The contribution of cyclooxygenase-1 and -2 to persistent thromboxane biosynthesis in aspirin-treated essential thrombocythemia: implications for antiplatelet therapy

*Alfredo Dragani,1 *Silvia Pascale,1 Antonio Recchioni,2 Domenico Mattoscio,2,3 Stefano Lattanzio,2 Giovanna Petrucci,4 Luciana Mucci,4 Elisabetta Ferrante,2 Aida Habib,9 Franco O. Ranelletti,6 Giovanni Ciabattoni,7 Giovanni Davi,1,2 Carlo Patrono,4 and Bianca Rocca4

1Department of Hematology, Spirito Santo Hospital, Pescara, Italy; 2Center of Excellence on Aging, G. D'Annunzio University Foundation, Chieti, Italy; 3Department of Biomedical Sciences, University G. D'Annunzio, Chieti, Italy; 4Department of Pharmacology, Catholic University School of Medicine, Rome, Italy; 5Department of Biochemistry, American University of Beirut, Beirut, Lebanon; 6Department of Pathology, Catholic University School of Medicine, Rome, Italy; and 7Department of Drug Sciences, School of Pharmacy, University G. D'Annunzio, Chieti, Italy


*A.D. and S.P. contributed equally to this work.

An Inside Blood analysis of this article appears at the front of this issue.

© 2010 by The American Society of Hematology

We tested whether cyclooxygenase 2 (COX-2) expression and unacetylated COX-1 in newly formed platelets might contribute to persistent thromboxane (TX) biosynthesis in aspirin-treated essential thrombocythemia (ET). Forty-one patients on chronic aspirin (100 mg/day) and 24 healthy subjects were studied. Platelet COX-2 expression was significantly increased in patients and correlated with thiazole orange–positive platelets (r = 0.71, P < .001). The rate of TXA2 biosynthesis in vivo, as reflected by urinary 11-dehydro-TXB2 (TXM) excretion, and the maximal biosynthetic capacity of platelets, as reflected by serum TXB2, were higher in patients compared with aspirin-treated healthy volunteers. Serum TXB2 was significantly reduced by the selective COX-2 inhibitor NS-398 added in vitro. Patients were randomized to adding the selective COX-2 inhibitor, etoricoxib, or continuing aspirin for 7 days. Etoricoxib significantly reduced by approximately 25% TXM excretion and serum TXB2. Fourteen of the 41 patients were studied again 21 (± 7) months after the first visit. Serum TXB2 was consistently reduced by approximately 30% by adding NS398 in vitro, while it was completely suppressed with 50µM aspirin. Accelerated platelet regeneration in most aspirin-treated ET patients may explain aspirin-persistent TXA2 biosynthesis through enhanced COX-2 activity and faster renewal of unacetylated COX-1. These findings may help in reassessing the optimal antiplatelet strategy in ET. (Blood. 2010;115:1054-1061)

Introduction

Essential thrombocythemia (ET) is a myeloproliferative neoplasm characterized by high platelet generation, whose incidence is estimated to be around 1 to 2.5 per 100 000 subjects, although this estimate is likely to increase in the near future due to the continuous rise of “occasional,” asymptomatic diagnoses.1-4 ET usually occurs in 50- to 60-year-old patients and has a longer life-expectancy compared with other myeloproliferative neoplasms.4-7 However up to 60% of all ET patients experience a minor or major thrombotic event in their life, mainly arterial, such as myocardial infarction, stroke, or transient ischemic attack.8,9 Thrombotic complications significantly increase morbidity and impair survival.7,10,11

Annual thrombotic event rates range from 2% to 7% in ET patients treated with cyto-reductive agents with or without antiplatelet drugs,7,9,11-13 with estimates up to 13%, in the absence of cyto-reduction.12 In the Primary Thrombocythemia 1 randomized trial, the annual rate of a composite cardiovascular endpoint was 4% in patients randomized to anagrelide, and 2.7% in the hydroxyurea arm, on a background of aspirin (98% of enrolled patients were on 75 mg of aspirin daily).13 The annual recurrence rate of thrombosis after a first event has been estimated to be approximately 6% to 8% on antiplatelet drugs.14 Hemorrhagic complications are less frequent, approximately 0.33% per year,9 possibly due to an acquired von Willebrand-like defect, associated with the highest platelet counts.15,16

