Cyclooxygenase-2 Inhibitors Enhance Shear Stress-Induced Platelet Aggregation

Piet Borgdorff, PhD, Geert Jan Tangelder, MD, PhD, Walter J. Paulus, MD, PhD
Amsterdam, the Netherlands

OBJECTIVES
We aimed to investigate the effect of parecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, on in vivo shear stress-induced platelet aggregation in a rat model of arterial bypass with focal narrowing.

BACKGROUND
Long-term use of COX-2 inhibitors is associated with increased incidence of adverse cardiovascular events, especially in patients with a history of cardiovascular disease. These patients are at risk for thrombotic occlusion of arterial stenoses initiated by shear stress-induced platelet aggregation.

METHODS
To mimic the combination of a tight arterial stenosis and high shear stress in rats, an extracorporeal shunt from carotid to femoral artery was compressed by the rollers of a pump. Platelet aggregation was continuously measured by a photometric detector in the shunt.

RESULTS
Pretreatment with parecoxib (20 mg/kg) almost doubled shear stress-induced platelet aggregation (188% vs. 100% in control subjects, p = 0.0003). This was accompanied by a fall in plasma 6-keto-prostaglandin F1α from 100 ± 25 pg/ml to 36 ± 11 pg/ml (p < 0.0001). Enhanced platelet aggregation was also observed with high-dose aspirin (150 mg/kg) (146%; p = 0.02) but not with low-dose aspirin (25 mg/kg), which reduced aggregation (68%; p = 0.03). The effect of parecoxib was neutralized by low-dose (1 mg/kg) clopidogrel (from 188% to 92%; p = 0.0001), but not by low-dose aspirin (from 188% to 177%; p = NS).

CONCLUSIONS
In the presence of an arterial stenosis, COX-2 inhibitors enhance shear stress-induced platelet aggregation. This enhancement was prevented by low-dose clopidogrel but not by low-dose aspirin. Clopidogrel might therefore allow COX-2 inhibitors to be used without raising risk of thrombotic occlusion. (J Am Coll Cardiol 2006;48:817–23) © 2006 by the American College of Cardiology Foundation

Use of selective cyclooxygenase-2 (COX-2) inhibitors has been associated with increased risk of serious cardiovascular harm (1–3). Excess death, myocardial infarction, or stroke among users of COX-2 inhibitors arose mainly from patients with pre-existing cardiovascular risk (1,4,5). This suggests that new cardiovascular events during COX-2 inhibition result from destabilization of atheromatous plaques (6) or from thrombotic occlusion of pre-existing arterial stenoses. Tight arterial stenoses are prone to thrombotic occlusion because of the presence of elevated shear stress, which promotes platelet aggregation. Because COX-2 inhibitors block the basal production of prostacyclin (PGI2) (7–9) and because PGI2 is a strong inhibitor of platelet aggregation (10), COX-2 inhibitors could reinforce shear stress-induced platelet aggregation. So far, enhanced platelet aggregation after long-term use of COX-2 inhibitors has not been observed (9). These investigators, however, used in vitro aggregometry, which might have failed to detect an effect of PGI2-inhibition on platelet aggregation because the half-life of PGI2 does not exceed 3 min (11).

The present study therefore investigated the effect of COX-2 inhibitors on in vivo platelet aggregation in a recently developed rat model of arterial bypass with high shear stress (12,13). This model offers the advantage of a continuous in vivo assessment of platelet aggregation and of downstream vascular resistance and allows for detection of suppressed platelet aggregation by endogenous PGI2. The effect of selective COX-2 inhibitors on platelet aggregation was also compared with that of high- or low-dose aspirin. In addition, the present study investigated whether the effect of COX-2 inhibitors on platelet aggregation could be neutralized by co-administration of low-dose aspirin or of the adenosine diphosphate (ADP) receptor antagonist clopidogrel.

