Contribution of Cyclooxygenase-2 to Elevated Biosynthesis of Thromboxane A2 and Prostacyclin in Cigarette Smokers

Brendan F. McAdam, MD, MRCPI; Daniel Byrne, MSc; Jason D. Morrow, MD; John A. Oates, MD

Background—Cigarette smoking is highly pathogenic to the vasculature. In smokers, the biosynthesis of both thromboxane (Tx) A2 and prostacyclin is increased. We hypothesized that the excess in prostacyclin biosynthesis in smokers was derived from the inducible cyclooxygenase-2 (COX-2). We further hypothesized that if the overproduction of prostacyclin in smokers were restraining platelet activation, then inhibition of COX-2 would lead to an increase in the activation of platelets, with a corresponding increase in the biosynthesis of TxA2.

Methods and Results—Smokers and nonsmokers received rofecoxib 25 mg twice daily or placebo for 1 week each in random sequence. The systemic biosynthesis of TxA2 and prostacyclin was assessed by analysis of their respective urinary metabolites, 11-dehydrothromboxane B2 (Tx-M) and 2′,3-donor-6-keto-PGF1α (PGI-M). Serum TxB2 was measured as an indicator of platelet COX-1 activity. Results are expressed as mean±SE with median and range. The elevated PGI-M in smokers (189±25, median 174, range 85 to 390 pg/mg creatinine) was reduced by rofecoxib to 78±27, median 71.5, range 50 to 135 pg/mg creatinine (P=0.002), and in nonsmokers, PGI-M at baseline (115±10, median 107, range 67 to 198 pg/mg creatinine) fell to 56±15, median 50, range 34 to 125 pg/mg creatinine (P=0.001) with rofecoxib. The increased excretion of Tx-M in smokers (284±26, median 252, range 200 to 569 pg/mg creatinine) was reduced by 21% to 223±16, median 206, range 154 to 383 pg/mg creatinine by rofecoxib (P=0.04) but was not changed in nonsmokers. Levels of serum TxB2 were not different in smokers and nonsmokers and were unaffected by rofecoxib.

Conclusions—The increased prostacyclin biosynthesis in smokers is derived largely from the inducible COX-2. COX-2 also contributes to the increased biosynthesis of TxA2 in smokers, most likely from inflammatory cells. (Circulation. 2005;112:1024-1029.)

Key Words: thromboxane • inflammation • smoking • drugs • platelets

The effects of cyclooxygenase-2 (COX-2) inhibitors on the function of the vasculature and pathophysiology of vascular disease have been examined in a number of investigations.1–3 These studies have engendered considerations of the consequence of COX-2 inhibition that range from prothrombotic to antiatherogenic and which appear to be dependent on the experimental model, clinical circumstance, or duration of treatment.3–9 The demonstration that selective COX-2 inhibitors inhibit the biosynthesis of prostacyclin in humans has raised the question of whether enhanced platelet aggregation, mediated by COX-1, might result from reduction of this potent inhibitor of platelet activation.10,11

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To address this question, one approach would be to assess the effect of COX-2 inhibition on platelet activation in vivo in humans. Any enhancement of platelet activation by removal of prostacyclin might be expected to be most evident in pathological states in which platelet activation is increased. In some diseases associated with increased platelet activation, such as myocardial infarction and unstable angina,12,13 an investigation of the effect of COX-2 inhibition on platelet activation would not be feasible for many reasons, including the fact that antiplatelet treatment with aspirin is the standard of care in these clinical settings.14

Cigarette smoking is a proinflammatory risk factor for vascular injury with biochemical features similar to that of atherosclerosis, with increased generation of thromboxane (Tx) A2 and PGI2 in vivo together with elevation in plasma levels of C-reactive protein (CRP).15,16 We considered that this paradigm might afford an opportunity to assess the effect of COX-2 inhibition on platelet activation in vivo. Urinary excretion of the TxA2 metabolite, 2,3-donor-thromboxane B2, is increased in young, otherwise healthy smokers, and is reduced by low-dose aspirin (20 mg twice daily) to levels that approach those of aspirin-treated nonsmokers.15,17,18 This suggested that a substantial portion of the excess TxA2 biosynthesis in smokers is derived from platelets. In conjunction with the elevated production of TxA2 in cigarette smokers, prostacyclin biosynthesis also is increased, as is the
case with other diseases associated with vascular patho-
gy.12,13,15 Moreover, it is unknown whether this excess
prostacyclin biosynthesis in cigarette smokers is derived from
COX-2.

