Pharmacologic Inhibition of Thromboxane Synthetase and Platelet Aggregation: Modulatory Role of Cyclooxygenase Products

By Vittorio Bertelé, Anna Falanga, Marian Tomasiak, Chiara Chiabrando, Chiara Cerletti, and Giovanni de Gaetano

Dazoxiben, an imidazole-derived selective inhibitor of thromboxane A2 (TxA2) synthetase, prevented TxB2 synthesis in vitro in platelet-rich plasma from 16 normal subjects. Inhibition of TxB2 synthesis was accompanied by increased generation of PGE2, PGF2α, and PGD2, as shown by radioimmunoassay, thin-layer radiochromatography, and high-resolution gas chromatography-mass spectrometry. Even at dazoxiben concentrations (40–80 μM) above those inhibiting TxB2 synthesis, platelet aggregation induced by threshold concentrations of arachidonic acid was inhibited in only 4 of 16 subjects, referred to as responders. The remaining 12 individuals were defined as nonresponders. The aggregating effect of arachidonic acid and of the prostaglandin-endoperoxide analog U-46619 was potentiated by PGE2 and prevented by PGD2 at concentrations within the range of those detected in dazoxiben-treated platelet-rich plasma. The antiaggregating effect of dazoxiben was counteracted by PGE2 (in responders) and was potentiated by PGD2 (in nonresponders). Platelets from responders and nonresponders did not differ in the amount of immunoreactive PGE2 material or in their sensitivity to U-46619 or PGD2. It is concluded that inhibition of thromboxane synthetase does not per se prevent platelet aggregation. The functional result of thromboxane suppression appears to be modulated by an interplay of the prostaglandin-endoperoxides, PGE2 and PGD2, which are formed in excess.

A RACHIDONIC ACID (AA) induces human platelet aggregation. This requires oxidation of the precursor fatty acid to intermediate products, prostaglandin (PG) endoperoxides, which in turn are transformed into stable PGs: PGE2, PGD2, PGF2α, and thromboxane A2 (TxA2). It is believed by some authors that the latter metabolite is responsible for platelet aggregation initiated by AA. Drugs inhibiting TxA2 generation should therefore prevent platelet aggregation induced by AA. This is indeed the case for aspirin and other nonsteroidal antiinflammatory drugs that act on cyclooxygenase.

More recently, compounds with selective activity on TxA2-synthetase have become available. These are theoretically more advantageous than aspirin-like drugs, as when administered in vivo, they should only prevent TxA2 formation in platelets without affecting PG generation in other cells (for instance, the antiaggregating PG12 in vascular endothelium). TxA2 synthetase inhibition has therefore been proposed as an antithrombotic strategy. However, with dazoxiben, a selective Tx synthetase inhibitor, platelets from 2 of 3 healthy subjects were normally aggregated by AA despite complete inhibition of Tx synthesis. Similar observations have been reported with dazoxiben and other TxA2 synthetase inhibitors. Other investigators, in contrast, have observed prevention of both Tx synthesis and platelet aggregation. Whether TxA2 formation is essential for platelet aggregation by AA has therefore been questioned.

We have now extended our investigation to a larger number of individuals and confirm that, in the majority of cases, inhibition of TxA2 generation is not, in fact, accompanied by prevention of platelet aggregation. We therefore investigated the possible mechanism by which platelet aggregation induced by AA may occur in the absence of TxA2 generation. In particular, we examined the possible role of PG endoperoxides, PGE2 and PGD2, which are all produced in excess as a consequence of the pharmacologic blockade of TxA2 synthetase.

An attempt was also made to assess whether platelets that do not aggregate in response to AA when Tx synthesis has been inhibited differ in some way from platelets that react normally to AA in the absence of TxA2 production.

MATERIALS AND METHODS

Chemicals

Aggregating agents employed were AA (sodium salt, >99% pure; Sigma, St Louis, MO), adenosine-5'-diphosphate (ADP, sodium salt, Sigma), U-46619 [(1S)-hydroxy-11,9-(epoxymethano)prosta (5Z,13E)-dienoic acid] (Upjohn Co., Kalamazoo, MI), and thrombin (Topostasine, Roche, Milan, Italy). Stock solutions and dilutions of these agents were prepared as described.