In addition to the clinical evidence of a predominantly arterial thrombotic diathesis, several groups have reported evidence of platelet activation in vivo or ex vivo in ET.17,18 We have previously reported enhanced urinary excretion of thromboxane (TX) metabolites (TXMs) in untreated ET patients.19 TXM excretion is a validated index of in vivo platelet activation, and it has been reported to be consistently increased in different clinical settings at high cardiovascular risk.20

On the basis of the arterial thrombotic diathesis and of the evidence of ongoing platelet activation, low-dose aspirin (75-100 mg once daily) is currently used not only in secondary, but also in primary cardiovascular prevention of ET patients.4,6,9,21 Despite the absence of controlled trials formally assessing the efficacy and safety of aspirin in ET, low-dose aspirin is currently recommended in ET, independently of the underlying cardiovascular risk, including young patients without prior vascular events.5,6,9,21 This recommendation is mainly based on retrospective analyses and extrapolation from polycythemia vera,5,6,9 for which a randomized clinical trial of aspirin was performed.22 Aspirin achieves its antithrombotic effect by permanently and selectively inactivating platelet cyclooxygenase 1 (COX-1), thus blocking TXA2 biosynthesis.23 It has been...
shown that COX-2 is up-regulated during megakaryopoiesis,24 is expressed by normal megakaryocytes and circulating young platelets,24-29 and it is overexpressed in bone marrow megakaryocytes of ET patients.30 Under conditions of physiologic thrombopoiesis, platelet COX-2 does not appear to contribute to TXA2 production, as assessed by selective COX-2 inhibition; such as after bone marrow transplantation, platelet COX-2 can contribute to TXA2 production, as assessed by selective COX-2 inhibition in vitro.24 Thus, it is conceivable that, due to accelerated thrombopoiesis, ET platelets might express relatively higher levels of COX-2, whose contribution to in vivo TXA2 biosynthesis is currently unknown.

The present study was designed to evaluate in ET patients whether (1) the disease is associated with increased platelet COX-2 expression; (2) platelet COX-2 is enzymatically active, contributing to enhanced TXA2 biosynthesis; and (3) the residual TXA2 production in aspirin-treated patients can be fully suppressed by selective COX-2 inhibition and/or by further inactivation of COX-1.

**Methods**

**Design of the study**

We enrolled 41 ET patients diagnosed by established World Health Organization criteria.33 Exclusion criteria were: recent (< 6 mo) major vascular event (myocardial infarction or stroke), bleeding history, gastroduodenal ulcer, aspirin intolerance, obesity, diabetes mellitus, dyslipidemia, hypertension, ongoing or planned pregnancy, use of anticoagulants or antiplatelet agents other than low-dose aspirin, need for chronic nonsteroidal anti-inflammatory drugs (NSAIDs), platelet count more than 1 000 000/µL; hemoglobin, 93 ± 702/g; hematocrit, 40.1 ± 5%; leukocytes, 6100 ± 833/µL [means ± SD]).

The present study included a baseline evaluation, a randomized treatment of aspirin or etoricoxib (V1) and a reexamination of 14 patients.

Patients were studied on 2 separate occasions (V0 and V1), 1 week apart, before randomization and instructed to take aspirin at the same time of the day (late afternoon/evening). At V1 patients were randomized to either continue aspirin (Cardioaspirina; Bayer) 100 mg once daily or to add etoricoxib (Tauxib; Sigma-Tau) 90 mg, once daily for 7 days, to aspirin therapy. Patients were studied again after 7 days of randomized treatment (V2 visit). Blood and urine samples were collected at each visit after an overnight fast. Aspirin and etoricoxib were provided at randomization (V1) and immediately analyzed on FACSCalibur, with 30 000 events being counted in the CD61-positive gate.