METHODS
A detailed description of the experimental set-up has previously been published (12). In short, Wistar rats (330 to 450 g) were anesthetized with ketamine (60 mg/kg intramuscularly) and pentobarbital (35 mg/kg intraperitoneally, followed by 0.16 to 0.23 mg/kg/min intravenously), ventilated, and heparinized with 800 IU/kg. In rats, a dose of 800 IU/kg is needed to prevent clot formation in the extracorporeal system. Saline was infused throughout the experiment at a rate of 0.016 ml/min. Body temperature was kept at 37.5°C. All animal handling was in compliance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication No. 85-23, revised 1985).

An extracorporeal shunt (medical grade polyvinyl chloride tubing, 1.5-mm inside diameter, 65 cm long), primed with a colloid osmotic solution (Gelofusine, Braun, Melsungen, Germany), was placed between the proximal part of a carotid artery and distal part of a femoral artery. To...
prevent platelet activation by the contact or complement system, all tubing was coated with albumin. Part of the shunt consisted of a peristaltic polyvinyl chloride tube (Gilson, Viliers Le Bel, France) that was loosely positioned in a roller pump. To switch from autoperfusion to pump perfusion, the tube was gradually compressed by tightening the rollers of the pump with a calibrated screw until flow stopped. Then the pump was started, and its speed was adjusted to restore flow to the original level, as measured with an inline flow probe (1N, Transonic Systems Inc., Ithaca, New York).

Platelet aggregation downstream of the pump was continuously measured with a photometric device that detects an increase in light transmission when platelet aggregates pass through a glass capillary. Once the output signal of the photometric device exceeded a threshold, the signal was converted to a uniform spike and counted (Fig. 1). Platelet aggregation was quantified by the amount (n) of platelet aggregates (PA) per milliliter of shunted blood during a 10-min pump perfusion run and expressed as nPA/ml.

Although there is no aggregation during spontaneous flow, start of the pump immediately elicits strong platelet aggregation that is maximal during the first 3 to 5 min before leveling off to a lower value that persists as long as pump perfusion continues. This platelet aggregation is initiated by the time-varying shear stress resulting from compression of the tube by the rollers and does not result from plasticizers or factors released from the albumin-coating nor from ADP released from damaged erythrocytes, because there is no detectable hemolysis (13). The involvement of shear stress in this model has been demonstrated by the use of auranofin, which inhibits shear-induced platelet aggregation and blocked platelet aggregation in this model (14). Although platelet aggregation in this model can already be elicited, albeit less intensely, by a single and partial occlusion of the tube, the present study used a pump run to have a continuous and protracted assessment of platelet aggregation. At the tightening of the pump rollers used in the present experiments, shear stress exceeded by far 100 dynes/cm², the level known to elicit platelet aggregation (13). The calibrated tightening of the pump rollers guaranteed equivalent amounts of shear stress in each experiment.

The vascular bed of the cannulated femoral artery was used as a “bioassay” for the circulatory effects of upstream platelet aggregation. To this end, pulsatile femoral artery pressure and flow were averaged and femoral resistance was continuously calculated as the ratio of mean pressure over flow (15). Femoral pressure was measured via a T-piece at the distal shunt insertion site. The pressure-drop over the shunt was 5 to 8 mm Hg.

Plasma PGF levels were studied by measuring its stable metabolite 6-keto-prostaglandin F (PGF) with an EIA Biotrak system (Amersham Biosciences, Piscataway, New Jersey). The detection limit was 3.0 pg/ml. Basal control values were determined in arterial blood from untreated animals, whereas the effect of COX-2 inhibition was measured in experimental animals, 1 h after receiving parecoxib, just before the experiment.

Drugs. The following selective COX-2 inhibitors were used: parecoxib, which is the injectable form of valdecoxib (20 mg/kg; Dynastat, Pfizer, New York), and NS-398 (15 mg/kg; Sigma, St. Louis, Missouri). Clopidogrel (Sanofi-Synthelabo, Toulouse, France) was used to specifically block platelet adenosine 5'-diphosphate receptors (P2Y12). Parecoxib and clopidogrel were dissolved in saline and administered intravenously 1 h before the experiment. The NS-398 was given orally, 2 h before the experiment.