PGD2, PGE2, and EIP (9,11-epoxyiminopronsta-5,13-dienoic acid), all from Upjohn, were dissolved in ethyl alcohol, stored at −20°C, and diluted in 0.15 M Tris buffer, pH 7.4, just before use.
SQ-22536, 9-(tetrahydro-2-furyl) adenine (Squibb, Princeton, NJ), was dissolved in ethyl alcohol and diluted in distilled water. Aspirin (lysine acetylsalicylate, Flectadol Maggioni, Milan, Italy) and theophylline monohydrate (Carlo Erba, Milan, Italy) were freshly dissolved in distilled water. Dazoxiben [UK 37,248-01 (4-(2-[1H-imidazole-1-yl)ethoxy] benzoic acid hydrochloride] was dissolved in 0.15 M Tris buffer, pH 7.4.

Platelet Aggregation Studies

Platelet-rich plasma (PRP) was prepared as described\(^7\) from 16 healthy human volunteers (11 males and 5 females, 27–38 yr old) who had not taken drugs for at least 8 days before donating blood. Platelet aggregation tests were made in an Elvi 840 aggregometer (Elvi Logos, Italy).\(^7\) Inhibitors and proaggregating agents or their vehicles were added to PRP samples 1 min before aggregating compounds. The aggregation tests were followed for 3 min and were evaluated by the criteria\(^7\) of the threshold aggregating concentration (TAC) and threshold inhibitory concentration (TIC). TAC was defined as the smallest amount of aggregating agent producing more than 70% of light transmission for AA and U-46619 or inducing two waves of aggregation for ADP. TIC was defined as the smallest amount of inhibitor causing more than 90% inhibition of aggregation induced by TAC of each aggregating agent.

Arachidonic Acid Metabolism

Radioimmunoassays. TxB\(_2\) and PGE\(_2\) immunoreactive materials were measured in PRP samples stirred for 3 min in the presence of AA (at concentrations indicated in the Results section). Radioimmunoassays employed either highly specific rabbit antibodies against TxB\(_2\) (provided by J.B. Smith, Cardesa Foundation, Thomas Jefferson University, Philadelphia, PA)\(^19\) or anti-PGE\(_2\) rabbit antiserum (provided by C. Patrono, Catholic University, Rome, Italy).\(^20\)

Thin-layer radiochromatography (TLC). Analysis of AA metabolism by thin-layer chromatography was performed on washed platelets labeled with \(^{14}C\)-sodium arachidonate (Amersham) and stimulated with thrombin as described.\(^1\)

High-resolution gas chromatography-mass spectrometry. Three different individual PRP samples were incubated at 37\(^\circ\)C for 1 min with 40 \(\mu M\) dazoxiben or its solvent before addition of AA and further incubation for 3 min. PGE\(_2\), PGD\(_2\), and TxB\(_2\) were detected simultaneously in PRP samples using high-resolution gas chromatography coupled to mass spectrometry in the selected ion monitoring mode, as described by Chiabrando et al.\(^21\) Briefly, this method is based on single-step extraction of PG and TxB\(_2\) from acidified PRP samples (pH 3.5) on C18 reversed-phase cartridges (SEP-PAK C18, Waters, Millipore, Segrate, Italy), after addition of deuterated analogs as internal standards. This is followed by derivatization of functional groups and final analysis by high-resolution gas chromatography with selected ion monitoring using SE-54 WCOT persilanized Pyrex glass capillary columns (20 m long; internal diameter 0.35 mm). A LKB 2091-051 gas chromatography-mass spectrometer, equipped with a LKB 2130 computer system and a DANI 3800 gas chromatograph, were used as described.\(^22\)

Statistical Analysis

Student’s \(t\) test was used.

