Contribution of Hepatic Cytochrome P450 3A4 Metabolic Activity to the Phenomenon of Clopidogrel Resistance

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Background—Interindividual variability of platelet inhibition after aspirin or clopidogrel administration has been described. Additionally, aspirin resistance and clopidogrel resistance occur in some individuals. Because the prodrug clopidogrel is activated by hepatic cytochrome P450 (CYP) 3A4, we hypothesized that interindividual variability in clopidogrel efficacy might be related to interindividual differences in CYP3A4 metabolic activity.

Methods and Results—Platelet aggregation was measured before and after clopidogrel treatment in 32 patients undergoing coronary artery stent implantation and in 35 healthy volunteers. The erythromycin breath test was used to measure CYP3A4 activity in vivo in 25 of the healthy volunteers. Individual platelet aggregation was studied in 10 healthy volunteers after the coadministration of clopidogrel and rifampin (a CYP3A4 inducer). Clopidogrel nonresponders, low responders, and responders were defined by a relative inhibition of adenosine diphosphate (20 μmol/L)–induced platelet aggregation of $<$10%, 10% to 29%, and $\geq$30%, respectively. Among patients, 22% were clopidogrel nonresponders, 32% were low responders, and 47% were responders. Among volunteers, 16% were nonresponders, 12% were low responders, and 72% were responders. Percent platelet aggregation after clopidogrel inversely correlated with CYP3A4 activity ($r=-0.6$, $P=0.003$). Improved platelet inhibition in volunteers resistant to clopidogrel was observed with the coadministration of clopidogrel and rifampin.

Conclusions—Clopidogrel administration results in interindividual variability in platelet inhibition, which correlates with CYP3A4 metabolic activity. Measurement of antiplatelet drug efficacy with a point-of-care device and alternative antithrombotic strategies for aspirin or clopidogrel nonresponders and low responders could reduce the incidence of thrombotic events that continue to occur despite oral antiplatelet therapy. **(*Circulation*. 2004;109:166-171.)**

Key Words: drugs ■ platelets ■ pharmacology

Clopidogrel, a thienopyridine derivative similar to ticlopidine, is an inhibitor of platelet aggregation induced by adenosine diphosphate (ADP).[1] Clopidogrel was approved by the United States Food and Drug Administration (FDA) in 1997 for the reduction of myocardial infarction, stroke, and vascular death in patients with recent stroke, recent myocardial infarction, or established peripheral arterial disease after the Clopidogrel versus Aspirin in Patients at Risk of Ischemic Events (CAPRIE) trial[2] showed superior reduction of these events with clopidogrel compared with aspirin (annual risk, 5.3% versus 5.8%; $P=0.04$). Dual antiplatelet therapy (aspirin plus clopidogrel) for acute coronary syndromes was approved by the FDA in 2002 on the basis of the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) trial[3] results, which showed a significant reduction in the 9-month composite end point of cardiovascular death, nonfatal myocardial infarction, or stroke versus aspirin monotherapy (9.3% versus 11.4%, $P<0.001$). Additionally, the combination of aspirin and clopidogrel is the standard antiplatelet therapy for coronary stenting,[4–7] although it has not gained formal FDA approval status.

The elucidation of the pharmacological properties of clopidogrel has lagged behind the randomized clinical trial reports. A 75-mg once-daily clopidogrel dose was used in CAPRIE because it produced inhibition of ADP-induced platelet aggregation equivalent to ticlopidine 250 mg twice daily.[2] Only later were dosing studies published,[8,9] and this work continues.[10–11] Subsequently, the active metabolite of clopidogrel, a prodrug, was identified,[12] and its noncompetitive inhibition of the platelet P2 Y_{12} ADP receptor was

described.[13] We recently demonstrated that clopidogrel is activated in humans by the hepatic cytochrome P450 (CYP) 3A4 enzyme system and that its platelet inhibition efficacy could be perturbed by CYP3A4 inhibitors, inducers, and substrates.[14,15]

Interindividual variation in platelet inhibition by clopidogrel has long been recognized but only recently evaluated in peer-reviewed manuscripts.[16–18] Clopidogrel resistance, a concept related to aspirin resistance,[19–22] has also been described.[18,23,24] This phenomenon includes both clopidogrel nonresponders and low responders.[24] Possible mechanistic explanations include increased platelet reactivity before clopidogrel dosing,[17,18] drug–drug interactions inhibiting clopidogrel activation by CYP3A4,[14,15] platelet P2 Y_{12} receptor genetic polymorphisms,[25] or defects in signaling pathways downstream from the receptor. In the present study, we demonstrate that low baseline CYP3A4 activity, which decreases clopidogrel activation, is one mechanism for clopidogrel resistance, at least during the first days of treatment.

Methods

Study Protocols

The institutional review boards approved the protocols, and written informed consent was obtained from each subject before enrollment. In the first study, 32 consecutive patients undergoing elective coronary artery stent implantation received an oral loading dose of 300 mg of clopidogrel followed by 75 mg daily.[18] Exclusion criteria included history of bleeding diathesis, acute myocardial infarction within 48 hours, cerebrovascular event within 3 months, illicit drug or alcohol abuse, prothrombin time >1.5 times control, platelet count <100 000/mm^3, hematocrit <25%, creatinine >4.0 mg/dL, and thienopyridine or glycoprotein IIb/IIIa use before the procedure. All patients received at least 81 mg aspirin for 7 days before the procedure and were administered 325 mg on the day of the procedure and daily thereafter. Heparin was administered in the catheterization laboratory to all patients to achieve an activated clotting time >300 seconds. Glycoprotein IIb/IIIa inhibitors were withheld. Blood was collected in Vacutainer tubes (Becton Dickinson) containing 3.8% trisodium citrate. Samples were obtained at baseline before clopidogrel or heparin therapy and at 5 days after stent implantation. Platelet aggregation was measured by light transmission aggregometry.

