Selective Inhibition of Cyclooxygenase-2 Enhances Platelet Adhesion in Hamster Arterioles In Vivo

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Background—Selective inhibitors of cyclooxygenase-2 (Cox-2) are reported to cause cardiovascular side effects in patients at risk. However, direct proof of prothrombotic effects of these drugs is lacking. We investigated in the microcirculation in vivo whether selective inhibition of Cox-2 induces platelet activation.

Methods and Results—The behavior of fluorescence-labeled human platelets was studied in hamster arterioles (dorsal skinfold chamber) by intravitral microscopy. Transient platelet–vessel wall interactions (PVWIs), firm platelet adhesion to the vessel wall, and vessel occlusion after FeCl₃-induced wall injury were analyzed as platelet activation parameters. In vitro experiments in human umbilical vein endothelial cells (HUVECs) were performed to assess specific effects of Cox-2 inhibition on platelet adhesion under shear stress (16 dyn/cm²) and on endothelial release of 6-ketoprostaglandin (PG) F₁α. Selective inhibition of Cox-2 (NS-398, 0.5 mg/kg) increased platelet adhesion to the vessel wall in vivo (11.9±3.9 platelets/mm²; controls, 1.4±1.4 platelets/mm², P<0.05) and platelet adhesion after ADP stimulation in vitro. PVWIs were significantly enhanced in NS-398–treated animals, which were reduced by platelet pretreatment with aspirin (5 mg/kg) or iloprost (1 nmol/L). Inhibition of Cox-2 reduced levels of 6-keto-PGF₁α in vivo and in HUVECs supernatants. Time to occlusion after vessel wall injury was significantly shortened by NS-398 (125.4±13.6 seconds in NS-398–treated animals versus 270.8±46 seconds in controls; P<0.01).

Conclusions—Selective inhibition of Cox-2 reduces 6-keto-PGF₁α endothelial release, increases PVWIs, and increases firm platelet adhesion in hamster arterioles. Moreover, it leads to faster occlusion of damaged microvessels. Thus, selective inhibition of Cox-2 may trigger thrombotic events by diminishing the antiplatelet properties of the endothelium. (Circulation. 2004;110:2053-2059.)

Key Words: aspirin ■ prostaglandins ■ platelets ■ thrombosis ■ endothelium

Selective cyclooxygenase-2 (Cox-2) inhibitors have repeatedly been reported to increase the risk of cardiovascular thrombotic events. Although nonspecific Cox inhibitors also affect Cox-2, their additional inhibitor effect on Cox-1–mediated platelet thromboxane A₂ (TxA₂) production prevails and exerts an antithrombotic net effect. Specific inhibition of Cox-2, however, may not only favor thrombosis by leaving platelet TxA₂ formation unchanged but could also decrease synthesis of prostacyclin from the endothelium, thus unbalancing the physiological equilibrium between vascular TxA₂ and prostacyclin levels.

Both isoforms of Cox participate in endothelial prostacyclin synthesis. Whereas Cox-2 is generally thought to be inducible, several reports suggest that it is expressed constitutively in the endothelium and is a major source of prostacyclin in humans in vivo. Hence, selective inhibitors of Cox-2 could exert an indirect prothrombotic effect by preventing the endothelial synthesis of prostacyclin, which is a significant basis for the antiplatelet properties of the vascular endothelium.

In the present study, we investigated whether specific inhibition of Cox-2 exerts prothrombotic properties in vivo. To do so, we used the microcirculation of the hamster as a model of physiological platelet–vessel wall interaction (PVWI).

Methods

In Vivo Platelet Monitoring in Hamster Microcirculation

Dorsal skinfold chambers were implanted in golden Syrian hamsters as described. On the day of experiments (at least 24 hours after chamber implantation), hamsters bearing preparations that fulfilled the criteria for an intact microcirculation underwent jugular vein catheterization under short-term anesthesia as described. To obtain human platelets, venous blood was drawn from healthy volunteers who gave their informed consent and had not taken medication for 10 days. Platelet-rich plasma (PRP) was prepared from blood samples as described. Platelets were labeled by incubation of PRP with calcine-AM (5 µmol/L) for 30 minutes in the presence of iloprost (10 µg/mL). Thereafter, platelets were pelleted and resuspended in platelet buffer to obtain a final concentration of 400,000/µL.
Immediately before recording, 2×10⁴ calcein-labeled platelets were intravenously injected. Intravital fluorescence microscopy was performed with a modified microscope (Zeiss AxioTech Vario). Starting at 1 minute after platelet injection, movies were recorded with a digital camera (C8484, Hamamatsu; image frequency, 14.5/s) and analyzed with SimplePCI software (Compix). In every animal, 4 to 8 vessel segments were recorded for 30 seconds and analyzed. To obtain such pictures from awake animals, they were immobilized in plastic tubes of appropriate size, which were fixed on the custom-made microscope’s stage during experiments as described before. To inhibit Cox-2, animals received intraperitoneal injections of NS-398 (5 mg/kg) 2 hours before the experiments. Aspirin was either administered intravenously 2 hours before experiments (5 mg/kg) or incubated with platelets at a concentration of 10 μg/mL before their infusion for 5 minutes. In experiments with iloprost treatment of platelets, labeled platelets were incubated with iloprost for 5 minutes (final concentration, 1 μmol/L). All experiments were conducted in full accordance with the German animal protection law.