The purpose of the present study was to examine the
contribution of COX-2 to the enhanced formation of PG12 in
cigarette smoking, and we hypothesized that the excess in
prostacyclin biosynthesis in cigarette smokers is derived from
the inducible COX-2. To test this hypothesis, the effect of
rofecoxib, a highly selective inhibitor of COX-2, on the
urinary excretion of the prostacyclin metabolite 2,3-dinor-6-
keto-PGF1α (PGI-M) was examined. A further hypothesis was
that if prostacyclin is important in restraining platelet activa-
tion in smokers, then TxA2 metabolite excretion should
increase during COX-2 inhibition.

Methods

Study Population

Thirty-four healthy male smoking and nonsmoking volun-
tees between the ages of 20 and 40 years (mean age 27 years) were
screened for the study at the General Clinical Research Center (GCRC) at
Vanderbilt. The study was approved by the Institutional Review
Board at Vanderbilt, and written informed consent was obtained
from all volunteers. Smokers were selected who were consuming at
least 1 pack per day, and they were asked to continue to smoke at that
time during the study. All volunteers underwent screening medical
history, physical examination, and routine hematology and biochem-
istry tests. Volunteers were excluded if they had any clinically
apparent disease, including febrile illness, in the preceding 2 weeks
or if they had treatment with any drug, notably aspirin, nonsteroidal
antiinflammatory drugs, or steroids, within 2 weeks of study entry.
Abstention from aspirin or nonsteroidal antiinflammatory drugs was
confirmed by measurement of serum TxB2 drawn before each part of
the treatment schedules.

The study was conducted under double-blind, placebo-controlled
conditions with a crossover design. Seventeen healthy male smokers
(mean age 27 years) and 15 nonsmoking volunteers (mean age 28
years) were randomized to receive either rofecoxib 50 mg daily (25
mg twice a day). Each group received rofecoxib or equivalent
placebo for 1 week each, with crossover of treatment schedules after
a washout period of 1 week, so that all volunteers received placebo
for 1 week each, with crossover of treatment schedules after
the inducible COX-2. To test this hypothesis, the effect of
rofecoxib, a highly selective inhibitor of COX-2, on the
urinary excretion of the prostacyclin metabolite 2,3-dinor-6-
keto-PGF1α (PGI-M) was examined. A further hypothesis was
that if prostacyclin is important in restraining platelet activa-
tion in smokers, then TxA2 metabolite excretion should
increase during COX-2 inhibition.

Biochemical Analyses

**COX-1 Activity in Whole Blood**

Whole-blood samples without anticoagulant were drawn into a
serum for quantification of serum TxB2, which was assayed by
allowing the blood to clot in nonsiliconized glass tubes for 1 hour in
a water bath at 37°C. The serum was saved after centrifugation, and
levels were measured with gas chromatography/mass spectrometry.

**Urinary Prostaglandin Metabolite Excretion**

All samples were stored at −70°C until analysis was performed.
Biosynthesis of TxA2 and PG12 was assessed by measurement of
their major urinary metabolites, 11-dehydrothromboxane B2 (Tx-M)
and 2,3 dimer 6-keto-PGF1α (PGI-M). PGI1 and TxA2 metabolites and
serum TxB2, were measured by stable isotope dilution/negative
ion/chemical ionization, gas chromatography/mass spectrometry as
described previously.19,20

**C-Reactive Protein**

High-sensitivity CRP (hsCRP) was measured with a latex anti-CRP
monoclonal antibody kit with immunonephelometry (Dade Behring)
on a Behring nephelometer according to the manufacturer’s instruc-
tions. Intra-assay variation was <5%.

