

Functional and Biochemical Evaluation of Platelet Aspirin Resistance After Coronary Artery Bypass Surgery

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Background—Aspirin inhibits platelet activation and reduces atherothrombotic complications in patients at risk of myocardial infarction and stroke. However, a sufficient inhibition of platelet function by aspirin is not always achieved. The causes of this aspirin resistance are unknown.

Methods and Results—Patients undergoing coronary artery bypass grafting (CABG) have a high incidence of aspirin resistance. To evaluate functional and biochemical responses to aspirin, platelet-rich plasma was obtained before and at days 1, 5, and 10 after CABG. Thromboxane formation, aggregation, and α -granule secretion were effectively inhibited by 30 or 100 μ mol/L aspirin in vitro before CABG, but this inhibition was prevented or attenuated after CABG. Whereas the inhibition of thromboxane formation and aggregation by aspirin in vitro partly recovered at day 10 after CABG, oral aspirin (100 mg/d) remained ineffective. The inducible isoform of cyclooxygenase in platelets, COX-2, has been suggested to confer aspirin resistance. In fact, immunoreactive COX-2 was increased 16-fold in platelets at day 5 after CABG, but the COX-2 selective inhibitor celecoxib did not alter aspirin-resistant thromboxane formation. By contrast, the combined inhibitor of thromboxane synthase and thromboxane receptor antagonist terbogrel equally prevented thromboxane formation of platelets obtained before (control) and after CABG.

Conclusions—Platelet aspirin resistance involves an impairment of both in vivo and in vitro inhibition of platelet functions and is probably due to a disturbed inhibition of platelet COX-1 by aspirin. (*Circulation*. 2003;108:542-547.)

Key Words: aspirin ■ platelets ■ thromboxane ■ bypass surgery

Aspirin reduces myocardial infarction, stroke, and death in patients with cardiovascular disease, but the extent of protection is limited.¹ Several studies have suggested that a significant proportion of patients (up to 45%) may not respond to aspirin with sufficient inhibition of platelet function.² Recently, an insufficient inhibition of platelet aggregation by aspirin (denoted “aspirin resistance”) has been observed in 6% to 24% of patients with stable coronary artery disease,³ suggesting that aspirin nonresponders may obtain less benefit with respect to cardiovascular events.⁴

The mechanism of aspirin resistance is unknown. Because platelets contain, besides cyclooxygenase (COX)-1, a variable amount of COX-2,^{5,6} which is at least two orders of magnitude less sensitive to inhibition by aspirin than COX-1,^{7,8} it has been speculated that an insufficient platelet response to aspirin may be caused by thromboxane formation via platelet COX-2. Nevertheless, the mechanism of aspirin resistance remains unclear, probably because appropriate experimental models are not available.

A useful tool to further study aspirin resistance would be a population of patients with an exceptionally high incidence of aspirin nonresponders. Recent work from our laboratory has

shown that the majority of patients who undergo coronary artery bypass grafting (CABG) develop aspirin resistance within the first 10 days after surgery.⁹ Moreover, a metaanalysis of the Antiplatelet Trialist’s Collaboration¹⁰ indicated that patients after CABG do not benefit from antiplatelet (mostly aspirin) treatment.

Therefore, we further investigated the insufficient response of platelets to aspirin early after CABG. The aims were to (1) determine the sensitivity of platelet thromboxane formation to aspirin in vivo and in vitro, (2) study platelet cyclooxygenase isoenzyme expression and sensitivity to a selective COX-2 inhibitor, and (3) identify pharmacological strategies to restore a sufficient inhibition of platelet thromboxane formation. We show here that aspirin resistance is associated with an insufficient inhibition of platelet thromboxane formation, even when aspirin is added in vitro at suprapharmacological concentrations. COX-2 is markedly increased after CABG but appears not to contribute to aspirin resistance.

