

Monitoring of Clopidogrel Action: Comparison of Methods

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Background: Clopidogrel is a potent drug for prevention of adverse effects during and after coronary intervention. Increasing experience indicates that a significant proportion of patients do not respond adequately to clopidogrel. Because failure of antiplatelet therapy can have severe consequences, there is need for a reliable assay to quantify the effectiveness of clopidogrel treatment.

Methods: Of 24 healthy volunteers admitted to the study, 18 were treated for 1 week with clopidogrel (300-mg loading dose and 75-mg maintenance dose), and 6 with placebo. Platelet function was monitored by 2 assays, based on flow cytometry and enzyme immunoassay, that measure the phosphorylation status of vasodilator-stimulated phosphoprotein (VASP) and by aggregometry, flow cytometry of P-selectin, and the platelet function analyzer at baseline, on days 1–5, and on day 9 of treatment.

Results: Aggregometry and VASP phosphorylation revealed a loss of platelet response to ADP within 12 h after clopidogrel intake. The phosphorylation status of VASP correlated with the inhibition of platelet aggregation. In contrast, neither P-selectin expression nor PFA-100 closure time was a clear indicator of clopidogrel effects on platelets.

Conclusions: VASP phosphorylation assays are reliable for quantifying clopidogrel effects. Because the VASP assay directly measures the function of the clopidogrel target, the P2Y₁₂ receptor, the assay is selective for

clopidogrel effects rather than effects of other platelet inhibitors commonly in use.

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Since the approval of the P2Y₁₂ ADP-receptor antagonist clopidogrel (Plavix[®] or Iscover[®]) in 2002, the results of numerous studies have revealed the remarkable potency of the thienopyridines in preventing spontaneous platelet aggregation after percutaneous transluminal coronary angioplasty (PTCA)⁴ (1, 2). Dual therapy combining clopidogrel and acetylsalicylic acid [aspirin (ASA)] has become the standard antiplatelet therapy, but several clinical studies have revealed considerable individual variability in platelet responses to clopidogrel. Depending on the type of antiplatelet therapy regimen used, 5%–25% of patients with stable coronary artery disease do not respond adequately to thienopyridine therapy (3–5). Recent studies indicated that clopidogrel resistance might be associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction (6). At present, the mechanisms underlying clopidogrel resistance are still controversial. Possible explanations include increased reactivity of resting platelets, dysfunctional cytochrome P450 metabolism (either because of a hereditary disorder or interaction with other drugs, such as statins), genetic polymorphisms of the ADP receptor, differences in resorption, or a combination of these factors (7–10). Because the outcome of PTCA relies on subsequent effective antithrombotic therapy, a quantitative assay for assessing the success of clopidogrel treatment is required. Likewise, such an assay is necessary for estab-

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⁴ Nonstandard abbreviations: PTCA, percutaneous transluminal coronary angioplasty; ASA, acetylsalicylic acid (aspirin); FACS, fluorescence-activated cell sorting; PKA, cAMP-dependent protein kinase; P-VASP, phosphorylated vasodilator-stimulated phosphoprotein; EIA, enzyme immunoassay; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; FITC, fluorescein isothiocyanate; PRP, platelet-rich plasma; PBS, phosphate-buffered saline; MFI, geometric mean fluorescence intensity; PRL, platelet reactivity index; and PGE₁ and PGL₂, prostaglandin E₁ and I₂ (prostacyclin), respectively.

lishing effective clopidogrel dosages for use in prophylactic prevention of secondary cardiovascular events (11, 12).

Although the need to monitor clopidogrel efficacy has increased, no standard method is available at present. Most clinical studies on clopidogrel rely on standard platelet aggregometry to measure inhibition of ADP-induced aggregation (4, 5). In addition to aggregometry, cytometric determination of surface expression of platelet proteins, in particular P-selectin (CD62P), is commonly used to monitor platelet activation. Other methods include measurement of fibrinogen binding or platelet adhesion. These approaches assay only general functional platelet indices, are relatively unspecific, and are of limited clinical use because patients typically receive several drugs affecting platelet function (e.g., ASA). Consequently, deficient responses to clopidogrel may be obscured by concomitant treatments. A recently developed fluorescence-activated cell-sorting (FACS) method enables more specific assessment of clopidogrel's inhibition of its biochemical target, the purinergic receptor P2Y₁₂ (13–17). In platelets, the P2Y₁₂ receptor is coupled to the inhibitory G-protein G_i. Hence, activation of the P2Y₁₂ receptor causes inhibition of adenylate cyclase via G_i, with subsequently decreased cAMP-dependent protein kinase (PKA) activity and thus diminished protein phosphorylation of PKA substrates. P2Y₁₂ receptor activity can therefore be determined by measurement of the phosphorylation status of vasodilator-stimulated phosphoprotein (VASP), a known substrate of PKA (Fig. 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol51/issue6/>), and cGMP-dependent protein kinase. Initially, this was accomplished by Western blot analysis (18) and later by flow cytometry (FACS) (13) using a phosphospecific antibody directed against phosphorylated VASP [phospho-VASP (P-VASP)]. However, no systematic comparison of different methods for determining platelet responsiveness under comparable conditions have been published. We therefore performed a blind clinical study with healthy volunteers receiving clopidogrel or placebo, but no other medication, to compare various assays of platelet responsiveness, including 2 methods [FACS and enzyme immunoassay (EIA)] based on VASP phosphorylation for monitoring clopidogrel action.

Materials and Methods

VOLUNTEERS

We enrolled 24 healthy volunteers (Table 1) in the study after obtaining their written, informed consent. The baseline demographic characteristics of these healthy volunteers are given in Table 1. Of the 24 volunteers, 6 were randomized in a blinded manner to the placebo group. The study was approved by the ethics committee of the University of Würzburg. Volunteers had not ingested any medication affecting platelet function within the 2 weeks before onset of the study. The volunteers received a

Table 1. Demographic characteristics of the volunteers.

