Inhibition of Thromboxane Biosynthesis and Platelet Function by Indobufen in Type II Diabetes Mellitus

G. Davi, C. Patrono, I. Catalano, N. Custro, C. Giammarresi, A. Ganci, F. Cosentino, A. Notarbartolo

Indobufen is a reversible inhibitor of platelet prostaglandin G/H-synthase. To verify the dose dependence of the antiplatelet effect of indobufen on ex vivo and in vivo indexes of thromboxane (TX) biosynthesis and TXA₂-dependent platelet function, we studied nine patients with non–insulin-dependent diabetes mellitus (NIDDM). This was a randomized, double-blind, crossover study in which each patient was treated with three different daily regimens (50 mg BID, 100 mg BID, and 200 mg BID) of indobufen for 1 week, with a 7-day washout period between treatments. Urinary 11-dehydro-TXB₂ excretion averaged 58.2±21.8 ng/h at baseline. TX metabolite excretion was reduced dose dependently by indobufen: by 67% at 50 mg BID, 72% at 100 mg BID, and 81% at 200 mg BID. Platelet cyclooxygenase activity, ATP release, collagen-induced platelet aggregation, and bleeding time also were modified dose dependently by indobufen. Biochemical demonstration of suppressed platelet TXA₂ in vivo was accompanied by evidence of inhibited platelet function as assessed ex vivo. Under pathophysiological conditions, such as NIDDM, which are associated with enhanced TXA₂ synthesis, more than 95% suppression of platelet cyclooxygenase activity may be necessary to produce virtually maximal inhibition of platelet TXA₂ biosynthesis in vivo. (Arterioscler Thromb. 1993;13:1346-1349.)

KEYWORDS • thromboxane • diabetes mellitus • indobufen • platelet function • in vivo platelet activation

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Methods

Patient Selection

Nine patients with NIDDM (five women and four men; age range, 50 to 67 years) were studied on several occasions between May 1989 and February 1991. NIDDM was defined in accordance with the criteria of the American Diabetes Association. The mean±SD duration of NIDDM was 10.3±5.2 years (range, 2 to 19 years). All patients were hospitalized because of inadequate metabolic control, as assessed in our diabetes clinic; after treatment with oral hypoglycemic drugs. Consequently, insulin therapy was instituted on hospital admission.

All patients had a history or physical examination positive for evidence of macrovascular complications. Five patients had stable angina pectoris or had had a myocardial infarction, and four had peripheral vascular disease. Patients with coronary heart disease were in a stable phase, as judged on the basis of clinical symptoms, electrocardiographic (ECG) monitoring during exercise, and Holter monitoring. Patients with peripheral vascular disease were in Fontaine stage II (intermittent claudication, ankle-arm pressure index of less than 0.85, and no resting pain) with a constant level of pain while walking. Their disorder had been diagnosed on the basis of clinical symptoms, ability to walk a distance, bicycle ergometry, and Doppler echographic study of the lower limbs.

In none of the nine patients had vascular disease undergone detectable progression during the previous

Received from the Departments of Pharmacology (C.P.) and Hematology (G.D., F.C.), University of Chieti School of Medicine, and the Department of Medicine (I.C., N.C., C.G., A.G., A.N.), University of Palermo, Italy.

Correspondence to Prof Carlo Patrono, Università di Chieti "G. D'Annunzio," Facoltà di Medicina e Chirurgia, Via dei Vestini, 31, 66100 Chieti, Italy.
12 months, as judged by evaluation during outpatient visits. Moreover, all had mild-to-moderate symptoms compatible with a virtually normal lifestyle. Three patients were current smokers. Only one patient had arterial hypertension (170/100 mm Hg). Blood cholesterol level was 210.7±42.0 mg/dL. All patients continued to take cardiovascular medication during the study period (β-blockers, calcium channel blockers, or diuretic agents); they were asked to abstain from taking aspirin-like drugs. Patients with renal disease (creatinine clearance of less than 1.3 mL/s, serum creatinine level of more than 175 μmol/L, urinary albumin excretion of more than 0.3 g/d) as well as patients with a body mass index of more than 28 were excluded. At the time of study, all patients were being treated with three daily injections of insulin (intermediate-acting and regular insulin) that had begun 2 to 3 days earlier.

**Study Design**

This was a randomized, double-blind, crossover study in which each patient was treated with three different daily regimens (50 mg BID, 100 mg BID, and 200 mg BID) of indobufen (Ibustrin, Farmitalia Carlo Erba, Milan, Italy). Each dose was given orally for 1 week in identical tablets in a randomized sequence with a 7-day washout period between treatments.

Peripheral venous blood samples for platelet functional and biochemical studies were obtained after an overnight fast, at baseline, on the eighth day of each treatment period (ie, approximately 12 hours after the last dose), and at the end of each washout period.

Three consecutive 24-hour urine collections were performed at baseline and on the fifth, sixth, and seventh days of each treatment or washout period. Urine samples were frozen immediately and kept at −20°C until extraction.