Methods

Subjects and Treatment

The study has been conducted in agreement with the Declaration of Helsinki and was approved by a local ethical committee. One

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hundred ten patients with coronary three vessel disease, requiring an elective CABG procedure, have been screened for aspirin resistance, and 93 were included into the study. Informed written consent was obtained from each patient. Previous aspirin treatment was terminated 7 to 10 days before CABG and continued at day 1 after surgery (ASS 100 Protect, Bayer). None of the patients received additional cyclooxygenase inhibitors. Citrated venous blood was collected in the morning of the day before and at days 1, 5, and 10 after CABG. Blood was collected 1 hour before aspirin administration. Platelet counts were determined by automated counting.

Platelet Aggregation and Thromboxane Formation

Platelet aggregation was measured in platelet-rich plasma (PRP) by turbidimetry, as previously described.¹¹ The results are expressed as cm/min recorder deflection, representing the change in light transmission. The maxima of light transmission achieved similar results (not shown). Platelets were stimulated either by 1 mmol/L arachidonic acid or 1 μ g/mL collagen (each n=20). The sensitivity of platelets to aspirin in vitro was determined by addition of aspirin (30 and 100 μ mol/L) to PRP, 6 minutes before platelets were stimulated. Aggregation was recorded for 5 minutes, platelets removed by centrifugation, and the supernatants were used for determination of thromboxane B₂ by radioimmunoassay.¹²

To study COX inhibition by indomethacin and celecoxib, preferential inhibitors of COX-1 and COX-2,^{13,14} respectively, aspirin nonresponders were identified by less than 90% inhibition of thromboxane formation in the presence of 100 μ mol/L aspirin and 1 mmol/L arachidonic acid. These samples were obtained at days 5 to 10 after CABG. Indomethacin was assessed in PRP from 7 and celecoxib from 14 patients. The PRP of 8 additional nonresponders served to evaluate the effect of terbogrel, an equipotent inhibitor of thromboxane synthase and thromboxane receptors.¹⁵ Indomethacin, celecoxib, and terbogrel (each 1 μ mol/L) were added 5 minutes before PRP was stimulated by 1 mmol/L arachidonic acid and aggregation and thromboxane formation were determined as described. Previous measurements have shown that 1 μ mol/L celecoxib and terbogrel inhibit COX-2 and thromboxane synthase by >90%.

Platelet P-Selectin Expression

PRP from 3 nonresponders was used to measure α -granule secretion by flow cytometric detection of P-selectin (CD62P) expression. For comparison, PRP was obtained from 3 healthy subjects and 3 patients after CABG who were sensitive to aspirin. P-selectin was assessed without and with preincubation with 30 μ mol/L aspirin for 5 minutes at 37°C. The platelets were stimulated with 1 mmol/L arachidonic acid (5 minutes, 37°C) in the presence of 500 μ mol/L RGDS. Thereafter, aliquots of 25 μ L PRP were incubated with 5 μ L anti-CD62P-FITC (Beckman Coulter, Krefeld, Germany) for 30 minutes and diluted with 500 μ L Isotone for analysis on an EPICS-XL cytometer (Beckman Coulter), as described elsewhere.¹⁶

Platelet COX Isoenzymes

Platelets from 8 patients (nonresponders) were obtained 1 day before and at days 1, 5, and 10 after CABG to determine COX-1 and COX-2 by Western blot, as previously described.⁶ Briefly, platelet pellets were homogenized in RIPA buffer, the proteins separated by SDS-PAGE and electrotransferred to Immobilon-P membranes (Millipore Inc). The COX isoenzymes were immunodetected by COX-1- and COX-2-specific antibodies (rabbit polyclonal, Alexis) and peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). Chemiluminescence was visualized on Hyperfilm ECL (Amersham) and the relative band intensity determined densitometrically. The specificity of COX-2 immunodetection has previously been validated by flow cytometry.⁶