**Plasma Markers of Platelet Activation**

Plasma P selectin, CD40 ligand (R&D Systems), and platelet factor
4 (American Diagnostica) were measured with commercially avail-
able ELISA kits.

**Statistical Analysis**

We had designed the study to address 2 hypotheses: that the enhanced
systemic biosynthesis of prostacyclin in smokers was dependent
on COX-2 activity but also to determine, in particular, the
functional importance of COX-2−derived PGI1 in limiting platelet
activation in smokers in vivo. The sample size was designed before
GCRC approval and was based on the desire to detect a difference in
the primary response variable, the biosynthesis of prostacyclin,
between the placebo and active treatment schedules in both smokers
and nonsmokers. Our group has previously shown 1.5- to 2-fold
elevations in the biosynthesis of Tx-M and prostacyclin with smoking
20 cigarettes daily.15 In addition, we have previously shown that
rofecoxib, under steady state conditions, reduced prostacyclin me-
tabolite formation by 50% without alteration in Tx-M in healthy
elderly subjects.11

On the basis of these prior data, given variability in the analytic
estimates of 10% and assuming a common standard deviation of
difference in response to treatment of 40, we estimated that a sample
size of at least 12 in each group in a crossover design would have
>95% power to detect at least 50% change in PGI-M between
rofecoxib and placebo, using a 2-group t test with an ρ of 0.05. In
both smokers and nonsmokers. Thus, under double-blind, placebo-
controlled conditions, each volunteer acted as his own control. The
secondary response variable was a change in the biosynthesis of
TxA2. With this sample size, it was anticipated that this investigation
would have 80% power to detect a difference in means of ≥25% in
Tx-M between active treatment and placebo in smokers, assuming
a common standard deviation of differences of 68 with an independent
t test with 0.05 2-sided significance level.

Results are presented as mean and standard error (SE), with
median and range values. Statistical analyses were performed on a
personal computer with the statistical package SPSS for Windows
(version 13.0, SPSS). Continuous variables were not normally
distributed, and the data were screened for outliers by plotting
histograms. The differences in prostaglandins between smokers and
nonsmokers were assessed with the Mann-Whitney U test. Changes
in urinary eicosanoid formation, plasma markers of platelet activa-
tion, and CRP levels were compared between baseline and posttreat-
mant in smokers and nonsmokers and with placebo with the
Wilcoxon signed-rank test. The percentage reduction in urinary
excretion of TxB-M with rofecoxib and placebo between smokers and
nonsmokers was also examined with Wilcoxon signed-rank test.
These data were also screened for a treatment order effect. The
differences between smokers and nonsmokers and between the active
treatment groups over time were examined with a general linear
model repeated-measures ANOVA. All tests were 2 tailed. Probabi-
licity values <0.05 were considered statistically significant.

**Results**

**Clinical Results**

All volunteers tolerated the protocol without incident. There
were no adverse events. There was no effect of rofecoxib on
blood pressure in either the smokers or the nonsmokers.

**Systemic Biosynthesis of PGI1**

The urinary excretion of PGI-M, an index of systemic
prostacyclin biosynthesis, was significantly higher in smokers
than in nonsmokers at baseline (mean±SEM 199±26, median 180, range 92–410 pg/mg creatinine versus 119±10, median 111, range 58–187 pg/mg creatinine; P<0.002). These levels remained unchanged after placebo in both smokers and nonsmokers (data not shown). Consistent with prior studies in healthy volunteers, selective inhibition of COX-2 with rofecoxib resulted in a reduction in PGI-M from 115±10, median 107, range 67–198 pg/mg creatinine to 56±15, median 50, range 34–125 pg/mg creatinine (P=0.001), or 51% of total. When smokers received rofecoxib, PGI₁ formation was reduced from 189±25, median 174, range 85–390 pg/mg creatinine to 78±27, median 71.5, range 50–135 pg/mg creatinine (P=0.002), which indicates a COX₂–dependent component, or 59% of the total excretion (Figure 1). This difference between smokers and nonsmokers after rofecoxib was significant (P=0.03).