**Platelet immunophenotyping and reticulated platelets**

Platelet COX-2 expression was assessed by flow cytometry (fluorescein-activated cell sorter, FACS) and immunocytochemistry on washed platelets. For FACS analysis, platelets were collected in 3.8% sodium citrate and processed within 2 hours. Briefly, after centrifugation (550 rpm, 15 minutes), platelet-rich plasma was aspirated, incubated with 10µM prostaglandin E2 (Cayman Chemicals), and washed twice with Ca/Mg-free Dulbecco phosphate-buffered saline (PBS)/0.5% BSA. An aliquot (10^7 platelets) was permeabilized (BD FACS Permeabilizing Solution 2; BD Biosciences) for 10 minutes at room temperature, centrifuged, resuspended in Dulbecco PBS/bovine serum albumin and incubated for 3 hours at 4°C with the following antibodies: fluorescein isothiocyanate–conjugated mouse anti–COX-1 (Cayman Chemicals), phycoerythrin–conjugated anti–COX-2 (Cayman Chemicals), peridinin-chlorophyll-protein–conjugated mouse anti–CD61 (BD Biosciences), and isotype- and fluorochrome-matched irrelevant mouse immunoglobulin Gs as negative controls. Platelets were examined on a FACSCalibur cytometer (BD Biosciences) on a log scale. The platelet population was identified on the basis of forward and side scatter distribution and CD61 positivity, and 30 000 CD61-positive platelets were acquired and analyzed with the CellQuest software (BD Biosciences). Data were analyzed as mean fluorescence intensity (MFI). For each sample, the values of corresponding negative controls were subtracted from the positively stained sample and expressed as ΔMFI.

Reticulated platelets were determined by flow cytometry using the thiazole orange (TO) method as previously described.24 Nonpermeabilized washed platelets were kept in TO (Retic-Count; BD Biosciences) 10 minutes at room temperature or PBS as control, or incubated with anti–CD61 for 1 hour, then spun down, washed, and resuspended in PBS and immediately analyzed on FACSCalibur, with 30 000 events being counted in the CD61-positive gate.

Immunocytochemistry was performed on washed platelets as previously described,24 using an anti–CD61 monoclonal antibody (Dako) and/or an anti–COX-2 noncommercial polyclonal antibody.36 In single-stain experiments, COX-2 was revealed using the ABC peroxidase kit with a diaminobenzidine chromogen (ScyTech Laboratories). In double staining experiments, CD61 was detected with biotinylated secondary antibodies (Vector Laboratories) revealed with streptavidin horseradish peroxidase and AEC chromogen (ScyTech Laboratories), while COX-2 was detected with secondary antibodies revealed with alkaline phosphatase streptavidin (Vector Laboratories) and Sigma FAST 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chromogen (Sigma-Aldrich). Platelets were lightly counterstained with eosin. In each case, 6 different fields were analyzed by light microscopy (Axiostar microscope; Zeiss), counting 100 platelets per field, and positive platelets were recorded. A mean of 6 counts was considered for each sample.

Western blottings were performed on washed platelets as previously described.37 Briefly, platelets were washed as detailed for flow cytometry, lysed in ice-cold radioimmunoprecipitation assay buffer containing 1% Nonidet P-400-0.5% sodium deoxycholate/0.1% sodium dodecyl sulfate (SDS), protease inhibitors (Sigma-Aldrich), and mechanically homogenized. Samples were electrophoresed in 10% SDS polyacrylamide gel
under reducing conditions. Gels were blotted onto nitrocellulose membranes, which were incubated with monoclonal antibodies against COX-1 or COX-2.36 Positivity was revealed by anti–mouse horseradish peroxidase–conjugated antibodies (Calbiochem) and enhanced chemiluminescence detection reagent (GE Healthcare). Protein bands were visualized using Kodak BioMax light film (Sigma-Aldrich).