Time-Dependent Kinetics of Cyclooxygenase Inhibition by Aspirin

The time course of platelet inhibition by aspirin was determined by incubating PRP from 10 aspirin nonresponders after CABG with 10

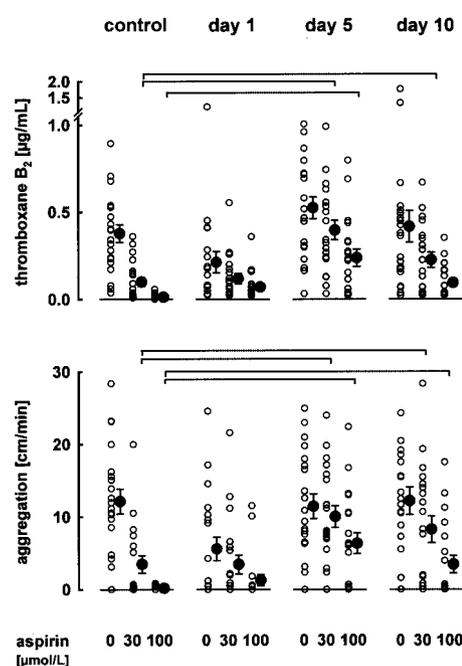


Figure 1. Arachidonic acid (1 mmol/L)-induced platelet thromboxane formation (top) and aggregation (bottom) in PRP of patients (n=20) before (control) and at the indicated times after CABG. The measurements have been performed without or with addition of aspirin in vitro (30 and 100 μ mol/L). Open symbols represent individual values; filled symbols, mean \pm SEM. Brackets indicate significant differences ($P < 0.05$).

μ mol/L aspirin at 37°C. This concentration is reached in plasma after oral treatment.¹⁷ After 15, 30, 45, and 60 minutes, aliquots were stimulated by 1 mmol/L arachidonic acid for 5 minutes and thromboxane was determined by radioimmunoassay.

Substances and Solutions

Aspirin was used in vitro as lysine salt (Aspisol, Bayer). The stability of aspirin during the measurements has been verified by colorimetric detection of aspirin hydrolysis under assay conditions. Collagen was obtained from Nycomed and arachidonic acid from Oxford Biomedical Research. Celecoxib was kindly provided by Dr Geisslinger, Pharmazentrum Frankfurt, University of Frankfurt, Germany. Terbogrel was a gift from Boehringer (Ingelheim, Germany). All other chemicals were from Merck or Sigma.

Statistical Analysis

All data are mean \pm SEM. Groups were compared by Student's *t* test and one-way analysis of variance with post hoc analysis (Dunnett), as appropriate. Event rates were compared by Fisher's exact test. A value of $P \leq 0.05$ was considered significant.

Results

Aspirin was well tolerated by all patients without thrombotic or bleeding complications. The mean platelet count before surgery (control) was $233 \pm 17 \times 10^3/\mu\text{L}$. Due to hemodilution during extracorporeal circulation, the platelet counts decreased to $165 \pm 17 \times 10^3/\mu\text{L}$ at day 1 after surgery ($P < 0.05$ versus control), recovered at day 5 ($277 \pm 22 \times 10^3/\mu\text{L}$), and further increased at day 10 ($417 \pm 25 \times 10^3/\mu\text{L}$; $P < 0.05$ versus control).

Platelet Thromboxane Formation

Arachidonic acid-stimulated (Figure 1) and collagen-stimulated (Table 1) thromboxane formation were compara-

TABLE 1. Platelet Thromboxane Formation After Stimulation With 1 $\mu\text{g}/\text{mL}$ Collagen

Inhibitor	Control	Time After CABG		
		Day 1	Day 5	Day 10
Aspirin (0 $\mu\text{mol}/\text{L}$)	78 \pm 9	24 \pm 5*	55 \pm 11	47 \pm 13
Aspirin (30 $\mu\text{mol}/\text{L}$)	34 \pm 4†	21 \pm 4†	34 \pm 8*	16 \pm 6
Aspirin (100 $\mu\text{mol}/\text{L}$)	4 \pm 1†	13 \pm 2*†	12 \pm 3*†	5 \pm 2†

Inhibition by 30 and 100 $\mu\text{mol}/\text{L}$ aspirin. Shown are measurements ($n=20$) before (control) and at the indicated times after CABG. All values are ng/mL.