### Analysis of Platelet Velocities and PVWI Patterns
From the resulting length of the platelet trace in still images, velocities of single platelets were calculated from the exposure time of each single picture. Firm platelet adhesion and transient PVWI were classified according to the time of PVWI or a single platelet velocity, respectively. Platelets were considered to have adhered firmly when they did not move for at least 30 seconds. The number of firmly adhering platelets was expressed per vessel area investigated. To analyze transient PVWI in each vessel, velocities of all platelets, except firmly adhering platelets were measured in 10 randomly chosen images from the 30-second observation period. These velocities were normalized to the maximum velocity (velocity of the fastest platelet) to exclude biasing influences of altered blood flow velocities. Velocity distributions were depicted in frequency histograms. Vessels with irregular flow were excluded from analysis.

### In Vivo Arterial Occlusion
For visualization of the vessel lumen, 50 μL of a 5% fluorescein isothiocyanate–labeled dextran solution (molecular weight, 150 000; Sigma) was infused into the jugular catheters before the vessel occlusion assays. After digitally recording the blood vessel and confirming regular blood flow in the analyzed arterioles, 30 μL of an FeCl₃ solution (25 mmol/L) was pipetted onto arterioles. Movies were recorded either until blood flow ceased or for at least 6 minutes, and the time until vessel occlusion occurred was monitored.

### In Vitro Platelet Aggregation
Platelet aggregation was measured in PRP by the turbidimetric method in a 2-chamber-aggregometer (ChronoLog 490) under continuous stirring at 1000 rpm at 37°C as described.

### Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated and cultivated in medium 199 containing penicillin/streptomycin and 10% newborn calf serum (Biomol) mixed with endothelial cell growth medium (PromoCell; 1:1, vol/vol) as previously described.

### Platelet Adhesion to HUVECs Under Flow
HUVECs were grown to confluence on collagen-coated glass plates, which were afterward placed in perfusion chambers. Fluorescence-labeled platelets (200 000/μL, resuspended in platelet buffer) were continuously superfused over the cell layer at calculated shear rates of 16 dyn/cm² (37°C, 10 minutes). CaCl₂ (final concentration, 2 mmol/L) and ADP (final concentration, 100 μmol/L) were added to superfusates immediately before entrance into the perfusion chamber. After washout of nonadherent platelets and fixation (by 2% formalin solution), the number of adherent platelets was analyzed by confocal microscopy (Zeiss LSM410) in 4 different randomly chosen regions of interest as described.

### 6-Keto-PGF₁α Immunoassay
The amount of 6-ketoprostaglandin F₁α (6-keto-PGF₁α) was assessed with a commercially available immunoassay kit (ELISA) according to the manufacturer’s instructions (R&D Systems). For in vivo measurements, ~300 μL of blood was drawn by cardiac puncture immediately before the anesthetized animals were humanely killed. Euthanization was performed by intravenous injection of an overdose of pentobarbital. The clotted blood samples were centrifuged in the presence of indomethacin (10 μg/mL, 1000g, 2 minutes). The supernatants were collected and stored at ~80°C until assay for 6-keto-PGF₁α. HUVECs were grown to confluence on collagen-coated, 24-well, cell-culture dishes. NS-398 (100 to 500 μmol/L), aspirin (50 to 500 μg/mL), or sham solutions were preincubated for 2 hours before histamine (10 μmol/L) was added for 5 minutes.

### Statistical Analysis
Data are expressed as mean±SEM. Data were analyzed by ANOVA, unpaired Student’s t test, or the Kolmogorov-Smirnov test (distribution of platelet velocities). Differences were considered significant at error probability levels of P<0.05.