	Clopidogrel group	Placebo group
n	18	6
Mean (SD) age, years	29.6 (4.9)	27.0 (3.1)
M/F, n	10/8	4/2
Mean (SD) body mass index, kg/m ²	21.9 (2.4)	22.5 (1.7)
Mean (SD) platelet count, × 10 ⁹ /L	267 (62)	256 (62)

loading dose of 4 tablets containing either 75 mg of clopidogrel (Plavix) or a placebo and continued taking 1 tablet of either clopidogrel or placebo daily for a 7 additional days. Blood was drawn before treatment, at 12 h after the first drug intake (loading dose), at 24-h intervals for the next 3 days, and finally 12 h after the last drug intake. We monitored the volunteers for adverse effects, such as bleeding, after clopidogrel administration but observed none.

REAGENTS

The ADP was from Sigma; U46619 (9,11-dideoxy-11 α ,9 α -epoxymethanoprostaglandin F_{2 α}), a stable thromboxane A₂ analog, was a product of Alexis; and iloprost (Ilomedin[®]) was from Schering. The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and complete[®] EDTA-free (protease inhibitor cocktail tablets) were purchased from Roche. Clopidogrel (Plavix; Sanofi-Synthelabo) was provided by a local pharmacy.

ANTIBODIES

Unlabeled and fluorescein isothiocyanate (FITC)-labeled VASP-16C2 antibodies directed against Ser-239-phosphorylated VASP were obtained from Nanotools, and IE273 anti-VASP, which recognizes VASP irrespective of its phosphorylation status, was from Immunoglob. FITC anti-human CD62P was purchased from BD Biosciences Pharmingen, and peroxidase-conjugated goat anti-mouse IgG was purchased from Chemicon.

PLATELET PREPARATION

At each visit by each volunteer, 60 mL of blood was collected in 15 mL of citrate buffer (100 mmol/L sodium citrate, 7 mmol/L citric acid, 140 mmol/L glucose, pH 6.5) and directly used for the P-VASP EIA and the P-VASP FACS assay. Platelet-rich plasma (PRP) and washed platelets were prepared from the citrate-anticoagulated whole blood as described previously (18). Briefly, PRP was separated by centrifugation for 20 min at 300g (Sigma 3K-1), removed with a plastic pipette, and transferred into polypropylene plastic tubes. For preparation of washed platelets, blood was collected in the citrate buffer described above, supplemented with 15 mmol/L EGTA. After preparation of PRP, the platelet suspension was centrifuged for 10 min at 500g, and the platelet pellet was resuspended in HEPES buffer (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4).

PLATELET AGGREGATION

Platelet aggregation was determined on a PAP-4C aggregometer (Biodata) as described previously (19). Briefly, 0.3-mL samples of PRP were transferred to siliconized glass cuvettes and stimulated under continuous stirring. Platelet aggregation was expressed as the maximum percentage change in light transmittance from baseline, with platelet-poor plasma used as a reference for 100% aggregation.

DETERMINATION OF P-SELECTIN EXPRESSION BY FLOW CYTOMETRY

We used a standard cytometric assay to measure P-selectin surface expression in whole blood without prior fixation with formaldehyde. Washed platelets (20 μ L) stimulated with ADP, U46619, or thrombin were stained with 10 μ L of FITC-conjugated anti-human CD62P and, after a 15-min incubation in the dark, were diluted with 1.5 mL of phosphate-buffered saline (PBS). All samples were analyzed at low flow rate on a Becton Dickinson FACSCalibur. The instrument settings were as follows: forward scatter, E00; side scatter, 337 V; fluorescence channel 1, 850 V. Platelets were differentiated from other cells on the basis of their scatter characteristics. Using CELLQuest software (Ver. 3.1f), we analyzed a total of 15 000 platelet events to obtain the mean fluorescence. Because there were no clopidogrel-associated effects on P-selectin expression, we carried out the experiments only in the first experimental set with 12 volunteers (9 in the clopidogrel and 3 in the placebo group).

PLATELET FUNCTION TEST WITH PFA-100[®]

The PFA-100 point-of-care platelet function analyzer (Dade Behring) simulates the process of platelet adhesion and aggregation *in vitro* by means of collagen test cartridges coated with epinephrine or ADP. Results are expressed as "closure time", i.e., the time it takes for the aperture of the cartridge to become congested as a result of blood clotting. Citrate-anticoagulated blood was aspirated into an ADP/collagen- or epinephrine/collagen-coated cartridge of a PFA-100 instrument. The aperture closing time was recorded in seconds. Because of the obvious absence of any clopidogrel effects, the PFA-100 measurements were carried out only with the first 12 volunteers.

MEASUREMENT OF VASP PHOSPHORYLATION

Incubation of platelets with prostaglandins (e.g., Iloprost) increases intracellular cAMP concentrations and PKA activation and, subsequently, VASP phosphorylation. Concomitant treatment of platelets with ADP counters the effects of prostaglandins by inhibiting adenylate cyclase, and thus PKA activity, leading to dephosphorylation of VASP (Fig. 1 in the online Data Supplement). Phosphorylation of VASP can be detected with the monoclonal antibody 16C2, which selectively recognizes Ser-239-phosphorylated VASP. PKA-dependent VASP phosphor-

ylation can be induced by incubation with prostaglandins. We used the highly stable prostacyclin analog iloprost (Ilomedin) instead of a labile native prostaglandin. Whole-blood samples were incubated with 40 nmol/L iloprost alone or with a mixture of iloprost and 5 μ mol/L ADP.