* $P<0.05$ vs control.

† $P<0.05$ vs aspirin (0 $\mu\text{mol}/\text{L}$).

ble before CABG and at days 5 and 10 thereafter, indicating that oral aspirin did not significantly inhibit platelet cyclooxygenase. A transient decrease of thromboxane formation at day 1 was probably caused by hemodilution.

When 30 and 100 $\mu\text{mol}/\text{L}$ aspirin was added in vitro to PRP collected before CABG (control), arachidonate-induced thromboxane formation was significantly inhibited to 25 \pm 5 and 5 \pm 1%, respectively (Figure 1). Aspirin in vitro (100 $\mu\text{mol}/\text{L}$) decreased thromboxane formation to 10% or less in most (17/20) of the samples.

This in vitro effect was remarkably attenuated after surgery. For example, at day 5 after CABG the in vitro addition of 30 and 100 $\mu\text{mol}/\text{L}$ aspirin decreased thromboxane formation only to 75 \pm 7% and 45 \pm 8%, respectively (each $P<0.05$ versus control before CABG). At this time, 100 $\mu\text{mol}/\text{L}$ aspirin reduced thromboxane formation to 10% or less only in 6/20 patients ($P<0.01$ versus control before CABG). The inhibitory effect of aspirin in vitro was restored in part, but not completely, at day 10. Similar results were also obtained for collagen-induced thromboxane formation (Table 1).

Platelet Aggregation

Arachidonic acid effectively induced platelet aggregation before CABG with some interindividual differences. As expected from the failure of oral aspirin to inhibit thromboxane formation, platelet aggregation was not significantly altered at days 5 and 10 after CABG (Figure 1, Table 1). Moreover, the inhibition of aggregation by aspirin in vitro (30 and 100 $\mu\text{mol}/\text{L}$) was remarkably attenuated at days 5 and 10 (Figure 1). Comparable results were obtained when aggregation was induced with collagen (not shown).

Platelet P-Selectin Expression

Arachidonic acid increased P-selectin expression in the PRP of healthy subjects ($n=3$) from 3.0 \pm 0.1 to 125 \pm 59 fluorescence units (Figure 2A). This was nearly prevented by 30 $\mu\text{mol}/\text{L}$ aspirin (9.8 \pm 7.5 U; Figure 2B). In the PRP of aspirin-resistant patients after CABG ($n=3$), arachidonic acid also stimulated P-selectin expression from 6.4 \pm 1.1 to 45.2 \pm 12.9 U (Figure 2C), but this was not inhibited by aspirin in vitro (49.0 \pm 16.6 U; Figure 2D). Arachidonic acid did not stimulate P-selectin expression in the PRP of aspirin-sensitive patients ($n=3$, 1.6 \pm 0.7 versus 1.9 \pm 0.9 U; Figure 2E), without further inhibition by aspirin in vitro (Figure 2F). Thus, aspirin resistance also applied to α -granule secretion.