### JAK2 mutational analysis

A quantitative real-time polymerase chain reaction (qRT-PCR)–based allelic discrimination assay was used to detect the V617F (JAK2) mutation, using TaqMan real-time technology on an AB7900. Genomic DNA was amplified in 40 cycles at a T annealing of 61°C, in a final volume of 25 µL containing 1× PCR Master Mix (Applied Biosystems), 900nM forward and reverse primers and 100nM each probe. For each DNA sample a control amplification was generated to test DNA amount. Allele relative frequency was calculated as previously described.38

### Statistical analyses

The sample size of the randomized intervention study was calculated to evaluate the impact of selective COX-2 inhibition on in vivo TXA₂ biosynthesis. We estimated that 20 patients per treatment arm (etoricoxib or aspirin) would be required to yield 85% power to detect a 20% reduction in urinary TXM in the etoricoxib arm versus aspirin plus aspirin vs aspirin alone (one-tailed α = 0.05). The percentage of the whole platelet population staining for TXM was positively correlated with COX-2 expression both in patients and controls (Figure 3A-B), while in the 14 control subjects only a nonsignificant trend toward a positive correlation was found (r = 0.3; P = 0.5) or in the whole studied population under study (Figure 3A), while in the 14 control subjects only a nonsignificant trend toward a positive correlation was found (r = 0.24; P = 0.10). Using immunocytochemistry of washed platelets and light microscopy, COX-2 expression in circulating platelets was consistently stronger and detectable in a significantly (P < 0.001) higher percentage of the whole platelet population in samples from patients (18.8% [IQR 16-29%]) compared with controls (6.5% [IQR 4-7%]; Figure 2 and data not shown).

We investigated whether COX-2, detectable in platelets by flow cytometry or immunocytochemistry, had a shift in molecular weight, as previously reported in a different clinical condition, characterized by enhanced platelet COX-2 expression.39 By Western blot analysis, we observed bands of the expected molecular mass (~72 kDa) in platelet extracts from both patients and controls (Figure 3A). Notably, antibodies used for flow cytometry, immunocytochemistry, and Western blotting were from different sources (poly- or monoclonal, commercial or not), and they all detected COX-2 expression in platelets.

In a subgroup of 16 patients (4 women, 12 men; mean age, 53.6 ± 12 years) and 14 healthy subjects (4 women, 10 men; mean age, 45.4 ± 15 years), we studied both platelet COX-2 expression and the fraction of TO-positive platelets. TO-positive, RNA-containing platelets represent the youngest fraction of peripheral platelets.46 The percentage of the whole platelet population staining for TO was positively correlated with COX-2 expression both in patients (r = 0.62, P = .007) and in the entire population under study (Figure 3B), while in the 14 control subjects only a nonsignificant trend toward a positive correlation was found (r = 0.42, P = .14, data shown in Figure 3B). There was no statistically significant correlation between the TO fraction and platelet COX-1 expression in patients (r = −0.19, P = .5) or in the whole studied subjects (r = −0.25, P = 0.3). Interestingly, neither platelet COX-2 expression nor TO-positive platelet fraction significantly correlated

---

### Results

**Phenotypic and enzymatic studies of platelets isolated from ET patients**

The main clinical and haematologic characteristics of the 41 patients are detailed in Table 1. Platelet COX-1, COX-2, and CD61 expression was studied by flow cytometry in patients before randomization, and in 24 age- and sex-matched healthy subjects. MFI values of platelet COX-2 (gated on the CD61* population) were significantly (P < .001) increased in patients compared with controls (Figure 1A-B), while the expression of CD61 and COX-1 was comparable in the 2 groups (CD61, 46 [IQR 30-85] vs 64 [IQR 44-95]; COX-1, 25.1 [IQR 17.5-35.7] vs 36.1 [IQR 24-46.4]; ΔMFI in patients and controls, respectively, P = .24; COX-1, 25.1 [IQR 17.5-35.7] vs 36.1 [IQR 24.4-46.4]; ΔMFI in patients and controls, respectively, P = .10). Using immunocytochemistry of washed platelets and light microscopy, COX-2 expression in circulating platelets was consistently stronger and detectable in a significantly (P < 0.001) higher percentage of the whole platelet population in samples from patients (18.8% [IQR 16-29%]) compared with controls (6.5% [IQR 4-7%]; Figure 2 and data not shown).