FLOW CYTOMETRIC ANALYSIS

Flow cytometric analysis of VASP phosphorylation was performed essentially according to the protocol of Schwarz et al. (13). Briefly, 100- μ L samples of citrate-anticoagulated blood were incubated at 37 °C for 1 min with iloprost alone or with iloprost plus ADP (Fig. 2 in the online Data Supplement). Reactions were stopped by addition of 50 μ L of 100 mL/L formaldehyde (methanol free). Samples were fixed for 5 min at room temperature. Subsequently, cell suspensions were diluted with 125 μ L of 4 mL/L Triton X-100 in PBS. After permeabilization for 10 min at room temperature, samples were further diluted with 5 mL of PBS, and 200 μ L of each sample was transferred to a FACS tube. The platelets were then stained with the P-VASP-specific antibody VASP-16C2-FITC (final concentration, 1.25 mg/L) and diluted with 800 μ L of PBS after a 20-min incubation in the dark. The samples were analyzed on a Becton Dickinson FACSCalibur with the same instrument settings as for the P-selectin measurements. We compared the geometric mean fluorescence intensities (MFIs) obtained with the FITC-labeled antibody 16C2 after incubation of platelets with iloprost alone or with iloprost plus ADP.

EIA

For EIA, we used the physiologic VASP binding partner zyxin as a capture molecule. Each sample was incubated with both the P-VASP-specific antibody 16C2 and the antibody IE273, which recognizes VASP regardless of its phosphorylation state. The results are reported as the P-VASP/VASP signal ratio to normalize for sample differences in platelet counts or protein content.

For sample preparation, we stimulated 0.1 mL of citrate-anticoagulated blood as described for the P-VASP FACS assay and stopped the reaction by mixing the sample in a 1:1 ratio with lysis buffer containing (in 5 mL) 40 mmol/L Tris, 150 mmol/L NaCl, 50 mmol/L EDTA, 50 mmol/L EGTA, 20 g/L desoxycholate, 20 mL/L Triton X-100, 1 g/L sodium dodecyl sulfate, 50 mmol/L sodium pyrophosphate, and 1 tablet of complete EDTA-free. We then transferred 100 μ L of each sample to a glutathione S-transferase-zyxin-coated 96-well protein immobilizer plate. After 1 h of shaking at ambient temperature, the plate was washed 4 times with 0.5 mL/L Tween 20 in PBS. The wells were then stained for 1 h with the anti-VASP antibodies IE273 (0.1 μ g/well) or 16C2 (0.5 μ g/well). The plate was washed 3 times, and 100 μ L/well of peroxidase-conjugated goat anti-mouse antibody (diluted 1:2000 in PBS) was added. After 1 h, the plate was washed 3 times and incubated with 100 μ L/well ABTS solution, which

was prepared according to the manufacturer's instructions. We measured the absorbance at 405 nm (A_{405}) 5 and 20 min after addition of ABTS on a Wallac Victor² 1420 Multilabel Counter. Each sample was measured in triplicate.

The intraassay imprecision (CV) for the EIA was 5.6% for sodium nitroprusside-treated, lysed whole human blood, used as the calibrator, measured with both antibodies (16C2 and IE273) on one microtiter plate [$R_{(PGE_1)} = 0.402$ (0.22); $n = 20$]. The interassay imprecision was 9.6% and was obtained by triplicate measurements of the same calibrator on 18 consecutive days. Blood samples can be kept for at least 2 h after collection at ambient temperature without significant changes in the platelet reactivity index (PRI). We tested lysed samples for stability at -20°C by repeated determination of the PRI, which remained unchanged over 3 month. The reagents can be stored at 4°C for weeks without changes, as indicated by test results in our laboratory and by data provided by Schering for Ilomedin.

EFFECT OF ASPIRIN ON VASP PHOSPHORYLATION

We examined the possible interference of ASA treatment with the VASP phosphorylation assay by measuring platelet aggregation and VASP phosphorylation at baseline and 12 h and 7 days after ASA intake in 6 healthy volunteers taking no medication other than 100 mg of ASA. Samples were obtained and analyzed as described above.

EVALUATION OF THE PRI

Platelet responsiveness to clopidogrel, assessed by VASP phosphorylation as a reflection of ADP stimulation of the P2Y₁₂ receptor, is reported as the PRI, the calculation of which was slightly different for the FACS and EIA assays. For FACS, we determined the $PRI_{(FACS)}$ from the MFI of platelets treated with prostaglandin E₁ (PGE₁) or prostaglandin I₂ (PGI₂)/Iloprost, respectively (measured with the FITC-labeled P-VASP antibody) in the absence [$MFI_{(PGE_1)}$] or presence of ADP [$MFI_{(PGE_1 + ADP)}$; Eq. 1] (14, 16):

$$PRI_{(FACS)} = \frac{MFI_{(PGE_1)} - MFI_{(PGE_1 + ADP)}}{MFI_{(PGE_1)}} \quad (1)$$