### Results
**Selective Inhibition of Cox-2 Enhances PVWI**
Mean diameters of all analyzed arterioles amounted to 54.8±1.1 μm. Within groups, mean vessel diameters were 54.9±2.5 μm in controls (38 vessels analyzed in 7 animals), 58.6±2.4 μm in NS-398–treated animals (42 vessels, 7 animals), 57.8±2.2 μm in aspirin-treated animals (24 vessels, 4 animals), 47.8±1.8 μm in animals receiving NS-398 followed by infusion of aspirin-treated platelets (4 animals, 21 vessels), and 51.4±2.4 μm in animals receiving NS-398 followed by infusion of iloprost-treated platelets (4 animals, 21 vessels; see the Table). Peak platelet velocity, as estimated from the highest platelet velocity within each vessel that was analyzed, reached a mean of 2.0±0.13 mm/s in controls, 1.7±0.09 mm/s in NS-398–treated animals, 1.9±0.15 mm/s in aspirin-treated animals (both not significantly different from control), and 1.4±0.08 or 1.3±0.08 mm/s in animals receiving NS-398 in combination with iloprost or aspirin.
respectively (4 animals in each group, 23 vessels in the NS-398/H11001 iloprost group, 21 vessels in the NS-398/H11001 aspirin group; both \( P<0.05 \) versus control, see the Table).

NS-398 significantly shifted the distributions of platelet velocities leftward, indicating enhanced PVWI in comparison with control animals (\( P<0.05 \)). Aspirin treatment resulted in a significant shift of PVWI distribution patterns rightward (\( P<0.05 \) versus control). When NS-398–treated animals were infused with either aspirin- or iloprost-pretreated platelets, the leftward shift in PVWI distribution patterns caused by NS-398 treatment was prevented (both \( P<0.05 \) versus NS-398; all data in the Table, all PVWI distribution patterns in Figure 1). Of note, in all groups treated with NS-398, including those animals that received aspirin- or iloprost-pretreated platelets after preincubation with NS-398, flow velocities were reduced in comparison with controls. However, platelet pretreatment with aspirin or iloprost prevented enhanced PVWI after inhibition of Cox-2 (Figures 1–3).

**Selecting Inhibition of Cox-2 Induces Firm Adhesion of Resting and Activated Platelets**

In control hamsters, virtually no firmly adherent platelets (0.9±0.9 platelets/mm\(^2\)) were observed in arterioles in vivo. Treatment with NS-398 induced significant platelet adhesion (10.2±4.1 platelets/mm\(^2\); \( P<0.05 \) versus control; Figure 2), which was fully prevented when platelets were pretreated with aspirin or iloprost before infusion (n=4 each; both \( P<0.05 \) versus NS-398). In vitro, platelets that had been pretreated with aspirin at a dose of 50 \( \mu \text{g/mL} \) (assumed to be equivalent to the in vivo dose of 5 mg/kg) showed decreased maximum aggregation and a prolonged lag phase between stimulation and the onset of shape change after stimulation with collagen. Aspirin pretreatment of platelets decreased aggregation to collagen (2 \( \mu \text{g/mL} \)) from 95.2±2% of maximum aggregation to 64.3±11% (n=4, data not shown; \( P<0.05 \)). The lag phase was increased by aspirin, from 47.5±2 to 71.3±9 seconds (n=4, data not shown; \( P<0.05 \)).

Activation-dependent (ADP, 100 \( \mu \text{mol/L} \)) platelet adhesion was studied under in vitro conditions, which allowed for controlled stimulation by ADP. Whereas unstimulated plate-
Platelets exhibited negligible firm adhesion in the absence of Cox-2 or endothelial nitric oxide synthase inhibitors in vitro, there was already significant adherence of platelets to sham-treated HUVECs (198.9±42.6/1000 mm², n=5). When HUVECs were pretreated with NS-398 (100 μmol/L, 1 hour), the number of adherent platelets amounted to 346.9±46.6/1000 mm² (n=5, all data in Figure 4; P<0.05 versus control), similar to the situation after preincubation of HUVECs with an inhibitor of endothelial nitric oxide synthase, N-nitro-L-arginine (100 μmol/L, 30 minutes, 425.8±49.6/1000 mm²; n=5, P<0.01 versus control, NS versus NS-398).