---

### Table 1. Baseline characteristics of patients

<table>
<thead>
<tr>
<th></th>
<th>All patients (n = 41)</th>
<th>Aspirin only (n = 21)</th>
<th>Aspirin plus etoricoxib (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>51 [43-63]</td>
<td>55 [44-61]</td>
<td>50.5 [41-64]</td>
</tr>
<tr>
<td>Males (n)</td>
<td>21</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>Time from diagnosis (y)</td>
<td>1 [0.5-4]</td>
<td>1.5 [0.5-4]</td>
<td>1 [0.5-3]</td>
</tr>
<tr>
<td>History of vascular events (n)</td>
<td>MI (5), ischemic stroke (3), TIA (1), vein thrombosis (3)</td>
<td>MI (3), ischemic stroke (1), TIA (1), vein thrombosis (1)</td>
<td>MI (2), ischemic stroke (2), vein thrombosis (2)</td>
</tr>
<tr>
<td>Hydroxyurea (n)</td>
<td>18</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>JAK2 mutation (n)*</td>
<td>23</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Platelet count at diagnosis (per µL)</td>
<td>623 000 [536 500-788 000]</td>
<td>640 000 [535 000-806 000]</td>
<td>625 500 [581 000-778 000]</td>
</tr>
<tr>
<td>Platelet count at study entry (V0; per µL)</td>
<td>444 000 [378 500-636 750]</td>
<td>465 000 [396 000-606 000]</td>
<td>426 500 [373 000-816 000]</td>
</tr>
<tr>
<td>Platelet count at V1 (per µL)</td>
<td>439 500 [355 500-644 500]</td>
<td>448 500 [377 000-577 000]</td>
<td>399 000 [351 000-889 000]</td>
</tr>
<tr>
<td>Platelet count at V2 (per µL)</td>
<td>458 500 [356 000-635 500]</td>
<td>457 000 [375 000-559 000]</td>
<td>458 500 [343 000-771 000]</td>
</tr>
<tr>
<td>Hematocrit at study entry (%)</td>
<td>42.0 [40-44]</td>
<td>42.3 [39.7-44.3]</td>
<td>42.1 [40.2-44.5]</td>
</tr>
<tr>
<td>WBC at study entry (per µL)</td>
<td>7080 [5475-8225]</td>
<td>6910 [5110-7350]</td>
<td>7275 [5950-8640]</td>
</tr>
</tbody>
</table>

All values are expressed as medians [IQR] due to the skewed distribution of some variables.

MI indicates myocardial infarction; TIA, transient ischemic attack; WBC, while blood cells; and MPV, mean platelet volume.

*JAK2 mutation screening was performed in 38 of 41 randomized patients, 20 in the etoricoxib arm and 18 in the aspirin-only arm.
with absolute platelet counts ($r = 0.15$ for COX-2 ΔMFI and $r = -0.08$ for the percentage of TO-positive platelets).

We assessed in vitro whether platelets from ET patients had detectable COX-2 enzymatic activity, as measured by whole blood TXB$_2$ production$^{34}$ in the presence of a selective COX-2 inhibitor, NS-398.$^{24}$ At V0, serum TXB$_2$ was measured after incubation of 1-mL blood samples with 1 μM NS-398 or vehicle (Figure 3C).

NS-398 significantly reduced serum TXB$_2$ values by approximately 30% compared with vehicle-treated samples, from 8 [IQR 4.9-20] to 5.6 [IQR 3-14.9] ng/mL.

In vivo and ex vivo TX measurements

Blood and urine samples were taken twice before randomization, at V0 and at V1, to assess the intrasubject variability of TX biosynthesis as well as to maximize adherence to low-dose aspirin intake before randomization.

No statistically significant differences in TXM excretion were found between the 2 repeated measurements 1 week apart: V0, 325 [IQR 243-404.7] pg/mg creatinine; V1, 322 [IQR 233-507] pg/mg creatinine, $P = .945$.