### Systemic Biosynthesis of TxA₂

The present study confirms previous work that TxA₂ biosynthesis is increased in smokers compared with nonsmokers.¹⁵ In nonsmokers, excretion of TxA₂ at randomization was 220±36 pg/mg creatinine (median [range] 177 [78–462] pg/mg creatinine), and during selective inhibition of COX-2, it was 210±32 pg/mg creatinine (168 [100–447] pg/mg creatinine), a change that was not significant (P=0.94). However, in smokers, rofecoxib reduced Tx-M from 284±26 pg/mg creatinine (median [range] 252 [200–569] pg/mg creatinine) to 223±16 pg/mg creatinine (206 [154–383] pg/mg creatinine; P=0.041; Table 1). There was no change in the biosynthesis of TxA₂ in either smokers or nonsmokers with placebo treatment (Table 1).

![Figure 1](image1.png)

**Figure 1.** Effect of selective COX-2 inhibition on biosynthesis of PGI₂ (PGI-M) in smokers and nonsmokers. There was a highly significant difference in PGI-M with rofecoxib treatment in both groups (for nonsmokers, P=0.001; for smokers, P=0.002).

### Serum TxB₂

There was no difference in serum TxB₂ between smokers and nonsmokers at baseline (195±12, median [range] 188 [98–280] versus 193±16, median [range] 184 [91–252] ng/mL; P=0.71) or before treatment randomization. Rofecoxib did not alter serum TxB₂ in either smokers (181±12, median [range] 179 [88–263] versus 189±13, median [range] 177 [106–279] ng/mL; P=0.78) or nonsmokers (187±14, 194 [97–242] versus 184±13, median 193 [85–233] ng/mL; P=0.56; Figure 4). There was no significant change in either group after administration of placebo (data not shown).

### Table 1. Effects of Selective COX-2 Inhibition With Rofecoxib or Placebo on TxA₂ Biosynthesis (Tx-M) in Smokers and Nonsmokers

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<tr>
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<th>Smokers</th>
<th>Nonsmokers</th>
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<tbody>
<tr>
<td></td>
<td>Before Treatment</td>
<td>After Treatment</td>
</tr>
<tr>
<td>Tx-M, pg/mg Creatinine</td>
<td>284±26</td>
<td>223±16*</td>
</tr>
<tr>
<td></td>
<td>252 (200–569)</td>
<td>206 (154–383)</td>
</tr>
<tr>
<td>Smokers</td>
<td>210±32†</td>
<td>177 (78–462)</td>
</tr>
<tr>
<td>Nonsmokers</td>
<td>168 (100–447)</td>
<td>218±36</td>
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</table>

Data are expressed as mean±SEM (top line) with median (range) on second line. *P=0.041 compared with baseline; †P=NS.

When expressed as percentage change from baseline, there was a significant mean 21% reduction (median 20%) in Tx-M with rofecoxib in smokers (P=0.01), but no change was observed with placebo. There was no significant percentage change in Tx-M with either placebo or active treatment in the nonsmokers (P=0.73; Figure 2). This percentage difference between smokers and nonsmokers with rofecoxib was significant (P=0.029). There was no treatment order effect. We also examined the variability around the change in TxA₂ biosynthesis with rofecoxib and show the mean (with 95% CIs) of the differences between the smokers and nonsmokers after treatment with rofecoxib, which remains statistically significant (Figure 3).
Plasma Markers of Platelet Activation

There was no difference in the plasma levels of ex vivo indices of platelet activity (CD40 ligand, P-selectin, and platelet factor 4) between smokers and nonsmokers at baseline or at randomization. Neither rofecoxib nor placebo altered the plasma levels of these indices in smokers or nonsmokers (Table 2; data for placebo not shown).