RESULTS

Effects of Dazoxiben on Platelet Function

AA Metabolism

Figure 1 shows the effect of 20 \(\mu M\) dazoxiben on platelet AA metabolism studied by radio-TLC. Concomitantly with complete suppression of the TxB\(_2\) peak, three peaks appeared that were absent or very small in control platelets. These peaks comigrated with authentic PGF\(_{2\alpha}\), PGE\(_2\), and PGD\(_2\); PGE\(_2\) was the highest. The peak corresponding to HHT was partially reduced by dazoxiben, whereas those comigrating with 12-HETE and unmetabolized AA were unmodified.

Figure 2 shows that reorientation of platelet AA metabolism toward PGF\(_{2\alpha}\), PGE\(_2\), and PGD\(_2\) was apparent at concentrations of dazoxiben (0.6–1 \(\mu M\)) that only partially inhibited TxB\(_2\) synthesis. Raising the dazoxiben concentrations to 80 \(\mu M\) did not affect PG synthesis, thus confirming the lack of effect of this drug on platelet cyclooxygenase or PG isomerases.

TLC data were supported by high-resolution gas chromatography coupled to mass spectrometry on three individual samples of PRP incubated with and without 40 \(\mu M\) dazoxiben and stimulated with TAC of AA (0.25–0.35 mM); the absolute amounts of TxB\(_2\),
PGE₂ and PGD₂ in the presence or absence of dazoxiben are reported in Table 1.

**TxB₂ and PGE₂ Immunoreactive Material**

After a 3-min incubation of PRP with TAC of AA (0.2–0.7 mM), both TxB₂ and PGE₂ immunoreactive material could be detected. In 8 samples, TxB₂ averaged 48.4 ± 13.1 ng/10⁸ platelets, and PGE₂ averaged 13.5 ± 3.7 ng/10⁸ platelets (mean ± SEM). Dazoxiben (0.1–20 µM) induced concentration-dependent inhibition of TxB₂ generation. The IC₅₀ value was about 0.6 µM, and complete (>95%) inhibition was obtained with 40 µM dazoxiben. In the latter condition, PGE₂ immunoreactive material rose to 78.5 ± 15.6 ng/10⁸ platelets (n = 8).

**Aggregation Induced by AA**

In 12 of 16 individual PRP samples tested, platelet aggregation induced by TAC of AA was not affected by any concentration of dazoxiben (up to 1 mM). TxB₂ generation (measured by RIA) was completely prevented in all 12 subjects at concentrations of dazoxiben between 20 and 80 µM; these subjects were considered to be nonresponders to dazoxiben.

In the remaining 4 individual PRP samples, all from male donors, platelet aggregation induced by TAC of AA was inhibited by dazoxiben at concentrations between 40 and 80 µM. In all 4 subjects, TxB₂ synthesis was completely prevented at concentrations of dazoxiben between 20 and 80 µM. These 4 subjects were considered to be responders to dazoxiben.

At intervals from 1 day to 6 mo, at least one further blood sample was collected from all 16 volunteers. In PRP from the 12 subjects designated as nonresponders, dazoxiben never inhibited aggregation induced by TAC of AA. In PRP from the four subjects designated as responders, dazoxiben occasionally did not inhibit the aggregation induced by TAC of AA.

As summarized in Table 2, the inhibitory effect of dazoxiben on platelet aggregation was overcome by raising the AA concentrations above the TAC. Under these conditions, dazoxiben still inhibited TxB₂ generation by more than 90%. The antiaggregating activity of dazoxiben also disappeared when the TAC of AA was unchanged, but dazoxiben concentrations lower than the TIC were used. In the latter condition, dazoxiben was slightly less effective as an inhibitor of TxB₂ synthesis. However, the absolute amount of TxB₂ generated in the presence of the lower dazoxiben concentration was less than in control platelets challenged with subthreshold (nonaggregating) concentrations of AA. Thus, lowering the dazoxiben concentration resulted in restoration of aggregation despite the presence of nonaggregating levels of TxA₂.