Similarly, serum TXB$_2$ values did not differ between the 2 baseline visits, being 8 [IQR 4.9-20] ng/mL at V0 and 7.5 [IQR 4-11.7] ng/mL at V1 ($p = .31$). Both the rate of TXA$_2$ biosynthesis in vivo, as reflected by urinary TXM excretion, and the maximal biosynthetic capacity of blood platelets, as reflected by serum TXB$_2$, were markedly increased in ET patients compared with values measured with the same methodology in 48 healthy volunteers treated with the same dose of aspirin for 1 to 8 weeks (Figure 4).$^{32}$

On multiple regression analysis including TXM as the dependent variable and haematologic parameters (leukocytes, hemoglobin, hematocrit, erythrocytes, polymorphonucleates, and platelet counts) as well as serum TXB$_2$ as independent factors, serum TXB$_2$ was the sole predictor of TXM excretion ($n = 82$, $\beta = 1.48$, SE = 0.6, $t = 2.13$, $P = .036$). In addition, serum TXB$_2$ was weakly, but significantly correlated with the platelet count ($r = 0.31$, $P = .002$).

As expected, cytoreductive treatment with hydroxyurea (HU) was associated with statistically significant reductions in both platelet (HU-treated patients, 381 000 [IQR 329 500-535 000] platelets/μL, $n = 38$ determinations; non-HU patients, 496 000 [IQR 437 000-864 000] platelets/μL, $n = 44$ determinations, $P = .004$) and leukocytes counts (HU-treated patients, 5720 [IQR 5155-7070] leukocytes/μL, $n = 38$ determinations; non-HU patients, 7520 [IQR 7040-8640] leukocytes/μL, $n = 44$ determinations, $P = .009$). HU treatment was also associated with a 20% statistically significant increase in TXM excretion (HU treated, 371 [IQR 267-642] pg/mg creatinine, $n = 37$ determinations; non-HU treated, 303 [IQR 202-395] pg/mg creatinine, $n = 44$ determinations, $P = .025$). The apparent association between HU treatment and increased TXM excretion was not significant when compared with the same methodology in a control population.$^{32}$

As expected, cytoreductive treatment with hydroxyurea (HU) was associated with statistically significant reductions in both platelet (HU-treated patients, 381 000 [IQR 329 500-535 000] platelets/μL, $n = 38$ determinations; non-HU patients, 496 000 [IQR 437 000-864 000] platelets/μL, $n = 44$ determinations, $P = .004$) and leukocytes counts (HU-treated patients, 5720 [IQR 5155-7070] leukocytes/μL, $n = 38$ determinations; non-HU patients, 7520 [IQR 7040-8640] leukocytes/μL, $n = 44$ determinations, $P = .009$). HU treatment was also associated with a 20% statistically significant increase in TXM excretion (HU treated, 371 [IQR 267-642] pg/mg creatinine, $n = 37$ determinations; non-HU treated, 303 [IQR 202-395] pg/mg creatinine, $n = 44$ determinations, $P = .025$). The apparent association between HU treatment and increased TXM excretion was not significant when compared with the same methodology in a control population.$^{32}$
add etoricoxib, a highly selective COX-2 inhibitor, on top of aspirin, for 7 days. Urinary TXM and serum TXB2 were measured before and after a week (V2) of randomized treatment. One dropout occurred at day 3 of etoricoxib intake for causes unrelated to the study drug (flu-like syndrome and need of NSAID therapy). No adverse effects of the randomized treatment were recorded during the study.

Etoricoxib caused a statistically significant 25% reduction in TXA2 biosynthesis in vivo, as reflected by lower TXM excretion at V2 compared with V1 (Figure 4). It also determined a similar reduction in whole blood TXA2 production ex vivo, as reflected by decreased levels of serum TXB2 (Figure 4). This finding is consistent with the results of adding the selective COX-2 inhibitor NS-398 in vitro to whole blood samples from the same patients (Figure 3C).

Patients continuing aspirin alone showed no statistically significant changes between V1 and V2 in either urinary TXM excretion or whole blood TXB2 production (Figure 4).

Altogether, the results of etoricoxib dosing in vivo (Figure 4) and NS-398 in vitro (Figure 3C) showed that selective COX-2 inhibition was unable to completely suppress residual TXA2 biosynthesis in aspirin-treated ET patients.