For EIA, the $PRI_{(EIA)}$ was calculated from the ratio of the signals detected with the IE273 antibody, which recognizes VASP irrespective of its phosphorylation status [total VASP; $A_{405(VASP)}$], and the 16C2 antibody, which recognizes Ser-239-phosphorylated VASP [$A_{405(P-VASP)}$], after PGE₁ (or PGI₂) treatment of platelets in the absence [$R_{(PGE_1)}$; Eq. 2A] and presence of ADP [$R_{(PGE_1 + ADP)}$; Eq. 2B]. $R_{(PGE_1)}$ represents the relative amount of VASP phosphorylation through PKA and $R_{(PGE_1 + ADP)}$ the relative amount of P-VASP after PKA inhibition by ADP stimulation. If the ADP receptor is completely blocked, $R_{(PGE_1 + ADP)}$ is almost equal to $R_{(PGE_1)}$. The $PRI_{(EIA)}$ represents the

relative amount of VASP dephosphorylation attributable to ADP stimulation, thus reflecting platelet responsiveness to ADP stimulation (Eq. 2C):

$$R_{(PGE_1)} = \frac{A_{405(P-VASP, PGE_1)}}{A_{405(VASP, PGE_1)}} \quad (2A)$$

$$R_{(PGE_1 + ADP)} = \frac{A_{405(P-VASP, PGE_1 + ADP)}}{A_{405(VASP, PGE_1 + ADP)}} \quad (2B)$$

$$PRI_{(EIA)} = \frac{R_{(PGE_1)} - R_{(PGE_1 + ADP)}}{R_{(PGE_1)}} \quad (2C)$$

If inhibition of the platelet P2Y₁₂ receptor by clopidogrel occurs, ADP fails to cause a decrease in VASP phosphorylation and the PRI converges to 0. In contrast, if treatment is ineffective (low responder), dephosphorylation of VASP by ADP still occurs, and the PRI reaches values of ~ 0.5 or greater.

STATISTICS

Results are reported as means (SD), and P values < 0.05 were considered significant. We compared the data between groups at different time points by one-way ANOVA (SigmaStat software) and calculated Pearson correlations.

ROLE OF THE SPONSOR

The design of the study and the experimental procedures were developed in close cooperation with vasopharm BIOTECH. vasopharm BIOTECH also assisted in determination of assay characteristics and data analysis. R. Schinzel from vasopharm BIOTECH substantially contributed to the preparation of the manuscript.

Results

EFFECT OF CLOPIDOGREL ON ADP-INDUCED PLATELET AGGREGATION

The mean ADP-induced decrease in iloprost-stimulated aggregation was inhibited to 40% of baseline 12 h after clopidogrel loading and to 31% of baseline 60 h after clopidogrel loading. We observed no inhibition in the placebo group (Fig. 1). A plot of individual aggregation values before ($t = 0$ h) and 12 h after clopidogrel administration ($t = 12$ h) is shown in Fig. 2. For 4 volunteers (1 in the placebo group and 3 in the clopidogrel group), aggregometry failed on day 0 (before treatment) or day 1, respectively, for technical reasons. The aggregation data for these volunteers were therefore omitted from the figures. According to the definition of Gurbel et al. (4), 4 of the remaining 15 clopidogrel-treated volunteers could be regarded as weak responders, which is in the range observed in other studies (4, 5). Clopidogrel had no effect on aggregation induced by collagen (1 g/L) or thromboxane A₂ (obtained with the thromboxane analog U46619 at $2 \mu\text{mol/L}$; data not shown).

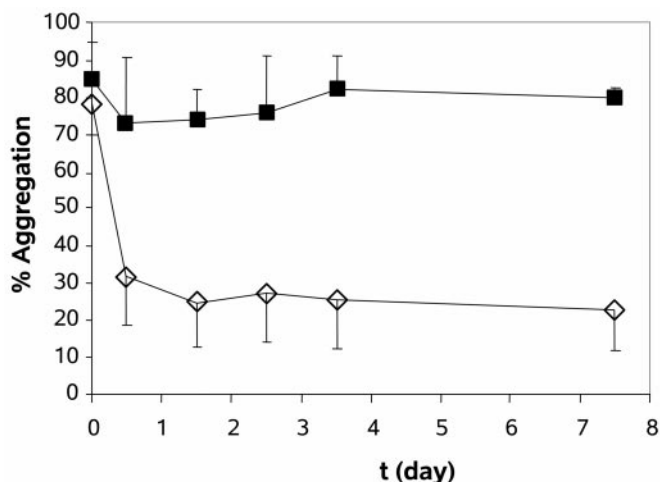


Fig. 1. Clopidogrel inhibition of ADP-induced (5 μ mol/L) platelet aggregation.

Platelet activation was measured in the clopidogrel-treated (\diamond ; 300 mg/day loading dose and 75 mg/day maintenance dose; n = 15) and control (\blacksquare ; n = 5) groups by standard aggregometry as described in the *Materials and Methods*.

EFFECT OF CLOPIDOGREL ON P-SELECTIN SURFACE EXPRESSION

We observed no clear difference between surface expression of P-selectin induced by 5 μ mol/L ADP at baseline (t = 0 h) and after clopidogrel administration at any time point (Fig. 3A). P-Selectin measurements failed to clearly distinguish between the placebo and clopidogrel groups of this study.

EFFECT OF CLOPIDOGREL ON IN VITRO BLEEDING TIME (PFA-100)

We observed no clear difference between closure time measured with ADP/collagen cartridges before (t = 0 h)

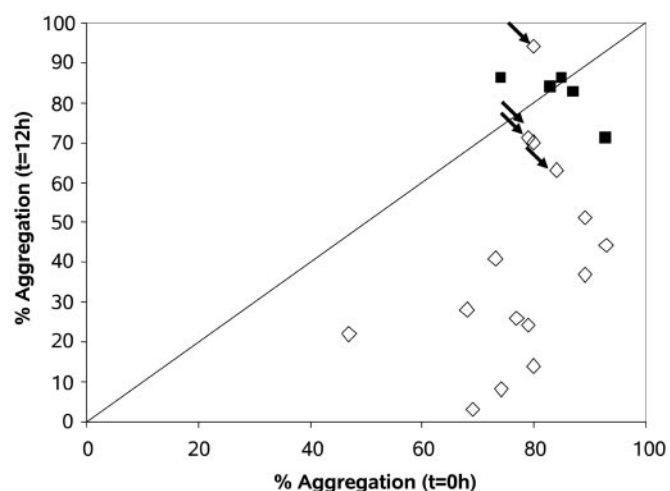


Fig. 2. Comparison of individual standard aggregation values before (t = 0 h; x axis) and 12 h after clopidogrel administration (t = 12 h; y axis) in response to ADP (5 μ mol/L) in the clopidogrel (\diamond) and placebo (\blacksquare) groups.