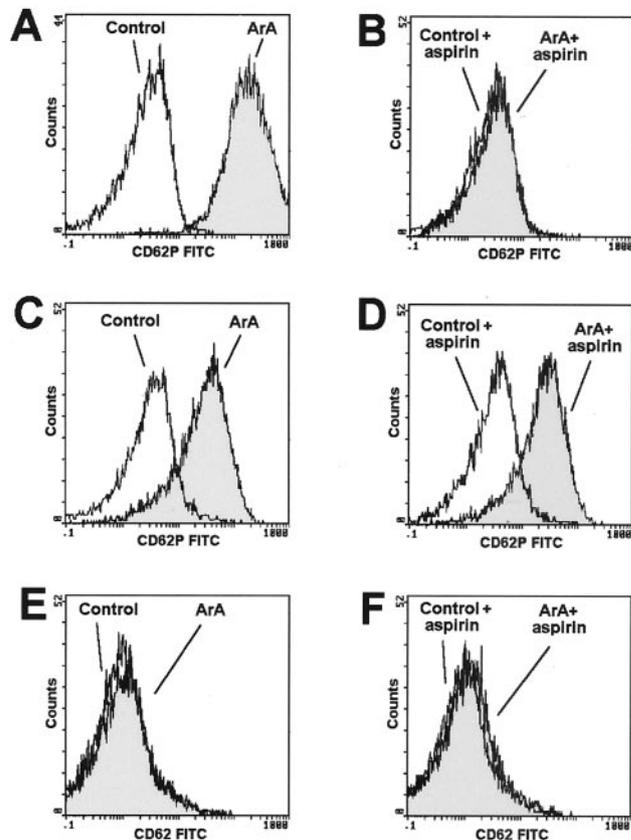


Figure 2. Arachidonic acid (1 mmol/L)-induced platelet P-selectin (CD62P) expression in PRP from a healthy subject and two patients after CABG. Each histogram is representative of measurements in 3 different individuals. Arachidonic acid (ArA, shaded) externalized P-selectin in PRP from the control subject (A), which was prevented by 30 $\mu\text{mol}/\text{L}$ aspirin (B). Arachidonic acid-induced P-selectin expression in PRP from a patient identified as aspirin nonresponder (C) was not inhibited (D). In the PRP from a patient with platelets responsive to aspirin, P-selectin expression was prevented by oral aspirin (E) without further inhibition by aspirin in vitro (F).

Platelet COX Isoenzymes

Before CABG, mainly COX-1 was detected in platelet protein extracts, whereas there were only traces of COX-2 (Figure 3). There were no major changes of COX-1 after CABG. However, COX-2 immunoreactivity increased at day 5 after CABG about 16-fold over the control before CABG ($P<0.05$). COX-2 returned to almost normal at day 10 after CABG.

Inhibitors of Arachidonic Acid Metabolism

The selective COX-2 inhibitor celecoxib (1 $\mu\text{mol}/\text{L}$) did not significantly inhibit arachidonic acid-induced thromboxane formation (control, 769 \pm 139 ng/mL; celecoxib, 686 \pm 109 ng/mL) in aspirin-resistant PRP collected after CABG. Similar results were obtained for platelet aggregation.

Indomethacin (1 $\mu\text{mol}/\text{L}$) effectively inhibited platelet thromboxane formation before CABG. However, this inhibition was markedly attenuated in aspirin-resistant platelets after CABG (Figure 4), indicating that the observed resistance toward aspirin was paralleled by an impaired response to indomethacin.

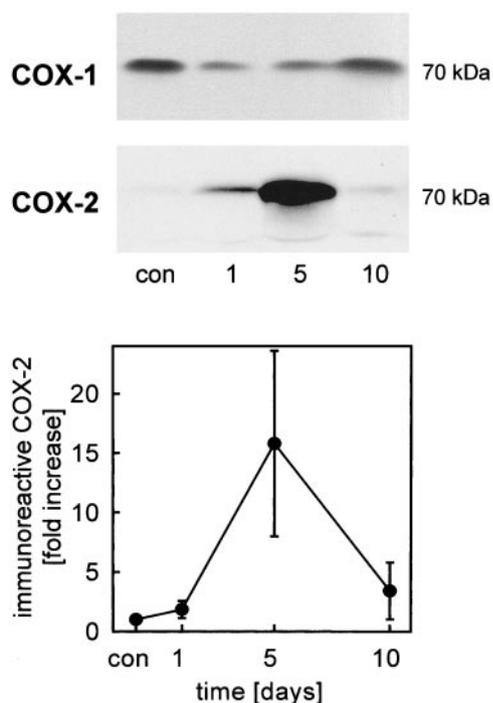


Figure 3. Top, Platelet COX-1 and COX-2 in a patient undergoing CABG. Immunoblots were performed before CABG (con) and at the indicated times (days) thereafter. Bottom, Time-dependent increase in platelet COX-2 determined by densitometry (n=8).