Informed consent was obtained from each patient, and the protocol was approved by the institutional review board.

**Platelet Studies**

Peripheral venous blood was obtained between 8 AM and 9 AM and collected in tubes containing 3.8% sodium citrate (1 mL/9 mL blood). Platelet-rich plasma and platelet-poor plasma were prepared as described previously.

Platelet aggregation was measured in an Elvi 840 (Logos, Milan) aggregometer according to the method of Born. For each agonist, the threshold aggregating concentration (TAC) was defined as the concentration of the agonist that caused 50% to 60% increase in light transmittance within 3 minutes. Serum TXB₂ was measured as described previously.

Platelet ATP secretion in platelet-rich plasma was measured in a Lumi-Aggregometer (ChronoLog Corp, Havertown, Pa) in the presence or absence of luciferin-luciferase (Sigma Chemical, St Louis, Mo) at 37°C with a stirring speed of 900 rpm. The ATP used for standardization was obtained from Sigma Chemical and was added at the end of each aggregation. Collagen was used at a final concentration of 2 μg/mL. Chronolume was obtained from ChronoLog Corp.

Bleeding time was determined using an automatic template device (Simplate II, General Diagnostics, Morris Plains, NJ). The incisions were placed in the longitudinal direction on the volar surface of the upper forearm. One operator carried out all of the bleeding time determinations throughout the studies in the same room with a relatively constant temperature.

**Urinary 11-Dehydro-TXB₂ Assay**

Immunoreactive 11-dehydro-TXB₂ was extracted from 20-mL aliquots of each urine sample (pH was adjusted to 4.0 to 4.5 with formic acid) on Sep-Pak C-18 cartridges (Waters Associates, Milford, Mass) and eluted with ethyl acetate. The eluate was subjected to silicic acid column chromatography and further eluted with a benzene–ethyl acetate–methanol solution (60:40:30, vol/vol/vol). The overall recovery as determined by the addition of 11-dehydro-[^3]H]-TXB₂, averaged 80±6%. Immunoreactive 11-dehydro-TXB₂ eluted from silicic acid columns was assayed at a final dilution of 1:15 to 1:100, as described elsewhere.

**Statistical Analysis**

The data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. A one-way analysis of variance was performed. Subsequent pairwise comparisons were made with the Wilcoxon matched-pairs sign-rank test. Moreover, the correlations between platelet function parameters were assessed by stepwise regression analysis and multiple linear regression. To this effect, the logarithm of the ratio between posttreatment and basal measurements was regressed against the indobufen dose. All values are given as mean±SD. Statistical significance was considered to be indicated by P<.05.

**Results**

Serum TXB₂, a measure of the maximum cyclooxygenase-dependent biosynthetic capacity of blood platelets, averaged 282±76 ng/mL at baseline, 303±52 ng/mL at the end of the first washout period, and 300±73 ng/mL at the end of the second washout period. The intrasubject coefficient of variation averaged 10±4.1%, as determined on the basis of these three separate measurements performed over a period of 4 weeks. As depicted in Fig 1, platelet cyclooxygenase activity was inhibited dose dependently by indobufen: by 85% at 50 mg BID, 91% at 100 mg BID, and 96% at 200 mg BID (y=−1.582−0.00525x; r=−.62; P<.001). These biochemical changes were associated with changes in platelet function, as detailed in the Table. Thus, collagen-induced platelet aggregation was inhibited with a statistically significant linear regression of changes in TAC over the dose of indobufen (y=0.07278+0.003896x; r=.60; P<.002). Arachidonate-induced platelet aggregation, ATP release, and bleeding time were modified significantly by indobufen at 50 to 100 mg BID, with no further changes at the highest dose.

The urinary excretion of 11-dehydro-TXB₂, a measure of in vivo TXA₂ biosynthesis, averaged 58.2±21.8 ng/h at baseline, 61.1±19.9 ng/h during the last 3 days of the first washout period, and 67.9±30.4 ng/h during the last 3 days of the second washout period. The corresponding excretion rate for age- and sex-matched healthy subjects averaged 19.3±5.6 ng/h (n=10; Reference 8). The intrasubject coefficient of variation averaged 28±10%, as determined on the basis of these nine separate control measurements performed throughout
INDEXES OF PLATELET FUNCTION IN PATIENTS WITH TYPE II DIABETES MELLITUS BEFORE, DURING, AND AFTER DAILY TREATMENT WITH INDOBUFEN