Possible low responders are indicated by arrows.

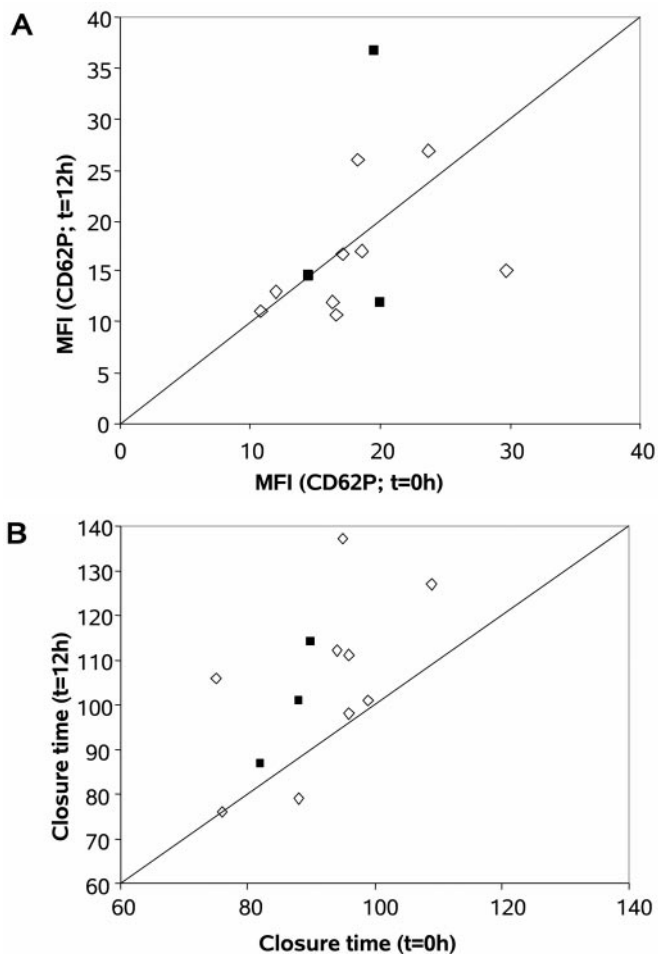


Fig. 3. P-Selectin surface expression in response to ADP (A), and closure time of ADP/collagen cartridges (B) before (t = 0 h; x axis) and 12 h after clopidogrel administration (t = 12 h; y axis).

\diamond , clopidogrel-treated group (n = 9); \blacksquare , placebo group (n = 3). (A), individual values for P-selectin (CD62P) surface expression in response to 5 μ mol/L ADP. (B), experiments were performed on a PFA-100 platelet function analyzer.

and at any time point during clopidogrel administration (Fig. 3B). As for P-selectin, this assay could not distinguish between the placebo and clopidogrel groups in our study. We obtained the same results with epinephrine/collagen-coated cartridges (data not shown).

MEASUREMENT OF VASP PHOSPHORYLATION

We measured VASP phosphorylation by 2 assay formats (FACS and EIA) based on the monoclonal antibody 16C2, which is specific for VASP phosphorylated at Ser-239 (20). The mean (SD) P-VASP/VASP ratio measured by EIA increased from 0.042 (0.14) to 0.80 (0.14) (P < 0.001) in the presence of 40 nmol/L iloprost. This represents an increase in cAMP concentration and, thus, inhibition of platelets. ADP (5 μ mol/L) decreased VASP phosphorylation by a mean of 55 (16)%. In the FACS assay, iloprost increased the P-VASP signal 3- to 4-fold [MFI, 9.8 (1.4) vs 34.7 (5.4)]. In the presence of 5 μ mol/L ADP, the signal induced by iloprost was reduced by a mean of 51 (14)%.

PRI values measured by both assay formats (FACS and EIA) showed a correlation (r^2) of 0.750 ($r = 0.866$; 95% confidence interval, 0.821–0.89; Fig. 4). The statistical test for the null hypothesis of no correlation gave a $P < 0.001$.

EFFECT OF CLOPIDOGREL ON VASP PHOSPHORYLATION
Twelve hours after clopidogrel loading, the mean ADP-induced decrease in iloprost-stimulated VASP phosphorylation was attenuated significantly. We observed a clear effect with both the FACS assay [$PRI_{(FACS, t=0)} = 0.67$ (0.11) vs $PRI_{(FACS, t=12)} = 0.31$ (0.17); $P < 0.001$; Fig. 5A] and the EIA assay [$PRI_{(EIA, t=0)} = 0.48$ (0.14) vs $PRI_{(EIA, t=12)} = 0.20$ (0.14); $P < 0.001$; Fig. 5B]. The maximum effect occurred 48 h after clopidogrel loading, with no additional significant changes at days 4–9. Attenuation of clopidogrel-induced antiplatelet effects commenced 2 days after cessation of clopidogrel administration (day 11). In volunteers receiving placebo, the ADP-induced activation of iloprost-treated platelets was not affected. Individual platelet responses in the placebo group differed considerably, and the PRI ranged from 0.57 to 0.95 (Fig. 6). P-VASP measurements and aggregation correlated ($r = 0.81$; $r^2 = 0.67$). The statistical test for the null hypothesis of no correlation gave a $P < 0.001$ (Fig. 7).