The combined thromboxane synthase inhibitor/thromboxane receptor antagonist terbogrel (1 $\mu\text{mol/L}$) almost abolished thromboxane formation (Table 2) and inhibited aggregation in aspirin-resistant PRP (Table 2, Figure 5A). Moreover, flow cytometric detection of platelet P-selectin expression showed that terbogrel largely prevented arachidonic acid-induced α -granule secretion (Figure 5B).

Time-Dependent Inhibition of Platelet COX by Aspirin

Before CABG, 10 $\mu\text{mol/L}$ aspirin inhibited thromboxane formation within 15 minutes to 10% or less (Figure 6). In aspirin-resistant PRP, there was a significant delay of the

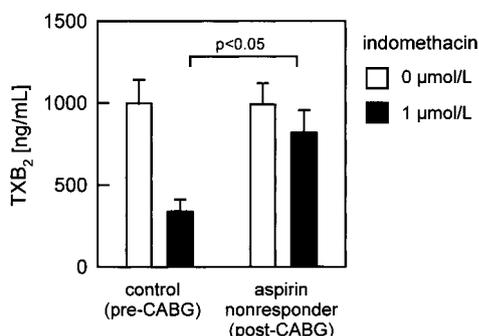


Figure 4. Effect of indomethacin on arachidonic acid (1 mmol/L)-induced thromboxane formation in PRP before (control) and after CABG. Measurements have been performed with PRP from patients who developed aspirin resistance after CABG (n=7).

TABLE 2. Effect of Terbogrel on Platelet Aggregation and Thromboxane Formation Induced by Arachidonic Acid (1 mmol/L) in PRP From Patients After CABG Who Were Previously Identified as Aspirin Nonresponders (n=8)

Parameter	Control	Aspirin (100 $\mu\text{mol/L}$)	Terbogrel (1 $\mu\text{mol/L}$)
Aggregation, cm/s	5.15 \pm 0.7	4.5 \pm 0.6	0.9 \pm 0.2*†
Thromboxane, ng/mL	717.0 \pm 158.6	341.1 \pm 37.4*	10.0 \pm 2.4*†

* $P < 0.01$ vs control.

† $P < 0.05$ vs aspirin.

inhibition of thromboxane formation. Nevertheless, an extended incubation achieved nearly complete inhibition of thromboxane formation. Control experiments determined the activity of plasma aspirin esterase, but no significant changes have been observed in aspirin-resistant patients (not shown).

Discussion

This study shows that platelets from patients after CABG insufficiently respond to aspirin in vitro, even at concentrations (30 and 100 $\mu\text{mol/L}$) that exceed those in plasma after oral antiplatelet treatment.¹⁸ Aspirin resistance involves, besides thromboxane formation, an impaired inhibition of platelet aggregation and an increased expression of P-selectin, a marker of α -granule secretion associated with the progression of atherosclerosis.¹⁹

We have previously proposed to classify aspirin resistance into three major categories,²⁰ of which one (type 1) includes the inhibition of platelet thromboxane formation in vitro but not in vivo (pharmacokinetic type). Type 2 is characterized by the inability of aspirin to inhibit platelet thromboxane formation in vivo and in vitro (pharmacodynamic type). Type