AA-induced platelet aggregation was inhibited in all 16 PRP samples by EIP, a PGH₂/TxA₂ receptor antagonist, at concentrations between 9 and 30 µM. The TIC of EIP reduced TxB₂ generation by 72.6% ± 3.6% (n = 10). AA-induced platelet aggregation in both responder and nonresponder PRP samples was completely prevented by 100 µM aspirin (data not shown).

**Aggregation Induced by U-46619 and ADP**

In all subjects, platelet aggregation induced by either U-46619, a stable endoperoxide analog and TxA₂ mimic, or ADP was not modified by dazoxiben at any concentration used. In contrast, EIP prevented aggregation induced by U-46619 and ADP (second wave). All individuals were considered to be nonresponders to dazoxiben as regards the ADP-induced second wave of aggregation.

**Modulatory Role of Cyclooxygenase Products**

**PG Cyclic Endoperoxides**

Because endoperoxides may accumulate when TxA₂ synthetase is inhibited by dazoxiben, we investigated whether sensitivity of platelets to the aggregatory activity of the endoperoxide analog U-46619 was dif-

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### Table 1. Quantitative Profiling of Prostaglandins and Thromboxane B₂ Measured in PRP by High-Resolution Gas Chromatography-Mass Spectrometry

<table>
<thead>
<tr>
<th></th>
<th>TxB₂ (ng/10⁸ Platelets)</th>
<th>PGE₂ (ng/10⁸ Platelets)</th>
<th>PGD₂ (ng/10⁸ Platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.1</td>
<td>18.0</td>
<td>ND</td>
</tr>
<tr>
<td>36.9</td>
<td>15.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>26.3</td>
<td>17.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dazoxiben</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(40 µM)</td>
<td>ND</td>
<td>67.5</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>42.0</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>50.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Detection limit</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

*Values are ng/10⁸ platelets. ND, not detectable.*

### Table 2. Effect of Dazoxiben on Platelet Aggregation and TxB₂ Generation Induced by AA in PRP From Four Responders

<table>
<thead>
<tr>
<th></th>
<th>Platelet Aggregation</th>
<th>TxB₂ (ng/10⁸ Platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Dazoxiben</td>
<td>Control</td>
</tr>
<tr>
<td>&lt;TAC*</td>
<td>—</td>
<td>10.0 ± 2.2</td>
</tr>
<tr>
<td>TAC†</td>
<td>TIC</td>
<td>46.0 ± 8.3</td>
</tr>
<tr>
<td>TAC</td>
<td>&lt;TIC</td>
<td>46.0 ± 8.3</td>
</tr>
<tr>
<td>&gt;TAC</td>
<td>TIC</td>
<td>79.9 ± 14.4</td>
</tr>
</tbody>
</table>

*AA was used at three concentrations (µM): <TAC (0.25 ± 0.05), TAC (0.47 ± 0.03), >TAC (0.64 ± 0.07). Dazoxiben was used at two µM concentrations: TIC (64.4 ± 16.9), <TIC (47.5 ± 12.5). Platelet aggregation was indicated as positive (+) when light transmission was more than 70% and as negative (−) when it was less than 10%.

Mean ± SEM; n = 4.

*Threshold aggregating concentration.
†Threshold inhibitory concentration.
different in responders and nonresponders. The TAC of U-46619 were 450, 480, 750 and 840 nM in 4 responders and 300, 450, 480, and 600 nM in 4 nonresponders ($p > 0.05$).

$PGE_2$

As reported in Figs. 1 and 2 and Table 1, Tx synthetase blockade was accompanied by a marked increase of $PGE_2$. This increase, however, did not distinguish between 4 responders and 4 nonresponders, as similar amounts of $PGE_2$-immunoreactive material ($74.0 \pm 23.3$ and $83.1 \pm 23.9$ ng/10$^8$ platelets) were produced in PRP from the two groups when platelets were challenged with AA in the presence of dazoxiben.

However, addition of 20–100 nM $PGE_2$ to PRP from a responder reversed the inhibitory effect of dazoxiben on AA-induced platelet aggregation (Fig. 3).