We next examined whether aspirin-insensitive TXA2 production in ET and its COX-2-dependent component were stable over time. Fourteen of the 41 ET patients, without major clinical complications or changes in therapy during follow-up, were studied again 21 (± 7) months after their first visit. No statistically significant changes were found in serum TXB2 levels over this interval, with a significant correlation between the 2 sets of repeated measurements (r = 0.685, P = .009, vs V1; r = 0.655, P = .015, vs V0). Moreover, consistently with the results of the first in vitro experiment, we found a statistically significant reduction in serum TXB2 concentration by approximately 30%, after the addition of NS-398 to whole blood in vitro (Figure 5).

**Effects of aspirin in vitro**

In light of the pathogenesis of ET affecting megakaryocytes and the platelet-producing mechanism, we explored whether residual TXA2 production was sensitive to the addition of aspirin in vitro. At variance with NS-398, aspirin (50 μM) added to whole blood samples of ET patients was able to completely suppress TXA2 production (Figure 5) to levels similar to those measured in aspirin-treated healthy volunteers, suggesting a major role for unacylated platelet COX-1 in this phenomenon.

Finally, the absolute reduction in serum TXB2 obtained with aspirin or NS-398 was directly correlated with the basal levels of whole blood TXB2 production (aspirin-treated samples: r = 0.991, n = 12, P < .001; NS-398-treated samples: r = 0.784, P < .001, n = 13).

**Discussion**

Our study demonstrates that low-dose aspirin, given at the dosing interval uniformly recommended for cardiovascular prevention, is unable to fully inhibit serum TXB2 ex vivo and TXM excretion in vivo in the majority of patients with ET. In fact, the urinary TXM excretion rate while on aspirin was approximately 2.5-fold higher in ET patients compared with the rate measured in healthy subjects. We recently reported a median TXM excretion rate of 135.5 [IQR 106-170] pg/mg creatinine (n = 120 determinations) in 48 aspirin-treated healthy subjects, compared with 338 [IQR 247-466.5] ng/mL in ET patients.
pg/mg creatinine (n = 103 determinations) in 41 ET patients under the same antiplatelet regimen in the present study (Figure 4). These levels are also higher than those reported in patients with stable coronary artery disease treated with the same aspirin regimen. Serum TXB₂ levels in aspirin-treated ET patients were also above the median values of healthy subjects given the same aspirin regimen (shown in Figure 4). In fact, only 23 of 104 serum TXB₂ measurements in ET patients were below this threshold. Although we could not obtain pre-aspirin measurements, the data of the present study are nevertheless consistent with the less-than-complete inhibition (≤90%) of serum TXB₂ that we previously reported in a limited number of untreated ET patients who were given aspirin of 50 or 100 mg, at a time when antiplatelet therapy was not uniformly prescribed.

In light of previous evidence of COX-2 expression in mature megakaryocytes from normal and ET bone marrow specimens, and of in vitro data showing that platelet COX-2 might contribute to TXA₂ biosynthesis in hematopoietic hyperregenerative conditions (eg, recovery after bone marrow transplantation), we sought to explore the contribution of platelet COX-2 to TXA₂ biosynthesis in ET. Etoricoxib, a highly selective COX-2 inhibitor, was used as a pharmacologic tool to test this hypothesis in vivo, along with experiments adding a selective COX-2 inhibitor, NS-398, in vitro to blood samples from aspirin-treated ET patients. The choice of a 7-day treatment period was dictated by safety considerations as the shortest interval compatible with the achievement of steady-state drug levels. The urinary excretion rate of TXM, which is a validated index of whole body TXA₂ biosynthesis in vivo, as well as the level of serum TXB₂, which reflects the maximal biosynthetic capacity of thrombin-stimulated COX activity in platelets, were further but incompletely lowered by selective COX-2 inhibition.

