Effects of Cyclooxygenases Inhibitors on Vasoactive Prostanoids and Thrombin Generation at the Site of Microvascular Injury in Healthy Men

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Objective—Balance between vasoactive prostanoids that contribute to homeostasis of the circulatory system can be affected by cyclooxygenases inhibitors. Results of a recent large clinical trial show that myocardial infarction was more frequent among patients with rheumatoid arthritis treated with the selective cyclooxygenase-2 inhibitor rofecoxib compared with those treated with naproxen. Whether this difference was attributable to deleterious cardiovascular effects of rofecoxib or cardioprotective effects of naproxen has not been determined. We tested the hypothesis that naproxen, contrary to rofecoxib, exerts antithrombotic effects.

Methods and Results—Forty-five healthy men were randomized to receive a 7-day treatment with rofecoxib (50 mg/d), naproxen (1000 mg/d), aspirin (75 mg/d), or diclofenac (150 mg/d). Formation of thromboxane, prostacyclin, and thrombin in the bleeding-time blood at the site of standardized microvascular injury was assessed before and after treatment. Naproxen, like aspirin, caused significant reduction of both thromboxane and prostacyclin, whereas diclofenac depressed prostacyclin synthesis but had no effect on thromboxane formation. Naproxen and aspirin significantly suppressed thrombin generation. Diclofenac showed a similar tendency, which did not reach statistical significance. Rofecoxib had no effect on any variables measured.

Conclusions—In healthy men, naproxen exerts an antithrombotic effect at least as potent as aspirin, whereas rofecoxib does not affect hemostatic balance. (Arterioscler Thromb Vasc Biol. 2003;23:1111-1115.)

Key Words: myocardial infarction ■ risk factors ■ cyclooxygenase inhibitors ■ prostaglandins ■ thrombin

The discovery of 2 isoforms of cyclooxygenases (COX-1 and COX-2) led to the introduction of coxibs, selective COX-2 inhibitors, into therapy.1 They proved to combine strong anti-inflammatory properties with fewer gastrointestinal side effects than traditional nonsteroidal anti-inflammatory drugs (NSAIDs).2-3 FitzGerald et al4 drew attention to the effects of coxibs on the synthesis of the vasoactive eicosanoids. They demonstrated that coxibs diminished excretion of prostacyclin (PGI₂) metabolites into urine without affecting either excretion of thromboxane A₂ (TXA₂) or platelet blood aggregability. These results suggested that PGI₂ is synthesized by COX-2 in endothelial cells and that its biosynthesis can be depressed by coxibs, leaving unopposed activity of COX-1 in platelets. The issue soon became of clinical relevance with the publication of the VIGOR trial, which compared the selective COX-2 inhibitor rofecoxib with the traditional NSAID naproxen in patients with rheumatoid arthritis. Patients treated with rofecoxib showed an increase in incidence of myocardial infarction compared with the naproxen group. Meta-analysis of studies concerning cardiovascular safety of rofecoxib gave conflicting results.5-6 Vane7 saw 3 possible explanations for the VIGOR trial results: a chance effect, a prothrombotic effect of rofecoxib, or the aspirin-like, protective activity of naproxen. We undertook this study to test these hypotheses in healthy men.

In our study, we used a microquantitative analytical technique that permits the precise and sensitive characterization of eicosanoids and thrombin formation at the site of direct platelet-vascular wall interface. This technique has been successfully applied for demonstration of inhibition of PGI₂ and TXA₂ biosynthesis by aspirin, characterization of the sequence of coagulant reactions following vascular injury, assessing resistance to aspirin, and in studies of various drugs on blood coagulation.8-15 This ex vivo model might better reflect the in vivo situation than models using human endothelial umbilical vein cell cultures16 or coronary artery injury.17 In the model used in this study, the full contact of injured endothelium with whole blood (platelets, leukocytes, and coagulation factors) is preserved at the site of skin incision. Oozing blood is collected directly to the anticoagulant medium, which contains indomethacin blocking the COX-1 activity.
activity in platelets. The injured endothelium and flowing blood might correspond to the platelet-wall interactions going on during the atherosclerotic plaque formation or rupture, although the flow is laminar rather than turbulent and muscle cells are absent.

Methods

Design of the Study

In this double-blind study, healthy men were randomly allocated into 4 treatment groups. Each group was treated for 7 consecutive days with 1 of the following NSAIDs: rofecoxib (25 mg twice daily; n=18), naproxen (500 mg twice daily; n=9), aspirin (75 mg daily; n=9), or diclofenac (75 mg twice daily; n=9). Rofecoxib and naproxen doses corresponded to those used in the VIGOR study, diclofenac was given in the most often prescribed dose, and aspirin was given in the minimal therapeutic dose sufficient for platelet function inhibition. The skin-bleeding time with blood collection was performed on the first day, before the drug administration, and was repeated on the 7th day, 4 to 6 hours after the morning drug dose.