Moreover, anti-$PGE_2$ antiserum turned nonresponder PRP into responder. As shown in Fig. 4, the antiserum neutralized about 75% of the $PGE_2$ formed in excess after inhibition of Tx synthesis.

$PGE_2$ (20–100 nM), inactive by itself on platelet aggregation, markedly potentiated the aggregating effect of AA or U-46619 (Fig. 5, left panel). In contrast, $PGE_2$ did not potentiate AA, but did enhance U-46619-induced aggregation of “aspirinated” platelets (thus unable to generate platelet endoperoxides) (Fig. 5, right panel).

$PGD_2$

As reported in Figs. 1 and 2 and Table 1, Tx synthetase blockade was accompanied by increased PGD$_2$ generation. Because PGD$_2$ is a potent inhibitor of platelet aggregation, we tested whether platelets from responders were more sensitive to the antiaggregating effect of PGD$_2$ than platelets from nonresponders. This was not the case, as the average TIC of PGD$_2$ was $21.5 \pm 2.9$ nM in responders and $26.7 \pm 7.1$ nM in nonresponders ($p > 0.05$). However, PGD$_2$ at nanomolar concentrations turned the PRP from a nonresponder into a responder (Fig. 6).

PGD$_2$ inhibits platelet aggregation by stimulation of adenylate-cyclase and subsequent increase of intracellular cyclic-AMP. Figure 7 (right panel) shows that in fact the inhibitory effect of PGD$_2$ was prevented by SQ-22536, an inhibitor of adenylate-cyclase. This figure (left panel) shows that SQ-22536 counteracted the inhibitory activity of dazoxiben in responder PRP.

On the other hand, phosphodiesterase inhibitors,
such as theophylline, by preventing c-AMP degradation, potentiate the antiaggregating effect of adenylate-cyclase activators. Figure 8 shows that theophylline, at concentrations that did not affect platelet aggregation, turned a nonresponder PRP to dazoxiben into a responder.

In additional experiments (not shown here), lower concentrations of theophylline did not potentiate dazoxiben, except in the presence of ineffective concentrations of PGD$_2$.

**DISCUSSION**

This study shows that selective inhibition of platelet TxA$_2$ synthesis need not result in prevention of platelet aggregation. Dazoxiben, an imidazole derivative and selective inhibitor of TxA$_2$ synthetase, completely prevents platelet TxB$_2$ synthesis in vitro at concentrations between 20 and 80 $\mu$M. At these concentrations, however, platelet aggregation induced by AA was only inhibited in 4 of 16 individual PRP samples. Aggregation in the remaining 12 samples was invariably and repeatedly unaffected, even by concentrations of dazoxiben as high as 1 mM. This confirms and extends previous observations by ourselves$^6$ and others.$^8,25$

Based on structure–activity studies with different endoperoxides and TxA$_2$, Raz et al.$^7$ also concluded that conversion of PG endoperoxides to TxA$_2$ is not an essential biochemical step in the induction of platelet aggregation by endoperoxides and/or AA. In contrast, Fitzpatrick et al.$^{12}$ suggested that endoperoxides must be converted to TxA$_2$ in order to induce platelet aggregation. This conclusion was mainly based on the observation that the synthetic prostaglandin analog, 9,11 azoprost-5,13-dienoic acid (azo analog I), inhibited both TxA$_2$ synthetase and platelet aggregation. Further studies, however, have shown that azo-analog I is not only a TxA$_2$ synthetase inhibitor, but also a PGH$_2$/TxA$_2$ receptor antagonist.$^{26}$ It is conceivable, therefore, that the inhibitory effect of this compound on platelet aggregation was not due to its TxA$_2$ synthesis inhibiting capacity. Azo analog I thus behaves like another synthetic prostaglandin analog used in the present study, 9,11-epoxyimino prosta-dienoic acid.$^{27}$ This compound inhibited TxA$_2$ synthesis less effectively than dazoxiben, but prevented platelet aggregation induced by AA, ADP (second wave), or U-46619 in all 16 subjects.