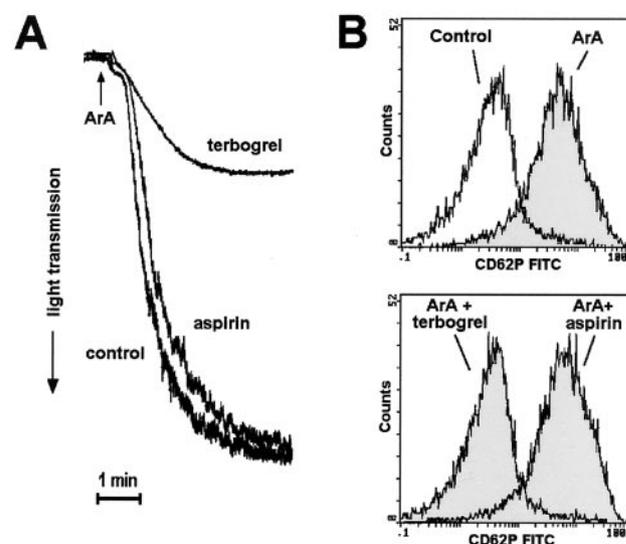


Figure 5. A, Effect of aspirin (30 $\mu\text{mol/L}$) and terbogrel (1 $\mu\text{mol/L}$) on arachidonic acid (1 mmol/L, ArA)-induced platelet aggregation. Original registrations with PRP from an aspirin nonresponder (representative of measurements in 8 patients). B, Arachidonic acid-induced P-selectin (CD62P) expression in control PRP and PRP stimulated by arachidonic acid (ArA, shaded histograms) from an aspirin nonresponder (top). Terbogrel, but not aspirin, prevented P-selectin expression (bottom). Histograms are representative of measurements in 3 patients.

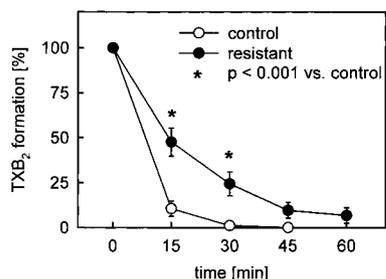


Figure 6. Time course of platelet inhibition by aspirin in PRP from control and aspirin-resistant patients ($n=10$) after CABG. Control PRP with a normal response to aspirin (before CABG) and aspirin-resistant PRP (after CABG) were incubated with $10 \mu\text{mol/L}$ aspirin for up to 60 minutes. After 15, 30, 45, and 60 minutes, arachidonic acid (1 mmol/L) was added and thromboxane formation determined as described.

3 involves thromboxane-independent platelet activation (pseudoresistance). According to this classification, the resistance to aspirin described here is consistent with type 2. The observed loss of platelet inhibition by aspirin *in vitro* excludes an impaired bioavailability as a cause of aspirin resistance. Moreover, the enhanced regeneration of new platelets after surgery with an increased fraction of active (not acetylated) cyclooxygenase does also not sufficiently explain aspirin resistance. The *in vitro* resistance observed here rather appears to result from an impaired interaction of aspirin with platelet cyclooxygenase.

It is long known that platelet thromboxane formation principally depends on COX-1, the constitutive isoform of cyclooxygenase.²¹ Nevertheless, careful analysis of COX isoforms in platelets also revealed a small amount of the inducible isoform COX-2,^{5,6} which is 170-fold less sensitive to inhibition by aspirin.²¹ A recent study demonstrated that an increased platelet turnover elevates platelet COX-2,⁸ and it has been speculated that platelet aspirin resistance may be caused by COX-2 in platelets by generating critical amounts of thromboxane despite aspirin treatment.

The present study shows an increased platelet COX-2 immunoreactivity after CABG, which may result from the increased platelet turnover after cardiopulmonary bypass. In fact, platelet counts increased to almost 80% above control within 10 days after CABG. COX-2 levels reached a maximum at day 5, which is well within this time. This augmented platelet turnover may have stimulated COX-2 expression in megakaryocytes, because COX-2 is a regulator of hematopoiesis²² and the COX-2 promoter contains a recognition site for the transcription factor GATA-1, which contributes to megakaryocyte differentiation.²³ Moreover, cytokines (TNF- α and various interleukins) circulate at elevated concentrations within the first days after cardiac surgery.²⁴ These may upregulate COX-2²¹ in megakaryocytes with a subsequent increase in platelets.