EFFECT OF ASPIRIN ON VASP PHOSPHORYLATION

Although aggregation was substantially reduced, we found no obvious ex vivo influence of ASA on VASP phosphorylation (Fig. 8). These results could also be verified by in vitro experiments testing the effect of 100 $\mu\text{mol/L}$ ASA on ADP-stimulated aggregation and VASP dephosphorylation (data not shown).

Discussion

MONITORING OF CLOPIDOGREL ACTION

Variable responses to clopidogrel can be explained by increased reactivity of resting platelets, differences in

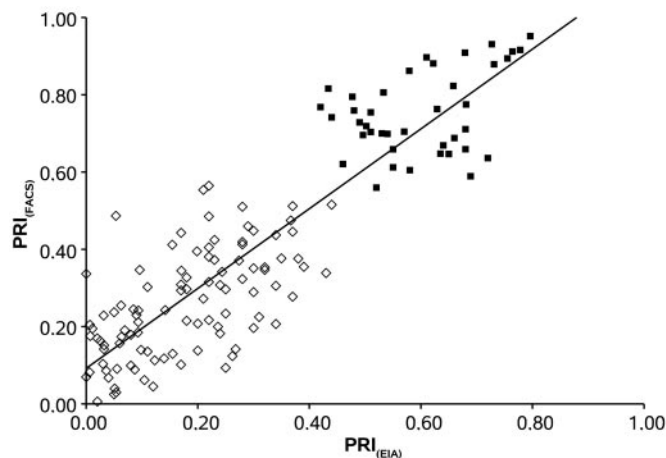


Fig. 4. Correlation of individual PRI values calculated with data obtained from FACS [$PRI_{(FACS)}$] and EIA [$PRI_{(EIA)}$] analysis in the clopidogrel (\diamond) and placebo (\blacksquare) groups [$r = 0.866$ (95% confidence interval, 0.821–0.89); $P < 0.001$].

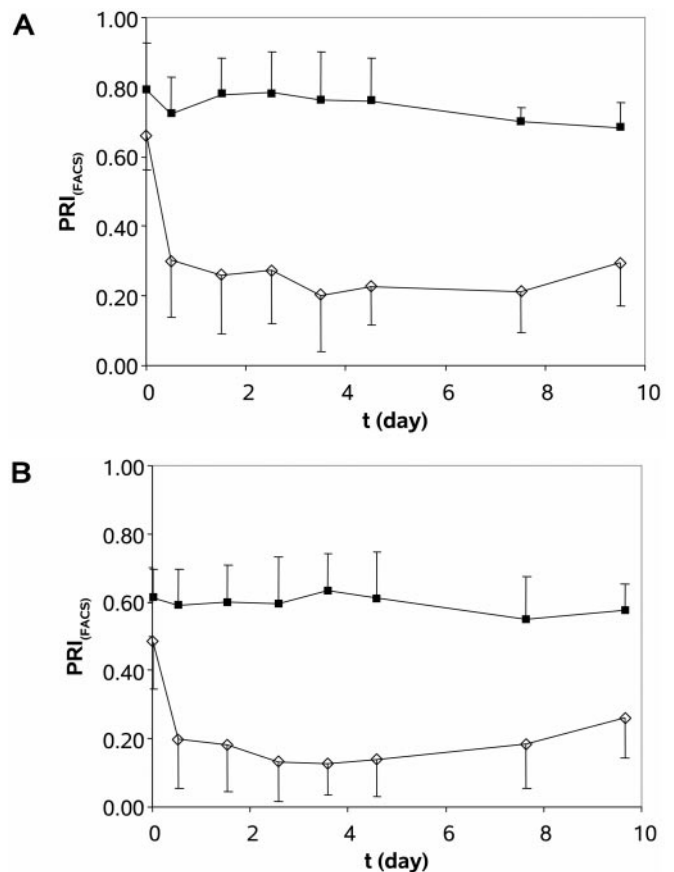


Fig. 5. Clopidogrel blockage of ADP-induced ($5 \mu\text{mol/L}$) platelet activation.

As a marker of platelet responsiveness, the PRI was determined in the clopidogrel-treated (\diamond ; 300 mg/day loading dose and 75 mg/day maintenance dose; $n = 18$) and placebo (\blacksquare ; $n = 6$) groups by FACS [$PRI_{(FACS)}$; A] or EIA [$PRI_{(EIA)}$; B] as described in the *Materials and Methods*. $P < 0.001$ for placebo vs clopidogrel group.

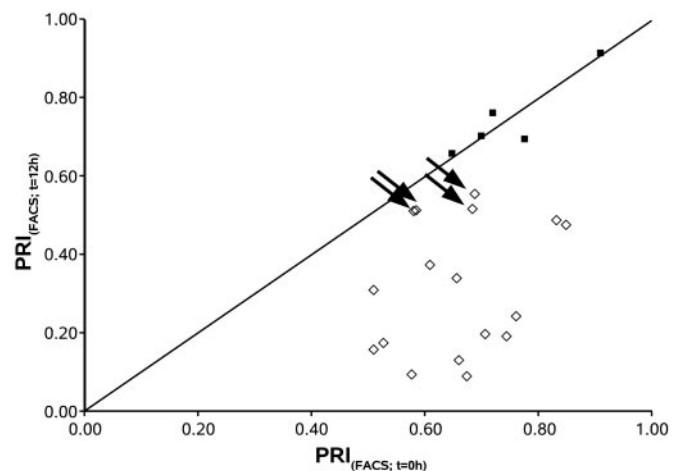


Fig. 6. Comparison of individual $PRI_{(FACS)}$ values before ($t = 0$ h; x axis), and 12 h after clopidogrel administration ($t = 12$ h; y axis) in response to ADP ($5 \mu\text{mol/L}$) in the clopidogrel (\diamond) and placebo (\blacksquare) groups.