Aspirin (Aspegic, Lorex Synthelabo, Maarssen, the Netherlands) was injected as a low dose (25 mg/kg) intraperitoneally on the day before the experiment or as a high
dose (150 mg/kg) intravenously 1 h before the start of pump perfusion. The lower dose has been shown to block rat platelet thromboxane (TXA₂) formation for several days (16) but to block endothelial PGI₂ formation for 6 h only (17). It is equivalent to the clinically used low-dose acetylsalicylic acid (ASA) in the prophylaxis of myocardial infarction. Aspirin (150 mg/kg) was used to non-selectively block the production of both platelet thromboxane (COX-1) and endothelial PGI₂ (COX-2) in the rat (17).

**Statistics.** Values in the text are expressed as mean ± SD, and in the figures as mean ± SEM. The nPA/ml values under different conditions were compared with one-way analysis of variance followed by Dunnnett's Multiple Comparison Test. Time series data of vascular resistance under different conditions were analyzed with two-way analysis of variance for repeated measurements. Differences were considered statistically significant if p < 0.05.

**RESULTS**

Pretreatment with parecoxib (n = 6) nearly doubled shear stress-induced platelet aggregation (Fig. 2A). The amount of aggregation during the first 10 min after pump start was 26.9 ± 5.3 x 10³ nPA/ml, compared with 14.3 ± 3.2 x 10³ nPA/ml in control rats (n = 6; p = 0.0003). Plasma 6-keto-PGF₁₀, the stable metabolite of PGI₂, was reduced from 100 ± 25 pg/ml to 36 ± 11 pg/ml (p < 0.0001) (Fig. 3). Enhanced platelet aggregation was also found after pretreatment with NS-398 (19.4 ± 4.6 x 10³ nPA/ml, n = 3, p = 0.04 vs. control) and, as shown in Figure 2B, after non-selective COX-2-inhibition with high-dose aspirin (20.9 ± 5.7 x 10³ nPA/ml; n = 6, p = 0.02 vs. control). In contrast, low-dose aspirin reduced platelet aggregation (9.7 ± 3.5 x 10³ nPA/ml; n = 6, p = 0.01 vs. control) (Fig. 2B).

Platelet aggregation also affects downstream vascular resistance in the extracorporeally perfused hind limb. Under control conditions, pump-induced platelet aggregation causes a triphasic change of vascular resistance: a short initial decline is followed by a 4–5 min lasting rise and a long-lasting fall (Fig. 4). The maximal rise in vascular resistance (from 64 ± 11 mm Hg/ml/min to 89 ± 16 mm Hg/ml/min) was observed after 2 min of pump-induced platelet aggregation and resulted from an increase in mean femoral artery pressure (from 127 ± 16 mm Hg to 173 ± 32 mm Hg) at unaltered mean femoral artery flow (2.0 ± 0.4 ml/min). This triphasic response was previously shown to be related to increasing and decreasing plasma concentrations of serotonin released from activated platelets (12). The constrictive phase of this response was significantly reinforced by parecoxib (p = 0.04) (Fig. 4A) and by high-dose aspirin (p = 0.03) (Fig. 4B), consistent with enhanced upstream platelet aggregation and increased release of serotonin. Low-dose aspirin had no significant effect (Fig. 4B).

Excess aggregation induced by parecoxib could not be corrected by co-administration of low-dose aspirin (Fig. 5A). The amount of aggregation in this condition (25.4 ± 5.5 x 10³ nPA/ml, n = 6) did not significantly differ from that with parecoxib alone (p = 0.32) or from that with high-dose aspirin alone (p = 0.19). The enhancing effect of COX-2 inhibition was, however, neutralized by clopidogrel in a dose as low as 1 mg/kg (Fig. 5B; 13.2 ± 1.4 x 10³ nPA/ml; n = 6, p = 0.48 vs. control). Higher doses of clopidogrel caused further reduction of shear-induced platelet aggregation (5.5 ± 0.4 x 10³ nPA/ml at 5 mg/kg [n = 2] and 4.9 ± 0.1 x 10³ nPA/ml at 25 mg/kg [n = 2]), and a dose of 50 mg/kg prevented pump-induced aggregation (1.2 ± 1.4 x 10³ nPA/ml, n = 4).

