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, 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-dinor-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 pathol-
gy.\textsuperscript{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 PGI\textsubscript{1} 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-PGF\textsubscript{1\alpha} (PGI-M) was examined. A further hypothesis was that if prostacyclin is important in restraining platelet activation in smokers, then TxA\textsubscript{2} metabolite excretion should increase during COX-2 inhibition.

Methods

Study Population
Thirty-four healthy male smoking and nonsmoking volunteers 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 rate during the study. All volunteers underwent screening medical history, physical examination, and routine hematology and biochemistry 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 TxB\textsubscript{2} 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 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-PGF\textsubscript{1\alpha} (PGI-M) was examined. A further hypothesis was that if prostacyclin is important in restraining platelet activation in smokers, then TxA\textsubscript{2} 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 syringe for quantification of serum TxB\textsubscript{2}, 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 \(-70^\circ\text{C}\) until analysis was performed. Biosynthesis of TxA\textsubscript{2} and PGI\textsubscript{1} was assessed by measurement of their major urinary metabolites, 11-dehydrothromboxane B\textsubscript{2} (TxB-M) and 2,3-dinor-6-keto-PGF\textsubscript{1\alpha} (PGI-M). PGI\textsubscript{1} and TxA\textsubscript{2} metabolites and serum TxB\textsubscript{2} were measured by stable isotope dilution/negative ion/chemical ionization, gas chromatography/mass spectrometry as described previously.\textsuperscript{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 instructions. 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 available 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 PGI\textsubscript{1} 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 TxA\textsubscript{2} and prostacyclin when smoking 20 cigarettes daily.\textsuperscript{15} In addition, we have previously shown that rofecoxib, under steady state conditions, reduced prostacyclin metabolite formation by 50% without alteration in TxB-M in healthy elderly subjects.\textsuperscript{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 \(\alpha\) 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 TxA\textsubscript{2}. With this sample size, it was anticipated that this investigation would have 80% power to detect a difference in means of \(\pm 25\%\) in TxB-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 prostanoids between smokers and nonsmokers were assessed with the Mann-Whitney U test. Changes in urinary eicosanoid formation, plasma markers of platelet activation, and CRP levels were compared between baseline and posttreatment 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. Probability values \(<0.05\) were considered statistically significant.

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 PGI\textsubscript{1}
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, PGI2 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 COX-2–dependent component, or 59% of the total excretion (Figure 1). This difference between smokers and nonsmokers with 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 Tx-M 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).

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).

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

<table>
<thead>
<tr>
<th>Tx-M, pg/mg Creatinine</th>
<th>Before Treatment</th>
<th>After Treatment</th>
<th>Before Treatment</th>
<th>After Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rofecoxib</td>
<td>284±26</td>
<td>223±16*</td>
<td>279±25</td>
<td>272±22†</td>
</tr>
<tr>
<td></td>
<td>252 (200–569)</td>
<td>206 (154–383)</td>
<td>246 (188–474)</td>
<td>247 (211–428)</td>
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<tr>
<td>Placebo</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>220±36†</td>
<td>210±32†</td>
<td>218±36</td>
<td>206±32†</td>
</tr>
<tr>
<td></td>
<td>177 (78–462)</td>
<td>168 (100–447)</td>
<td>184 (113–386)</td>
<td>190 (94–423)</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SEM (top line) with median (range) on second line.

*P=0.041 compared with baseline; †P=NS.
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 this gene for the prostacyclin receptor, the rate of spontaneous vascular thrombosis is not altered, but there is an increase in both thrombosis22 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 antiinflammatory 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**

![Figure 3. Variability of effect of rofecoxib on Tx-M between smokers and nonsmokers expressed as mean with 95% CIs. P<0.05.](image)

![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.](image)

Before Treatment | After Treatment | P
--- | --- | ---
**P-selectin, ng/mL**
3.9±0.6 | 35.8±7 | 0.5
34 (7–61) | 31 (9.8–55)
**CD40 ligand, μg/L**
3.8±1.1 | 3.5±0.8 | 0.5
2 (0.3–12) | 2.5 (0.3–12)
**Platelet factor 4, ng/mL**
9.9±0.6 | 7.8±0.9 | 0.1
9 (6–15.8) | 9 (6–14)

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.
Administration of this highly selective COX-2 inhibitor did not increase TxA2 metabolite (Tx-M) excretion in smokers, but rather, Tx-M was reduced by rofecoxib in smokers to a greater extent than in nonsmokers. Thus, TxA2 biosynthesis that is derived from COX-2 is increased in cigarette smokers.

Rofecoxib did not reduce the levels of serum TxB2, thus excluding the platelet as a source of COX-2–derived TxA2.29 Biosynthesis of TxA2 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 TxA2 biosynthesis45 and are the probable source of the TxA2 produced by COX-2 in cigarette smokers.

The formation of TxA2 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 P Gh in monocyte-platelet aggregates can also generate TxA2.36

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.26,37 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 TxA2 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 TxA2 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 TxA2 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 TxA2 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 TxA2 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 TxA2. Indeed, rofecoxib lowered TxA2 metabolite levels in smokers to a greater extent than in nonsmokers, which indicates that TxA2 biosynthesis via COX-2 is increased in cigarette smokers.

Acknowledgments

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Disclosure

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