Selective Inhibition of Cox-2 Induces Rapid Arterial Occlusion
To investigate whether the NS-398–dependent increase in platelet adhesion to the vessel wall would ultimately result in an accelerated thrombotic vessel occlusion under pathological conditions, the vessel wall was damaged by locally adding a solution of FeCl₃ on the adventitial side. This experiment was performed in 4 control hamsters and in 6 hamsters treated with NS-398. In all hamsters treated with NS-398, vessel occlusion occurred during the 6-minute recording time (times to occlusion in seconds, 106, 91.5, 96, 137, 176, and 146; Figure 5).
A

Figure 6. 6-Keto-PGF$_{1\alpha}$ concentrations in serum in vivo and in supernatants of HUVECs. A, Serum levels of 6-keto-PGF$_{1\alpha}$ were significantly lower in animals treated with NS-398 (5 mg/kg) and showed trend toward reduction in aspirin-treated animals (5 mg/kg). B, In cultured HUVECs, NS-398 (100 μmol/L) as well as aspirin (50 μg/mL) prevented histamine-induced increase in 6-keto-PGF$_{1\alpha}$. NS-398 also reduced basal concentrations below control. *Significantly different vs control at P<0.05; ##significantly different vs histamine at P<0.01. Abbreviations are as defined in text.

Discussion

Our experiments demonstrate that selective inhibition of Cox-2 results in an increase in transient platelet interactions with the vessel wall in vivo, resulting in significant firm platelet adhesion that normally does not take place in these intact arterioles. This is likely due to a reduction of endothelial prostacyclin release because Cox-2 inhibition paralleled the diminished serum levels of 6-keto-PGF$_{1\alpha}$. This enhanced interaction may facilitate thrombotic occlusion of damaged vessels, as demonstrated by the FeCl$_3$-induced vessel damage. Thus, although obtained in a different species, these data substantiate hypotheses derived indirectly from clinical or experimental observations with Cox-2 inhibitors in humans.\textsuperscript{2,8,16–19} They extend the original observations obtained in dogs by Hennan and colleagues\textsuperscript{16} by clarifying the mechanism underlying prothrombotic and pro-occlusive effects of selective Cox-2 inhibition in vivo.

The pronounced proadhesive effects of NS-398 even in intact arterioles are most likely due to decreased prostacyclin synthesis by the endothelium in vivo. Similar to what has been observed in humans,\textsuperscript{8,9,17} we measured decreased systemic levels of 6-keto-PGF$_{1\alpha}$ in the sera of animals treated with a selective Cox-2 inhibitor. Systemically decreased levels of 6-keto-PGF$_{1\alpha}$ in patients or animals treated with selective inhibitors of Cox-2, however, do not allow us to draw conclusions about the source of prostacyclin synthesis, because aside from the endothelium, Cox-2 in other tissues, such as the renal medulla, is also inhibited.\textsuperscript{20} Therefore, we specifically analyzed 6-keto-PGF$_{1\alpha}$ levels in supernatants of cultured endothelial cells after Cox-2 inhibition. Both selective inhibition of Cox-2 and the nonspecific blockade of endothelial Cox by aspirin significantly reduced 6-keto-PGF$_{1\alpha}$ release after histamine stimulation, confirming an important role for Cox-2 in endothelial prostacyclin biosynthesis. It is not surprising that aspirin, when given intravenously as in our experiments, equally affected platelet and vascular Cox activities, because aspirin only becomes a rather selective platelet Cox-1 inhibitor when given orally at low doses.\textsuperscript{21} The assumption that Cox-2 blockade exerted its proadhesive effect by decreasing endothelial prostacyclin synthesis is supported by findings showing that selective inhibitors of Cox-2 exert neither inhibitory\textsuperscript{9,22,23} nor augmenting\textsuperscript{24} effects on platelet aggregation ex vivo, suggesting that the enhanced platelet adhesion in our study is unlikely to be due to direct effects of NS-398 on platelets. The latter is further substantiated by a lack of NS-398 effects after pretreatment of the platelets with iloprost.

It is not surprising that inhibition of Cox-2–dependent prostacyclin biosynthesis may exert prothrombotic effects, because in contrast to nonspecific Cox inhibitors, specific Cox-2 inhibitors do not inhibit platelet TxA$_2$ synthesis. The resulting imbalance of vascular prostacyclin and TxA$_2$ effects has been shown to induce enhanced urinary 2,3-dinor-TxB$_2$, not only abolished the histamine-induced increase but also reduced the 6-keto-PGF$_{1\alpha}$ levels below those of unstimulated control cells (NS-398, 21.2±2 ng/mL; P<0.01 versus histamine, P<0.05 versus control; aspirin, 24.2±3 ng/mL; P<0.01 versus histamine, NS versus control; Figure 6B).