**Figure 2.** Enhancement of pump-induced platelet aggregation by parecoxib (A) and by high-dose aspirin (B), as well as attenuation of pump-induced platelet aggregation by low-dose aspirin (B).

**Figure 3.** Parecoxib (20 mg/kg) reduces the plasma level of the prostacyclin (PGI₃) metabolite 6-keto-prostaglandin F₁₀, from 100 ± 25 pg/ml (n = 7) to 36 ± 11 pg/ml (n = 15). *p < 0.0001.

**Figure 4.** Platelet aggregations per ml of blood measured during pump-induced platelet aggregation in control and after pretreatment with parecoxib (A) and high-dose aspirin (B), as well as attenuation of pump-induced platelet aggregation by low-dose aspirin (B).
Also the parecoxib-induced increase of vasoconstriction could, similarly to the enhancement of platelet aggregation, be prevented by pretreatment with low-dose clopidogrel (1 mg/kg) (Fig. 4A).

DISCUSSION

In an experimental rat model of arterial bypass with focal narrowing and high shear stress, the present study observed: 1) enhanced platelet aggregation and vasoconstriction of the downstream vascular bed after pre-treatment with the selective COX-2 inhibitors parecoxib and NS-398; 2) enhanced or reduced platelet aggregation with, respectively, high- or low-dose aspirin; and 3) correction of the parecoxib-induced enhancement of platelet aggregation by co-administration of low-dose clopidogrel but not by co-administration of low-dose aspirin.

COX-2 inhibitors and platelet aggregation. Prostacyclin is known as one of the strongest platelet inhibitors, especially in high shear stress conditions (10). Binding of PGI₂ to its platelet receptors increases cyclic adenosine monophosphate (18), which reduces cytosolic calcium, an essential mediator of shear stress-induced platelet aggregation (19) (Fig. 6). The enhanced shear stress-induced platelet aggregation after pretreatment with COX-2 inhibitors observed in the present study is explained by reduced binding of PGI₂ to platelet receptors because of lower plasma PGI₂ concentrations. Although endothelial release of PGI₂ is often presumed to occur only during inflammation, a continuous basal release of PGI₂ has also been observed, especially in the lungs (20,21). Moncada and Vane (22) therefore hypothesized that even under basal conditions platelet activation is continuously depressed by endogenous PGI₂. The short half-life of PGI₂ and the use of in vitro aggregometry made it difficult to prove this hypothesis. With in vivo aggregometry, the present study shows that basal PGI₂ formation can be reduced by selective COX-2 inhibitors and that this reduction can importantly enhance shear stress-induced platelet aggregation. Prostacyclin also stimulates disaggregation of formed platelet aggregates (23), and COX-2 inhibitors can therefore elongate the lifespan of the aggregates. Furthermore, in the presence of upstream platelet aggregation, COX-2 inhibitors enhance the risk of ischemic organ damage by diminishing local tissue perfusion because of an accentuated rise of vascular resistance. This
vasoconstrictive response to upstream platelet aggregation was previously shown to result from serotonin released by platelets (12).

Increased platelet reactivity after COX-2 inhibition was previously suggested by shortened vessel occlusion times in animals with endothelial arterial injury (24) and by prolonged duration of distal embolization after arteriolar puncture (25). Even in intact arterioles, PGI2 was important to prevent rolling or deposition of platelets (26,27). The present study did not expose platelets to damaged endothelium but subjected them to high shear stress as encountered in tight arterial stenoses. High shear stress induces agonist-independent platelet aggregation (Fig. 6). It triggers platelet activation by deformation of platelets and by conformational changes in von Willebrand factor (vWF), a large plasma multimer that exposes its A1-domain to bind to platelet glycoprotein (GP) Ib receptors (28–30). This binding triggers intracellular calcium mobilization and subsequent release of ADP and other substances from dense- and alpha-granules. Adenosine diphosphate binds to P2Y1 receptors on neighboring platelets and on the original platelet, where it further enhances calcium mobilization. Adenosine diphosphate also binds to P2Y12 receptors, which activate platelet GP Ib/IIa receptors. Activation of GP Ib/IIa receptors leads to attachment of adhesive proteins such as fibrinogen and vWF (31,32). This is essential for formation of platelet aggregates and is further potentiated by release of vWF from alpha-granules (28).