To explore whether residual TXA₂ production in whole blood was fully suppressible, we added aspirin in vitro at a concentration that fully inhibits both COX-1 and COX-2. From a direct comparison of the results obtained with aspirin versus NS-398 in vitro, we can conclude that there is a fraction of enzymatically active COX-1 in platelets isolated from ET patients taking 100 mg of aspirin once daily, and this pool of unacetylated enzyme is normally sensitive to aspirin. We observed a weak positive correlation (r = 0.31) between the platelet count and whole blood TXB₂ production. On this basis, we can reasonably assume that the increased platelet mass would not account for incomplete inhibition of COX-1 activity by low-dose aspirin in ET. In fact, the dose of 100 mg represents at least a 3-fold excess over the lowest dose of aspirin necessary and sufficient to fully inactivate platelet COX-1 and protect against thrombosis, in healthy subjects and patients with a normal platelet count, that is, 30 mg daily. Because our ET patients had a 2-fold increase in platelet count at the time of study (Table 1), this is unlikely to require a higher dose of aspirin than 100 mg to produce saturation of its antiplatelet effect. Therefore, an alternative explanation would be required to reconcile our in vitro, ex vivo, and in vivo findings. Based on enhanced
thrombopoiesis that characterizes ET, it is conceivable that the duration of platelet COX-1 suppression by low-dose aspirin given once daily is shortened as a consequence of enhanced turnover of the drug target. Because of the short half-life of intact acetylsalicylic acid in the human circulation,46 a 24-hour dosing interval is compatible with full acetylation of platelet COX-1 in subjects with a normal platelet lifespan given daily doses in excess of 30 mg daily,4,46-47 by virtue of the irreversible nature of COX-1 inactivation. Faster platelet regeneration in the majority of ET patients, as suggested by the abnormal serum TXB2 levels in 78% of all samples, accompanied by expression of unacetylated COX-1 and COX-2 in newly formed platelets seem to provide a biologically plausible explanation for aspirin-insensitive TXA2 biosynthesis described in the present study. This hypothesis is also consistent with recent data showing an association between enhanced platelet turnover, expressed as TO-positive platelet fraction, and thrombosis occurrence in ET.48 The extent to which a higher formation of aspirin-insensitive TXA2 biosynthesis due to accelerated renewal of the drug target, and the lessons learned by the study of ET could be applied to other clinical conditions characterized by enhanced platelet turnover, such as type 2 diabetes mellitus and coronary artery bypass grafting, where evidence of aspirin “resistance” has been reported.39,50

Acknowledgments

The authors are grateful to Prof Stefania Basili for statistical advice and to Daniela Basilico for expert editorial assistance. This work is dedicated to the memory of Drs Nicola Maggiano and Jacques Maclouf.

This study was supported by the European Commission FP6 funding (LSHMCT-2004-005033) to C.P., B.R., and G.D.

Authorship

Contribution: A.D. and S.P. recruited patients and performed all visits and follow-up reassessment; A.R., D.M., S.L., G.P., L.M., E.F., G.C., and B.R. performed experiments; A.H. contributed with noncommercial reagents and supervised analytical measurements; F.O.R. analyzed immunohistochemistry and made the figures; G.D. designed the research; and C.P. and B.R. designed the research and wrote the first draft. All authors contributed to the final version of the manuscript.

Conflict-of-interest disclosure: G.D. has received research grant support from Bayer, Sanofi-Aventis, and Servier. C.P. has received research grants from Bayer and Servier and lecture and consulting fees from AstraZeneca, Bayer, Eli-Lilly, Schering-Plough, Sanofi-Aventis, and Servier. B.R. has received honoraria from Bayer and Merck and lecture fees from Nycomed, CSL-Behring, AstraZeneca, and Bristol-Myers Squibb. The remaining authors declare no competing financial interests.

Correspondence: Bianca Rocca, Department of Pharmacology, Catholic University School of Medicine, Largo F Vito 1, 00168 Rome, Italy; email: b.rocca@tiscali.it.

References


The contribution of cyclooxygenase-1 and -2 to persistent thromboxane biosynthesis in aspirin-treated essential thrombocythemia: implications for antiplatelet therapy

Alfredo Dragani, Silvia Pascale, Antonio Recchiuti, Domenico Mattoscio, Stefano Lattanzio, Giovanna Petrucci, Luciana Mucci, Elisabetta Ferrante, Aida Habib, Franco O. Ranelletti, Giovanni Ciabattoni, Giovanni Davì, Carlo Patrono and Bianca Rocca