Subjects

Participants of the study were recruited from symptom-free, non-smoking, healthy volunteers, aged 20 to 30 years (mean, 23 years), who did not take any drug for at least 2 weeks. Arachidonic acid at a concentration of 900 μmol/L produced platelet aggregation in their platelet-rich plasma. Forty-five men who completed the study had no personal history of gastrointestinal disease, drug allergy, thrombotic disorders, or bleeding disorders.

The protocol was approved by the University Ethics Committee, and all subjects gave informed consent.

Model of Microvascular Injury

The eicosanoids studied and the tissue factor–initiated coagulation were evaluated in samples of bleeding-time blood, as described previously. Briefly, after compressing the upper arm with a sphygmomanometer cuff to 40 mm Hg, 2 standardized incisions were made on the forearm skin with use of a Simplate II device (Organon Teknica). The procedure was always performed by the same investigator. The blood shed was collected at 30 seconds and then at 1-minute intervals directly from the edge of the skin wound into microvippettes and then passed into Eppendorf tubes containing an anticoagulant mixture composed of 100 mmol EDTA and 60 μmol indomethacin in 0.9% NaCl. The tubes were centrifuged, and the supernates were removed, aliquoted, and stored at −80°C for additional analysis.

TXB2 and 6-keto-PGF1α were determined using a RIA Amersham assay. Thrombin-antithrombin complexes, reflecting thrombin generation, were determined by ELISA (Enzygnost TAT Micro, Dade) and expressed as nanomolar concentrations, whereas the eicosanoids concentrations were expressed as picograms per milliliter of oozing blood. When 150 mg indomethacin per 24 hours was given to 8 healthy men aged 20 to 25 years, the levels of 6-keto-PGF1α became depressed at least by 50% in each subject. For counting the ratio of 6-keto-PGF1α to TXB2, the nanomolar concentrations were used.

Statistical Analysis

Statistical evaluation was carried out using a personal computer and STATISTICA software (Statsoft Inc). The response was compared between treatment groups by an ANOVA model, including factors for treatment, period (repeated-measure factor), and time (repeated-measures factor). The logarithmic transformation as a variance-stabilizing transformation was used when needed. The between-treatment differences were summarized as least square means and 95% confidence intervals using the ANOVA model. The ANCOVA model, including factors for treatment, time, person (nested in treatment), and covariate (before treatment), was also used to adjust differential regression to the mean effects attributable to the imbal-

Results

Aspirin, naproxen, and diclofenac caused statistically significant increase in bleeding time (Figure I, available online at http://atvb.ahajournals.org) and in the volume of blood oozing from the bleeding time incisions. Platelet aggregation in response to arachidonic acid became inhibited. Rofecoxib had no effect on bleeding time, volume of blood, or platelet aggregation.

The median values of 6-keto-PGF1α for 3 NSAIDs recorded at 270 seconds were 119 pg/mL (1,3 quartile, 78; 138 pg/mL) before and 46 pg/mL (1,3 quartile, 39; 52 pg/mL) after the treatment, whereas for rofecoxib, they were 130 pg/mL (1,3 quartile, 87; 139 pg/mL) and 100 pg/mL (1,3 quartile, 79; 151 pg/mL), respectively. Similar data for TXB2 were 2956 (1,3 quartile, 1581; 5476 pg/mL) and 137 pg/mL (1,3 quartile, 90; 1304 pg/mL) for NSAIDs and 3012 (1,3 quartile, 2120; 4219 pg/mL) and 2432 pg/mL (1,3 quartile, 1618; 2993 pg/mL) for rofecoxib. In case of TAT, the median values obtained at the same time of blood sampling were 42 nmol/L (1,3 quartile, 26; 59 nmol/L) before NSAIDs and 20 nmol/L (1,3 quartile, 14; 26 nmol/L) after 7-day therapy, and 17 nmol/L (1,3 quartile, 14; 28 nmol/L) and 16 nmol/L (1,3 quartile, 12; 18 nmol/L) for rofecoxib, respectively.

Aspirin blocked TXB2 and 6-keto-PGF1α formation in clotting blood (Figure 1). Naproxen significantly decreased both 6-keto-PGF1α and TXB2 levels. Diclofenac displayed a weak trend toward diminution of TXB2 and depressed 6-keto-PGF1α levels. In the rofecoxib group, the levels of both PGI2 and TXA2 metabolites were almost identical before and after 7 days of treatment. Because balance between the 2 eicosanoids studied maintains cardiovascular homeostasis,7,19 we calculated their ratio at 60-second intervals (Figure 2). Both aspirin and naproxen shifted the ratio toward PGI2 metabolite, whereas rofecoxib and diclofenac had no such effect. Aspirin and naproxen produced a significant fall in thrombin generation. Diclofenac showed similar tendency, which did not reach statistical significance. Rofecoxib did not affect thrombin generation (Figure IV, available online at http://atvb.ahajournals.org).