6-Keto-PGF$_{1\alpha}$ Production

The stable prostacyclin analogue 6-keto-PGF$_{1\alpha}$ was measured in animal sera and in supernatants of HUVECs with and without selective Cox-2 inhibition. In vivo, serum levels were effectively reduced by NS-398 treatment (from 974.9±193 pg/mL in controls, n=4, to 416.6±60 pg/mL in NS-398–treated animals, n=4; P<0.05 versus control), whereas aspirin did not induce a significant reduction (608.2±191 pg/mL, n=4; Figure 6A). In HUVECs, the 6-keto-PGF$_{1\alpha}$ concentration in the supernatant after histamine treatment (10 μmol/L, 5 minutes) amounted to 48.7±6 ng/mL, which was significantly higher than in control cells (32.2±3 ng/mL; P<0.05, n=7 each). Preincubation of cells with either NS-398 (100 μmol/L, 2 hours, n=4) or aspirin (50 μg/mL, 2 hours, n=5)
excretion and neointima formation after balloon-induced vessel injury in mice deficient in the prostacyclin receptor.\textsuperscript{25} In accordance with this view, aspirin significantly decreased PVWI under basal conditions and prevented enhanced firm adhesion and PVWI under Cox-2 inhibition, indicating that the prothrombotic effects of decreased endothelial prostacyclin release can be compensated for by simultaneous inhibition of Cox-1–dependent \textit{TxA}$_2$ formation in platelets. These adverse effects of selective Cox-2 inhibition may be even more pronounced in situations of disturbed endothelial function.

It is tempting to speculate that our findings of enhanced platelet activation may form a basis for the reports of an increased risk for myocardial infarction after inhibition of Cox-2, as initially reported in a subanalysis of the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial. In that trial, in which the risk of gastrointestinal bleeding was studied, patients who met the criteria for secondary cardiovascular prophylaxis with aspirin but who had not taken it before randomization received the Cox-2 inhibitor rofecoxib instead of a nonselective Cox inhibitor or aspirin.\textsuperscript{12} In this subgroup of patients, an increased relative risk of 4.89% for experiencing myocardial infarction was subsequently estimated.\textsuperscript{1} It remained unclear whether the increased cardiovascular risk was merely due to the lack of inhibitory effects of selective Cox-2 inhibitors on platelet aggregation\textsuperscript{26} or whether the differences between treatment groups, who received either the nonselective Cox inhibitor naproxen or rofecoxib, were caused by an additional decrease in the antiadhesive properties of the vascular endothelium.\textsuperscript{1,2}

It is interesting to compare the efficiency and selectivity of the selective Cox-2 inhibitor that we used in this study with Cox-2 inhibitors that are in current clinical use. In terms of efficiency, the comparability is highlighted by the degree of reduction of prostacyclin metabolites in serum or urine caused by the substances. Clinically used doses of rofecoxib (50 mg/d), as applied in the VIGOR trial, resulted in \approx 74% inhibition of urinary excretion of prostacyclin metabolites in humans that even exceed the 57% inhibition reached by NS-398 in hamster sera in our study.\textsuperscript{27} Lower doses of rofecoxib still reach \approx 70% inhibition of Cox-2–dependent prostanoid synthesis.\textsuperscript{23} Celecoxib at 400 mg decreases human urinary excretion of prostacyclin metabolites by \textgreater 80% 6 to 12 hours after treatment.\textsuperscript{9} When comparing the selectivity of the mentioned substances for Cox-2, ex vivo assays show that at least the selectivity of rofecoxib is similar to that of NS-398, because both are 100-fold more effective in inhibiting Cox-2 than in inhibiting Cox-1, whereas the specificity of celecoxib is \approx 10-fold lower.\textsuperscript{5,28,29}

Of note, we did not observe thrombosis in intact arterioles of healthy hamsters, which is in line with meta-analyses of clinical trials on selective Cox-2 inhibitors that did not confirm an increased risk for thrombotic events in predominantly healthy populations.\textsuperscript{1,26} However, the proadhesive effects of selective Cox-2 inhibition in intact arterioles of even healthy animals and the rapid occlusion of injured vessels argue in favor of cautious use of these compounds in patients at cardiovascular risk.

Extrapolation of experimentally obtained data to the actual situation in humans is limited, and conclusions should be drawn carefully, especially because the small number of experiments performed in an animal study like ours cannot compare with large patient populations who are overseen in clinical studies. Whether the prothrombotic effects of selective Cox-2 inhibition that we observed in our study are actually paralleled by limited cardiovascular safety of these drugs in clinical practice can only be answered by prospective trials. The realization of such studies has repeatedly been postulated.\textsuperscript{6,30,31}

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