If TxA$_2$ is not necessary for platelet aggregation, what is the mechanism by which AA induces platelet aggregation? The most likely explanation is that PG endoperoxides or other cyclooxygenase products may be sufficient to induce aggregation by AA, when TxA$_2$ synthesis was inhibited.$^6,8,15,25$

The results reported here support this, offering evidence that, besides endoperoxides, both PGE$_2$ and
PGD$_2$ may play a modulatory role on platelet aggregation when TxA$_2$ synthetase is blocked. As in previous studies, both prostaglandins may be synthesized in excess in dazoxiben-treated platelets. The present study shows, by the use of high-resolution gas chromatography-mass spectrometry, that both prostaglandins are indeed produced in this condition. PGE$_2$ may potentiate the aggregatory activity of endoperoxides (see Fig. 5), and PGD$_2$ may prevent it (see Fig. 6). PGE$_2$ was previously shown to stimulate ADP-induced platelet aggregation.

The possible role of endoperoxides in mediating AA-induced aggregation in dazoxiben-treated platelets is supported by the observation that aspirin prevented AA-induced aggregation of platelets whose Tx synthesis had already been completely blocked by dazoxiben, so, presumably, aspirin does not act by preventing TxA$_2$ synthesis.

Results reported in Fig. 5 indicate that the aggregating effect of endoperoxides is strongly potentiated by nanomolar concentrations of PGE$_2$. These are within the range of PGE$_2$ detectable in dazoxiben-treated platelets (see Table 1). Thus, the combination of these compounds, both produced in excess when TxA$_2$ synthetase is blocked, may be responsible for AA-induced platelet aggregation in dazoxiben-treated platelets.

This combined effect could be counteracted, or reduced, by PGD$_2$. Because this prostaglandin stimulates adenylate cyclase, its antiaggregating activity can be more easily shown in the presence of compounds inhibiting phosphodiesterase, such as theophylline (see Fig. 8). The amount of PGD$_2$ that inhibits AA-induced platelet aggregation is within the range of concentrations of this PG produced in dazoxiben-treated platelets (see Table 1).

Despite the evidence that PG endoperoxides, PGE$_2$, and PGD$_2$, modulate platelet function in the absence of TxA$_2$, we were unable to explain the responder and nonresponder behavior to dazoxiben. No difference could be found in the sensitivity to this drug of platelets from either group. Platelets from responders and nonresponders appeared equally sensitive to the aggregating effect of U-46619, an endoperoxide analog and TxA$_2$ mimic. The amount of immunoreactive PGE$_2$ generated in the presence of dazoxiben was very similar in responder and nonresponder platelets, and they showed the same sensitivity to inhibition of platelet aggregation by PGD$_2$.

Difficulties in identifying characteristics that clearly distinguish responders and nonresponders were also encountered by Heptinstall et al. and are increased by the fact that the former may behave the same way as the latter if slight modifications are made to the experimental conditions, for instance, in the concentration of AA used (see Table 2).

Thus, although on the basis of repeated determinations dazoxiben had a fairly constant effect in PRP challenged with threshold concentrations of AA, the division of the subjects into responders and nonresponders might be arbitrary. It could depend, at least in some subjects, on the experimental conditions used, rather than reflecting a truly repeatable qualitative difference between subjects. Nonetheless, different effects of dazoxiben on platelets under different experimental conditions may be relevant for a better understanding of the role of AA metabolites in the physiopathology of platelet function.

In conclusion, inhibition of TxA$_2$ synthetase is usually not accompanied by apparent modification of platelet aggregation. In some particular individuals, the complex balance between PG endoperoxides, PGE$_2$ and PGD$_2$, and their interactions with specific receptors might result in prevention of platelet aggregation associated with TxA$_2$ synthesis inhibition. Thus, TxA$_2$ synthesis is not necessary for platelet aggregation initiated in vitro by AA.

The question as to whether drugs inhibiting TxA$_2$ synthesis exert an antithrombotic effect will only be answered by in vivo investigations and by carefully conducted clinical trials.

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