Despite the increase in platelet COX-2 after CABG, thromboxane synthesis was not prevented by the potent and selective COX-2 inhibitor celecoxib, indicating that COX-2 does not produce functionally relevant amounts of thromboxane. This is at variance to data recently published by Rocca and coworkers,⁸ who reported that the preferential

COX-2 inhibitor NS-398 decreased platelet thromboxane formation to a higher extent in patients with increased platelet COX-2. The same study, however, reported a nearly complete inhibition of thromboxane production in patient blood by $50 \mu\text{mol/L}$ aspirin, a concentration that would be expected to preferentially inhibit COX-1.^{7,14}

A recent study identified new variants of COX-1 and denoted one of them, COX-3'.²⁵ Whether COX-3 occurs in platelets is currently unknown. Nevertheless, a role of COX-3 for aspirin resistance is not likely because the IC_{50} values of aspirin for COX-1 and COX-3 inhibition are similar (3 to $10 \mu\text{mol/L}$) and much lower than aspirin's IC_{50} for COX-2 inhibition ($>1000 \mu\text{mol/L}$).²⁵

To further support the role of an impaired interaction between aspirin and platelet cyclooxygenase, it was of interest to study the effect of indomethacin, a structurally different compound, which, in contrast to aspirin, is a reversible inhibitor. Remarkably, the inhibition by indomethacin was also attenuated after CABG, which supports the contention that aspirin resistance is caused by an alteration at the level of the COX-1 enzyme.

Provided that a reduced sensitivity of cyclooxygenase accounts for aspirin resistance, COX-independent inhibition at the level of thromboxane synthase and thromboxane receptors should remain effective. The guanidino derivative terbogrel inhibits both with an IC_{50} of about 10 nmol/L .¹⁵ Indeed, terbogrel prevented thromboxane formation, P-selectin expression, and effectively inhibited aggregation, which further supports an abnormal interaction of aspirin with platelet COX-1.

The irreversible acetylation of a specific serine moiety (position 530) of COX-1 by aspirin²¹ needs to proceed at a high rate, because the plasma half-life of aspirin is short (20 minutes).¹⁸ Accordingly, thromboxane formation by control platelets (before CABG) was largely inhibited within 15 minutes, which is within the half-life of aspirin in blood. In contrast, platelets from patients after CABG revealed a significantly delayed inhibition of cyclooxygenase by aspirin, probably not allowing for relevant platelet inhibition before conversion of aspirin to salicylate. The delayed inhibition kinetics, therefore, provides the enzymological correlate of the observed resistance to aspirin. This observation may suggest that aspirin resistance can be overcome by a prolonged administration, such as repeated doses per day.

Although the present work provides new insight into the mechanism of aspirin resistance, the biochemical process remains to be identified. Because the induction of COX-2 in platelets does not appear to be involved, one may speculate that platelet COX-1 undergoes a modification that prevents the aspirin-dependent acetylation of serine 530. For example, nitrosylation of platelet COX has been described and found to be associated with alterations of the enzyme's catalytic activity,²⁶ but it is unknown whether this also involves an impairment of COX acetylation by aspirin.

In summary, the present study demonstrates that platelet inhibition by aspirin is compromised within several days after CABG, probably due to an impaired interaction between aspirin and platelet cyclooxygenase (likely COX-1). The enzyme's time-dependent inactivation is critically delayed,

whereas COX-2, although elevated, appears not to be involved. The molecular mechanism is beyond the focus of this study and remains to be established. Aspirin resistance may be overcome by an intensified treatment protocol with aspirin or COX-independent interference with the thromboxane system, such as inhibition of thromboxane synthase and thromboxane receptors.

Acknowledgments

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