C-Reactive Protein

CRP levels were higher in smokers than in nonsmokers at randomization, but the difference was not statistically significant (1.1±0.3, median [range] 0.6 [0.3 to 3.4] versus 0.53±0.15, median [range] 0.3 [0.1 to 1.5] mg/L; P=0.053). Selective COX-2 inhibition did not alter the plasma levels of CRP in either smokers (1.1±0.3 to 1.12±0.4 mg/L, median [range] 0.4 [0.3 to 2.9] mg/L, P=0.8) or nonsmokers (0.5±0.15 to 0.41±0.4 mg/L, median [range] 0.2 [0.1 to 1.3] mg/L, P=0.3). There was no treatment effect with placebo on plasma levels of CRP (data not shown).

Discussion

These findings indicate that the excess in prostacyclin biosynthesis in cigarette smokers is derived predominantly from COX-2. A vascular origin for prostacyclin biosynthesis is inferred from the localization of prostacyclin synthase in males primarily in blood vessels.21

The consequences of blocking prostacyclin biosynthesis in smokers with COX-2 inhibitors may be considered in light of the expanding number of known functions of this eicosanoid.22–24 Prostacyclin is a potent inhibitor of platelet aggregation in vitro25; however, insight into the function of prostacyclin, particularly in the regulation of platelet function in vivo is only beginning to emerge. In mice with targeted disruption of the gene for the prostacyclin receptor, the rate of spontaneous vascular thrombosis is not altered, but there is an increase in both thrombosis25 and vascular proliferation2 after endothelial injury. Prostacyclin also regulates the function of monocytes and macrophages,26,27 key participants in the process of destabilization of atherosclerotic plaques.

Studies in a model of endothelial injury in the canine coronary artery have suggested that COX-2–derived prostacyclin inhibits the rate of thrombus formation during platelet-selective administration of aspirin.3 Consistent with the lack of any demonstrated contribution of prostacyclin to the regulation of platelet function under physiological conditions in mice, reduction of prostacyclin biosynthesis in normal humans with COX-2 inhibitors does not increase TxA2 biosynthesis.10,11 Therefore, we examined the human vascular disease induced by cigarette smoking, to determine whether reduction in prostacyclin biosynthesis by COX-2 inhibition in smokers could increase platelet activation and thereby further increase the pathologically elevated biosynthesis of TxA2.

In contrast to other selective COX-2 inhibitors, such as nimesulide, we chose rofecoxib as a pharmacological probe for COX-2 given its remarkable selectivity and longer duration of action.28 The dosing regimen that was employed was designed to achieve full systemic anti-inflammatory efficacy and ensure complete sustained inhibition of COX-2, because new COX-2 may be induced in response to the intermittent nature of smoking over the 24-hour period, and this may not be inhibited unless there are sustained plasma levels of the inhibitor, particularly at the end of the study period, when the urine final urine samples were taken, on average 12 to 16 hours after the last dose of study medication.

| Table 2. Effect of Treatment With Rofecoxib on Plasma Markers of Platelet Activation |
|----------------------------------|-----------------|------|-----------------|-----------------|------|-----------------|-----------------|------|
|                                  | Before Treatment| After Treatment | P   | Before Treatment| After Treatment | P   |
| P-selectin, ng/mL                | 37.9±6          | 35.8±7          | 0.5 | 36.1±9          | 39.3±8          | 0.65 |
| CD40 ligand, μg/mL               | 3.8±1.1         | 3.5±0.8         | 0.5 | 3.2±0.9         | 3.4±0.9         | 0.68 |
| Platelet factor 4, ng/mL         | 9.9±0.6         | 7.8±0.9         | 0.1 | 9.6±0.5         | 8.8±0.4         | 0.2  |

Data are expressed as mean±SEM (top line) with median (range) on second line. There were no differences in any of these parameters at randomization between smokers and nonsmokers and after treatment with rofecoxib.

![Figure 3](http://circ.ahajournals.org/fig/3.png)  
Figure 3. Variability of effect of rofecoxib on Tx-M between smokers and nonsmokers expressed as mean with 95% CIs. P<0.05.