Possible low responders are indicated by arrows. $P < 0.001$ for placebo vs clopidogrel group.

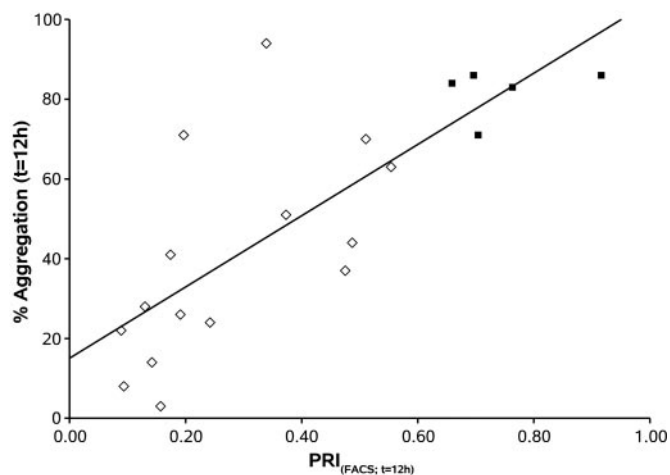


Fig. 7. Correlation of individual $PRI_{(FACS)}$ and ADP-induced ($5 \mu\text{mol/L}$) platelet aggregation values after 12 h of treatment in the clopidogrel (\diamond) and placebo (\blacksquare) groups ($r = 0.84$; $P < 0.001$).

cytochrome P450 metabolism, genetic polymorphisms of the ADP receptor, or a combination of these factors (7–10). Although the observed rate of adverse effects is lower than the incidence of clopidogrel resistance, recent studies have provided initial evidence that nonresponders might be at a higher risk of myocardial infarction (6, 14). Antiplatelet treatment is a key factor in the prevention of subacute stent thrombosis; therefore, monitoring of antiplatelet therapy has been considered important for patients undergoing PTCA (11, 12).

Clopidogrel resistance has been assessed by use of various indicators that reflect platelet function. Typically, inhibition of platelet aggregation, secretion, or adhesion is considered a marker of drug action, but there is no standard, accepted assay for clopidogrel efficacy. For that reason, the magnitude of clopidogrel resistance and the variability of platelet responses to clopidogrel remain uncertain. Generally, patients with $<10\%$ inhibition of platelet aggregation in response to clopidogrel have been considered resistant to its effects, and those with $<30\%$ inhibition have been considered weak responders (4, 5). For the P-VASP assay, the PRI approaches 1 in the absence of any clopidogrel effects. A PRI between 0.5 and 1 would be comparable to weak responses to clopidogrel, as described previously (14, 15).

PLATELET AGGREGATION

At present, conventional platelet aggregometry is the most widely used method to monitor ADP receptor antagonists. In healthy volunteers and in patients with stable coronary disease, clopidogrel administration produces a 35%–60% reduction in ADP-induced platelet aggregation (3–6, 21, 22). We obtained similar values in the present study.

In addition to the obvious technical difficulties associated with platelet aggregation measurements (e.g., sensitivity to sample handling and processing and difficulties

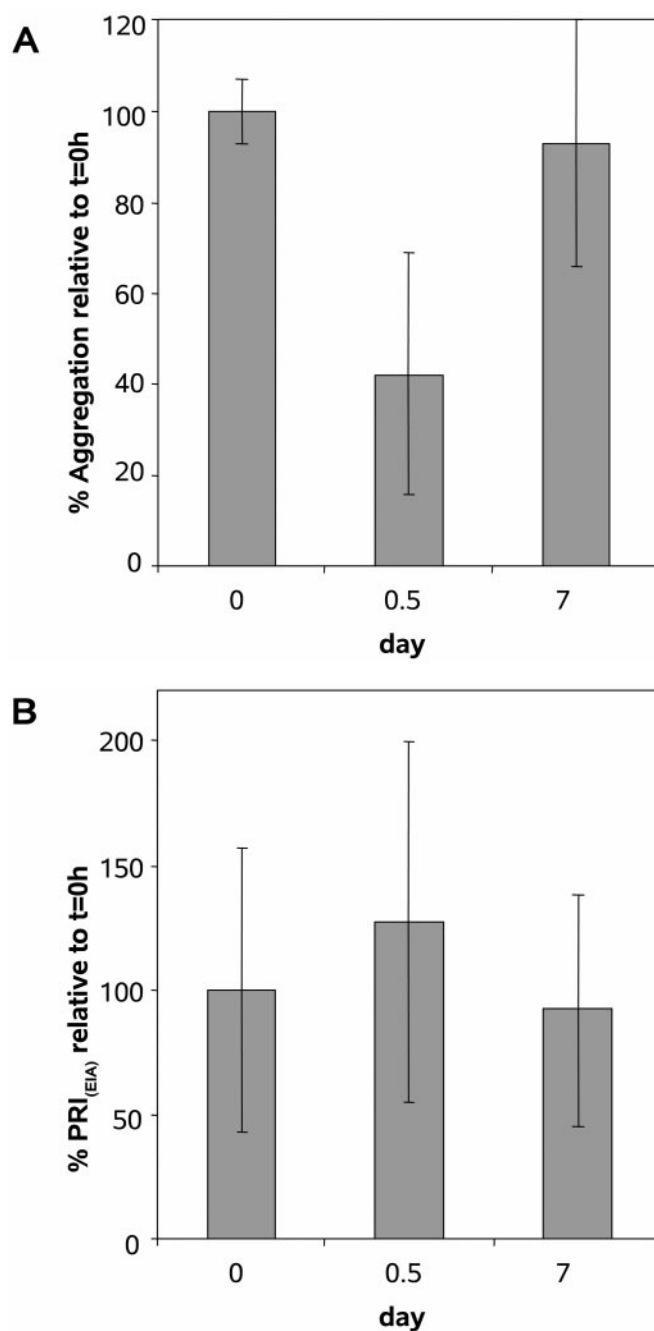


Fig. 8. ASA (100-mg single dose) inhibits ADP-induced ($5 \mu\text{mol/L}$) platelet aggregation (A) but has no effect on $PRI_{(EIA)}$ in platelets (B) from healthy volunteers.