<table>
<thead>
<tr>
<th>Indexes of Platelet Function</th>
<th>Baseline</th>
<th>Indobufen, 50 mg BID</th>
<th>Washout</th>
<th>Indobufen, 100 mg BID</th>
<th>Washout</th>
<th>Indobufen, 200 mg BID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen threshold, µg/mL</td>
<td>1.32±0.47</td>
<td>2.15±0.85*</td>
<td>1.37±0.28</td>
<td>3.24±1.73*</td>
<td>1.03±0.59</td>
<td>5.40±2.34f</td>
</tr>
<tr>
<td>Arachidonate threshold, mmol/L</td>
<td>0.61±0.15</td>
<td>0.97±0.17†</td>
<td>0.61±0.22</td>
<td>1.05±0.16†</td>
<td>0.62±0.20</td>
<td>1.02±0.19†</td>
</tr>
<tr>
<td>ATP release, (nmol/L)/mL</td>
<td>1.35±0.38</td>
<td>1.10±0.33</td>
<td>1.43±0.77</td>
<td>0.74±0.44§</td>
<td>1.52±0.59</td>
<td>0.71±0.34§</td>
</tr>
<tr>
<td>Bleeding time, s</td>
<td>307±29</td>
<td>380±50f</td>
<td>297±35</td>
<td>393±44†</td>
<td>290±37</td>
<td>403±47</td>
</tr>
</tbody>
</table>

Collagen threshold was determined in the platelet aggregation assay. ATP release was induced by collagen (2 µg/mL). Values are mean±SD.

*P<.02; **P<.005; †P<.001 vs baseline.
§P<.05; ‡P<.02; ¥<.01 vs 50 mg BID.

Blood samples were obtained between 8 AM and 9 AM, at baseline, on the eighth day of each treatment period (ie, approximately 12 hours after the last oral dose), and at end of each washout period.

Discussion

Inhibition of TXA2-dependent platelet function by aspirin is associated with a reduced risk of vaso-occlusive events. Aspirin dose dependently inhibits the cyclooxygenase activity of platelet prostaglandin G/H-synthase. At the doses (30 to 75 mg) used in recently completed clinical trials, aspirin causes more than 95% suppression of platelet cyclooxygenase activity, as reflected by serum TXB2, and 70 to 80% reduction in vivo TXA2 biosynthesis, as reflected by urinary TX metabolite excretion. The relation between the two has been explored by Reilly and FitzGerald and found to be nonlinear in healthy volunteers. Thus, more than 90% reduction in platelet biosynthetic capacity was required to produce a dose-dependent reduction in TX metabolite excretion, suggesting that a small residual enzymatic activity may be sufficient to sustain the very low level of TXA2 biosynthesis under physiological circumstances. Whether this applies to clinical conditions characterized by enhanced TXA2 biosynthesis has not been investigated previously.

Our study assessed the dose dependence of the effects of indobufen, a reversible inhibitor of platelet prostaglandin G/H-synthase, on ex vivo and in vivo indexes of TXA2 biosynthesis and TXA2-dependent platelet function. We chose a fourfold range of daily doses (50 to 200 mg BID) to the highest recommended therapeutic dose of the drug. Because indobufen pharmacokinetics dictates a twice-daily dosing regimen, we performed biochemical and functional measurements 12 hours after the last dose so as not to overestimate the early effects of lower doses. Thus, when such measurements are performed 2 hours after dosing, no clearcut dose dependence is found for the inhibition of platelet function by indobufen.

The basal measurements of urinary 11-dehydro-TXB2 performed in the present study confirm our earlier findings that indobufen dose dependently reduces TX metabolite excretion in patients with type II diabetes mellitus. Level of metabolite excretion is expressed as a percentage (mean±SD; n=9) of the level observed before indobufen administration. Each patient served as his or her own control. Three consecutive 24-hour urine collections were performed at baseline and on the fifth, sixth, and seventh days of each treatment or washout period. P<.02 for the comparison of 200 mg vs 100 mg; P<.01 for the comparison of 200 mg vs 50 mg.

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findings of enhanced TXA2 biosynthesis in patients with NIDDM with macrovascular complications. This was a relatively reproducible finding over the course of the study, as assessed repeatedly during the two washout periods.

Indobufen caused a dose-dependent suppression of TX metabolite excretion that was more than 80% at the highest dose only (Fig 2). This is comparable to the 80% reduction in 11-dehydro-TXB2 excretion found in patients with NIDDM after 1-week dosing with low-dose aspirin (50 mg/d). Biochemical demonstration of suppressed platelet TXA2 biosynthesis in vivo was accompanied by evidence of inhibited platelet function, as assessed ex vivo (Table). Bleeding time was prolonged only marginally by indobufen, a finding consistent with previous observations.23,24

We conclude that under pathophysiological conditions associated with enhanced TXA2 production, more than 95% suppression of platelet cyclooxygenase activity may be necessary to produce virtually maximal inhibition of platelet TXA2 biosynthesis in vivo. This can be obtained with repeated daily dosing with low-dose aspirin, as a consequence of cumulative inactivation of platelet prostaglandin G/H-synthase, as reported previously; or with the reversible inhibitor of the same enzyme, indobufen, at 200 mg BID, as a function of constant substrate competition during the dosing intervals, as demonstrated in the present study.

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