![Figure 4](http://circ.ahajournals.org/fig/4.png)  
Figure 4. Rofecoxib did not alter whole-blood COX-1–dependent formation of TxA2 (serum TxB2) in smokers or nonsmokers. P=NS for both smokers and nonsmokers.
Administration of this highly selective COX-2 inhibitor did not increase TxA2 metabolite (Tx-M) excretion in smokers, but rather, Tx-M was reduced in rofecoxib in smokers to a greater extent than in nonsmokers. Thus, Tx-A2 biosynthesis that is derived from COX-2 is increased in cigarette smokers.

Rofecoxib did not reduce the levels of serum Tx-B2, thus excluding the platelet as a source of COX-2-derived Tx-A2.29 Biosynthesis of Tx-A2 via the COX-2 pathway is known to occur in macrophages and the related cells that differentiate from circulating monocytes.30–34 These cells are the principal nonplatelet source of Tx-A2 biosynthesis35 and are the probable source of the Tx-A2 produced by COX-2 in cigarette smokers.

The formation of Tx-A2 from cells in the monocyte-macrophage cell line could potentially occur in circulating monocytes, pulmonary macrophages, vascular plaque, or any other site of smoking-induced inflammation. Transcellular metabolism of PGH2 in monocyte-platelet aggregates can also generate Tx-A2.36

Origin from a cellular participant in inflammation is consistent with other evidence that inflammation is a component of the pathophysiology induced by cigarette smoking, such as the elevated levels of the acute-phase reactants CRP and amyloid A.16,37 The inflammatory process in coronary plaques is a central feature of the pathophysiology of those plaques that are prone to disruption,38,39 and it is associated with systemic markers of inflammation, including CRP.40 As a component of that inflammation, COX-2 is expressed in macrophages within the plaques.41,42 The increased COX-2–derived Tx-A2 biosynthesis in smokers, therefore, raises the question of whether the vascular inflammation in smokers that engenders a high risk of coronary events also would lead to an elevation in Tx-A2 biosynthesis via COX-2 in plaques.43,44 COX-2–dependent biosynthesis by plaque macrophages would be of considerable interest in light of the fact that Tx-A2 stimulates basic fibroblast growth factor protein synthesis in vascular smooth muscle cells.45

A contribution of COX-2 to the biosynthesis of Tx-M also is germane to interpretation of the finding that elevated excretion of Tx-M in aspirin-treated patients with vascular disease portends an increased risk of a subsequent coronary event.46 Indeed, evidence for the biosynthesis of Tx-A2 from a nonplatelet source during aspirin treatment has been provided by Cippolone et al,47 who demonstrated in patients with unstable angina that the nonselective COX inhibitor indobufen lowered Tx-M levels more than did aspirin. This finding would be consistent with an origin of the Tx-M that eludes inhibition by aspirin from either COX-1 or COX-2.

In any future investigations that address COX-2 as a source of Tx-M in aspirin-treated patients, the dose of aspirin will be important, because Tx-M derived from nonplatelet sources declines progressively as the dose of aspirin exceeds the 80–to 100-mg/d level at which platelet Tx-A2 biosynthesis is ≥95% inhibited in normal individuals.12,17,18,48 Although the pharmacokinetics of low-dose aspirin produce a relatively selective acetylation of COX in the platelet, aspirin is not a COX-1–selective drug49,50 and therefore has the potential to inhibit COX-2 at higher doses.

In conclusion, prostacyclin biosynthesis in cigarette smokers was found to be derived largely from COX-2. This major reduction in prostacyclin biosynthesis during COX-2 inhibition in smokers, however, was not accompanied by an increase in the level of the urinary metabolite of Tx-A2. Indeed, rofecoxib lowered Tx-A2 metabolite levels in smokers to a greater extent than in nonsmokers, which indicates that Tx-A2 biosynthesis via COX-2 is increased in cigarette smokers.

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Disclosure

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References


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