Aggregation and PRI were measured at baseline ($t = 0$ h) and 12 h and 7 days after administration of ASA or clopidogrel, respectively ($n = 9$). Error bars, SD.

in standardization), aggregation may be induced or amplified by stimuli secreted from platelets and be partially inhibited by a loss of platelet responsiveness resulting from desensitization (10). These effects may impact platelet aggregation in an unpredictable manner and can result from both inappropriate conditions during platelet preparation and interference from other drugs. In particular,

all patients included in the studies reported on clopidogrel resistance also received ASA treatment. The fact that ADP- and collagen-induced platelet aggregation is affected to some extent by ASA suggests that ASA may exert its antiplatelet effects partially by mechanisms not directly related to cyclooxygenase inhibition (21). These effects may mask clopidogrel resistance. Consequently, platelet aggregometry is usually carried out before and after the first clopidogrel ingestion, if possible. The use of this approach to distinguish weak responders from patients who respond to clopidogrel treatment requires highly reproducible aggregometry.

PLATELET FUNCTION ANALYZER

In this study, we observed no significant effect of clopidogrel on cartridge closure time, an indicator of blood clotting, when we used epinephrine/collagen- and ADP/collagen-coated cartridges in a platelet function analyzer. This observation is in agreement with other studies showing limited suitability of the PFA-100 system for monitoring the efficacy of ADP receptor antagonists (23, 24). However, a long-term effect of clopidogrel on the closure time determined by PFA-100 after 5 days of treatment was reported recently (25). This effect, however, showed remarkable variability.

P-SELECTIN

Apart from aggregation, flow cytometry measurement of ADP-induced surface expression of P-selectin is frequently used for monitoring the effects of clopidogrel. The ADP concentrations used in these experiments, however, are usually much higher than what is needed for stimulation of aggregation (100 $\mu\text{mol/L}$ vs 5 $\mu\text{mol/L}$). Such high concentrations are typically required to obtain significant surface expression of P-selectin (4, 21). Therefore, platelet responses achieved by such nonphysiologically high concentrations of stimulant must be regarded, at least partially, as unspecific. In the present study, we stimulated platelets with moderate ADP concentrations (5 $\mu\text{mol/L}$), thus excluding secondary effects not directly associated with purinergic stimulation. In these experiments, no significant difference between the placebo and clopidogrel groups was evident. Hence, measurement of P-selectin may be inappropriate for the assessment of platelet resistance to thienopyridines in routine or bedside diagnostics.

VASP PHOSPHORYLATION AS MARKER OF CLOPIDOGREL ACTION

cGMP- and cAMP-dependent signaling pathways are the major inhibitors of platelet activation *in vivo*. Their signaling culminates in phosphorylation of substrates by cGMP-dependent protein kinase and PKA, respectively. The protein VASP, which is abundant in human platelets, is a common substrate of these kinases and is now an established and reliable marker of their activation (13–17). In most studies, VASP phosphorylation is determined by

Western blotting, which is neither quantitative nor suitable for the analysis of whole blood. In contrast, the FACS assay is superior for easy, precise measurement of VASP phosphorylation in platelets in whole blood (13). This assay has been useful for clinical studies as well. Further refinement of the P-VASP FACS assay has allowed monitoring of individual responses to clopidogrel in exploratory studies involving patients undergoing stent implantation (14–16).

In this study, we adopted the P-VASP assay for use in a chromogenic EIA. This assay simultaneously determines phosphorylated VASP and total VASP, facilitating data normalization by enabling correction for sample variations in cell counts and protein content. Our data indicate that P-VASP values obtained by FACS and EIA analysis correlate (Pearson correlation, $r = 0.866$ and $r^2 = 0.750$).

In our healthy volunteers, clopidogrel administration led to a 52% reduction in ADP-induced inhibition of adenylate cyclase in platelets, as measured by a decrease in VASP phosphorylation, and the clopidogrel and placebo groups were distinguished clearly. The decrease in P-VASP was correlated with ADP-stimulated platelet aggregation. Because we used 2 completely different techniques to determine the effects of clopidogrel, the correlation appears satisfactory. ASA did not interfere with VASP phosphorylation, whereas ADP-induced aggregation was markedly inhibited.

ADVANTAGES OF THE P-VASP ASSAY

The P-VASP assay is specific for inhibition of the P2Y₁₂ receptor and is, in contrast to aggregometry, not affected by ASA. A single FACS or EIA test is sufficient to determine platelet inhibition, obviating testing before treatment commences. Sample preparation is markedly simplified because the FACS and EIA tests are carried out with whole blood. Fresh blood samples can be measured hours after venipuncture without a significant affect on the results, and lysed samples can be stored for months.

LIMITATIONS OF THE P-VASP ASSAY

In the present study, we did not examine the effects of comedication and/or the presence of preactivated platelets, which present a more complex clinical situation. The clinical usefulness of the P-VASP assays is currently under investigation. Drugs affecting the NO/cGMP or cAMP pathways, and thus VASP phosphorylation, may complicate P-VASP analysis of thienopyridine effects. Despite this, we consider the P-VASP assay more specific, quantitative, and reliable than previous assays (e.g., aggregometry or P-selectin expression) available for analyzing platelet responses to thienopyridines (10).

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