High- versus low-dose aspirin and platelet aggregation. The present study observed enhanced platelet aggregation after non-selective COX inhibition with high-dose aspirin. In contrast, low-dose aspirin reduced platelet aggregation but was unable to prevent it (Fig. 2B). The reduction was most likely caused by COX-1 inhibition in platelets. Because aspirin blocks COX-1 irreversibly and because platelets are anucleate cells that cannot restore COX-1 by de novo protein synthesis, a small amount of aspirin can profoundly reduce thromboxane production of platelets. In contrast, nucleated endothelial cells can perform protein resynthesis and resume COX-2 mediated PGI2 production shortly after administration of low-dose aspirin. As indicated in Figure 6, thromboxane-A2 derived from COX-1 reinforces shear stress-induced platelet aggregation. Blockade of thromboxane-A2 formation with low-dose aspirin
does, however, not importantly diminish aggregation when shear stress exceeds 100 dynes/cm² (33,34). At high shear stress the vWF becomes more important, and its release from alpha-granules is not inhibited by aspirin (35). This explains the limited efficacy of low-dose aspirin in the present experimental model, in which shear stress exceeds 100 dynes/cm².

Prevention of parecoxib-enhanced platelet aggregation with low-dose clopidogrel but not with low-dose aspirin. Although low-dose aspirin significantly reduced shear stress-induced platelet aggregation, co-administration of parecoxib with low-dose aspirin did not modify the parecoxib-induced enhancement of aggregation. In contrast, this enhancement was prevented with a dose of 1 mg/kg clopidogrel. Higher doses of clopidogrel (5 and 25 mg/kg) resulted in further inhibition of aggregation, and a dose of 50 mg/kg completely abolished roller pump-induced platelet aggregation. Clopidogrel does not interfere with the production of thromboxane-A₂ but blocks platelet ADP-P2Y₁₂ receptors. Activation of these receptors is one of the final links in the cascade of shear-induced platelet aggregation (Fig. 6), and clopidogrel is known to effectively interrupt this mechanism in vitro (28,36) and in vivo (13).

Because in the present experiments low-dose clopidogrel effectively prevented parecoxib-induced enhancement of shear stress-induced platelet aggregation, clopidogrel could also be useful in patients with pre-existing cardiovascular disease to prevent cardiovascular complications when using COX-2 inhibitors. An estimate of the clinical oral dose of clopidogrel that could achieve an effect on shear stress-induced platelet aggregation comparable to the 1 mg/kg intravenous dose effective in rats can only be inferred from in vitro ADP-induced platelet aggregation. In vitro ADP-induced platelet aggregation has indeed been studied both in rats and in humans, in contrast to in vivo shear stress-induced platelet aggregation, which has only been studied in rats. A similar 50% inhibition of in vitro ADP-induced platelet aggregation is observed in rats after an intravenous dose of 5 mg/kg (37) and in humans after an oral dose of 5 mg/kg (38,39). Assuming the inhibitory effect of clopidogrel on platelet aggregation to be similar for aggregation induced in vitro by ADP and in vivo by shear stress, the intravenous dose of 1 mg/kg effective in the present rat experiments would correspond to a clinical oral dose of 1 mg/kg.

We conclude that both selective COX-2 inhibition with parecoxib or NS-398 and non-selective inhibition with high-dose aspirin enhance high shear stress-induced platelet aggregation. This enhancement can be prevented by a low dose of clopidogrel but not by low-dose aspirin. Co-administration of low-dose clopidogrel might therefore enable patients with cardiovascular disease to benefit from COX-2 inhibition without augmenting cardiovascular risk.

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References

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