to that of TXB₂.

Figure 1 illustrates the evolution of plasma 6-ketoprostaglandin F_{1α} before and following addition of collagen to citrated whole blood drawn at various moments of therapy. The findings are very similar to those observed following addition of dazoxiben to citrated blood *in vitro* (Defreyn *et al.*, 1982), namely a prompt rise of plasma 6-ketoprostaglandin F_{1α} in two steps: an initial "jump" (conversion by white cells of cyclic endoperoxides released by platelets) and a subsequent slope (production of prostacyclin from endogenous arachidonic acid by activated leukocytes). Both components are markedly enhanced during the ingestion of dazoxiben.

In three of the four patients platelet aggregation by threshold concentrations of arachidonic acid, collagen and even by adenosine-5'-diphosphate (namely the second wave of aggregation) was suppressed while taking dazoxiben 800 mg daily for several days. We had not observed inhibition of adenosine-5'-diphosphate induced platelet aggregation in human volunteers after a single oral dose of dazoxiben 200 mg. In the fourth patient (D), analysis of the aggregation curves was complicated by the occurrence of so-called spontaneous platelet aggregation, also during dazoxiben therapy.

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