Discussion

From the cardiovascular standpoint, the safety of COX-2 inhibitors has been the topic of much discussion and controversy.5,6 Concerns have been raised that selective blockade of COX-2 may impair endothelial function and promote cardiovascular disease,4 especially when coxibs are used at a high dose20 or aspirin is not given concomitantly in patients who meet criteria for its use as a preventive agent.2 These views seem to be supported by a recent experimental study. Smooth muscle cell proliferation in response to carotid artery endothelial injury was increased in mice lacking the prostacyclin receptor and decreased in mice lacking the thromboxane A2 receptor.19 However, prostacyclin receptor–deficient mice, which represent the complete elimination of prostacyclin receptor–mediated biologic effects of PGI2, may not reflect
the effects of selective COX-2 inhibition, because COX-1 also contributes to endothelial cell synthesis of PGI₂. On the other hand, coxibs may have a beneficial effect on cardiovascular events in atherothrombosis. COX-2 expression is upregulated in atherosclerotic plaques. Proinflammatory cytokines and growth factors enhance COX-2 expression in macrophages and monocytes in atherosclerotic plaque proximity. This leads to excessive production of proinflammatory eicosanoids (PGE₂, PGD₂, and TXA₂) and metalloproteinases. In parallel, the exaggerated expression of COX-2 observed in the endothelial cells and smooth muscles results in the upregulation of endothelial NO synthase and enhanced NO production. COX-2 inhibition may decrease vascular inflammation and improve plaque stability. A recent pilot study indicated that in patients with acute coronary syndrome without ST-segment elevation, treatment with a selective COX-2 inhibitor, meloxicam, together with heparin and aspirin was associated with a significant reduction in adverse outcomes compared with treatment with heparin and aspirin alone. Studies in the mouse have clearly shown that the inhibition of COX-2 or the deletion of the COX-2 gene in the arterial macrophage reduces atherosclerosis.

The drugs we studied have decreasing ability to inhibit COX-1 in vitro, with aspirin producing full inhibition of both isoforms, followed by naproxen and then diclofenac, with moderate preference toward COX-2 and rofecoxib, a highly selective COX-2 inhibitor. Because COX-1 in platelets is the major source of TXA₂, it is not surprising that both aspirin and naproxen strongly inhibited its formation whereas the effects of diclofenac were less accentuated and rofecoxib was deprived of such activity. The drugs we investigated in a
parallel fashion affected formation of prostacyclin, with aspirin being the strongest inhibitor and rofecoxib having no inhibitory activity. Similar to other authors,10,11 we expressed eicosanoid concentrations in picograms or nanograms per milliliter of blood; the conclusions of the study remained the same if data were expressed in picograms per second. Importantly, both naproxen and aspirin shifted the PGI2/TXA2 ratio in favor of prostacyclin (Figure 2). In addition, they both significantly depressed thrombin generation (Figure IV, available online at http://atvb.ahajournals.org).

Our data confirm the antithrombotic effects of aspirin.31 Because the drug inhibits irreversibly both COX-1 and COX-2 and only the nucleated cells can produce the new enzyme molecules, a single dose of aspirin leads to long-lasting depression of the TXA2 and decreases also PGI2 production. Of paramount importance is that in the model of microvascular injury after aspirin ingestion, the ratio of PGI2 to TXA2 was in favor of prostacyclin. Naproxen was shown as a potent and long-lasting (up to 8 hours) COX-1 inhibitor, which explains its antiplatelet efficacy.32 The drug only slightly decreased prostacyclin production, resulting in favorable balance of PGI2/TXA2. Diclofenac prolonged bleeding time and decreased prostacyclin generation but had no effect on thrombin generation and thromboxane levels. Rofecoxib did not affect any parameter measured. These results differentiate clearly the above 2 drugs from aspirin and naproxen, characterized by marked COX-1 selectivity. Our results indicate that coxibs do not necessarily lead to depressed prostacyclin production. This is in contrast to the conclusions of McAdam et al3 and Catella-Lawson et al,33 based on decreased excretion of urinary prostacyclin metabolite in healthy volunteers treated with celecoxib.

The population in this study was healthy and free of known cardiovascular risk factors. The results, therefore, should not be extrapolated leniently to patients with atherosclerosis, in whom prostanooids are known to play an important role in homeostasis of cardiovascular system.

Our results point to powerful antithrombotic effects of naproxen. They provide biochemical evidence supporting 3 recent case-control studies34–36 that demonstrate that patients treated with naproxen have a decreased incidence of myocardial infarction compared with patients receiving NSAIDs other than naproxen or those not receiving NSAIDs. Our data also corroborate observations37 that showed that neither rofecoxib nor naproxen, when used at the therapeutic doses, impairs endothelial vascular response in healthy volunteers. Finally, our results indicate that in human microvasculature, COX-1 and not COX-2 seems to be the source of prostacyclin.

Acknowledgments

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References

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A

Mean and 95% CI

PGF 1α [pg/ml]

Time

Diclofenac

Naproxen

Aspirin

Rofecoxib

p=0.009

p=0.047

p<0.001

p=0.565

B

Mean and 95% CI

Log 1XB 2

Time

Diclofenac

Naproxen

Aspirin

Rofecoxib

p=0.180

p<0.001

p<0.001

p=0.157
Mean and 95% CI

**Diclofenac**

- **p = 0.080**

**Naproxen**

- **p = 0.042**

**Aspirin**

- **p = 0.034**

**Rofecoxib**

- **p = 0.650**