In the second study, the metabolic activity of CYP3A4 was compared with platelet aggregation after a 450-mg loading dose of clopidogrel in 25 healthy volunteers. Exclusion criteria included history of platelet count <100 000/mm^3, bleeding disorder, or ingestion of any medications, alcohol, caffeine, tobacco, herbal remedies such as St John's wort, grapefruit juice, birth control pills, or charcoal-broiled food. An erythromycin breath test was performed before and 2 hours after ingesting clopidogrel to measure CYP3A4 activity.[26] Platelet aggregation was measured before and 4 hours after clopidogrel ingestion using a point-of-care aggregometer.

The third study analyzed individual platelet aggregation inhibition in 10 healthy volunteers who received a maintenance dose of clopidogrel 75 mg/d for 6 days, followed by a washout period of 14 days, followed by 4 days of rifampin 300 mg twice a day, followed by 6 days of both clopidogrel and rifampin.[14] Exclusion criteria were the same as in the second study. Platelet aggregation was determined with the point-of-care aggregometer on days 0, 6, 20, 24, and 30.

Platelet Aggregation

Platelet aggregation was measured in the first study using platelet-rich plasma stimulated with 20 μmol/L ADP and assessed with a Chronolog Lumi-Aggregometer (model 560-Ca, Chronolog) with the AggroLink software package.[27] After inverting the Vacutainer tube 3 to 5 times for gentle mixing, the blood-citrate mixture was centrifuged at 1200*g* for 2.5 minutes. The resulting platelet-rich plasma was kept at room temperature for use within 1 hour. The platelet count was determined in the platelet-rich plasma and adjusted to 3.5×10^8/mL with homologous platelet-poor plasma. Aggregation was expressed as the maximal percent change in light transmittance from baseline, using platelet-poor plasma as a reference. Inhibition of platelet aggregation was expressed as the absolute reduction in aggregation achieved by 20 μmol/L ADP after clopidogrel administration compared with baseline aggregation values before clopidogrel. Nonresponders were defined by a platelet inhibition of <10%, low responders were characterized by inhibition of platelet aggregation by 10% to 29%, and normal responders were ≥30% responsive.[24]

Platelet aggregation for the second and third studies was measured with the point-of-care MICROS cell counter (ABX Diagnostics) and the Plateletworks test platform (Helena Laboratories). The cell counter uses traditional electronic impedance cell counting principles.[28,29] In brief, a reference platelet count is performed on 1 mL of fresh whole blood in a Plateletworks tube containing K_3-EDTA as the anticoagulant. The sample is then passed through the cell counter and the platelet count is determined. This process is repeated with a second 1-mL sample of fresh whole blood in a Plateletworks tube containing both citrate and 20 μmol/L ADP. In the presence of ADP, platelets associate and aggregate. Because the aggregated platelets exceed the threshold limitations for platelet size, they are no longer counted as individual platelets. The ratio of the platelet count between the agonist and reference tubes is calculated as percent platelet aggregation. The results are available within 4 minutes. A previous study comparing this device to light transmission aggregometry using platelet-rich plasma demonstrated a correlation coefficient of 0.83 in 225 paired samples.[29]

Erythromycin Breath Test

The erythromycin breath test (Metabolic Solutions, Inc) was used to measure hepatic CYP3A4 activity in vivo in the second study.[26] A preinjection breath sample was obtained. An intravenous dose of [^{14}C-*N*-methyl]-erythromycin (3 μCi, 0.01 mmol of erythromycin) was then administered. Subsequently, a single breath sample was collected after 20 minutes. Quantitation of exhaled $^{14}CO_2$ provides a selective measure of the instantaneous hepatic CYP3A4 activity.[30]

Statistics

In patients undergoing stent implantation, paired 2-sample *t* tests were used to compare platelet aggregation between 0 and 5 days. Linear regression plot comparing the baseline CYP3A4 metabolic activity and postclopidogrel CYP3A4 activity with percent platelet aggregation before and after clopidogrel was performed using SPSS statistical analysis. In healthy volunteers taking rifampin, paired 2-sample *t* tests with Bonferroni's correction were used to compare platelet aggregation between 0, 6, 20, 24, and 30 days within each group. Nonparametric data that did not conform to a normal distribution were analyzed using Mann-Whitney *U* tests for unpaired data and Wilcoxon tests for paired data. All values were expressed as mean±SD. $P<0.05$ was considered statistically significant.

Results

Variable Clopidogrel Response

Interindividual variability in platelet inhibition was demonstrated in both patients and healthy volunteers. In patients, 7 of 32 (22%) were clopidogrel nonresponders, 10 of 32 (32%) were low responders, and 15 of 32 (47%) were responders (Figure 1). In healthy volunteers, 4 of 25 (16%) were clopidogrel nonresponders, 3 of 25 (12%) were low responders, and 18 of 25 (72%) were responders (Figure 2).

Clopidogrel Response Versus CYP3A4 Activity

Baseline CYP3A4 activity was randomly distributed, as measured by the erythromycin breath test, and was not related

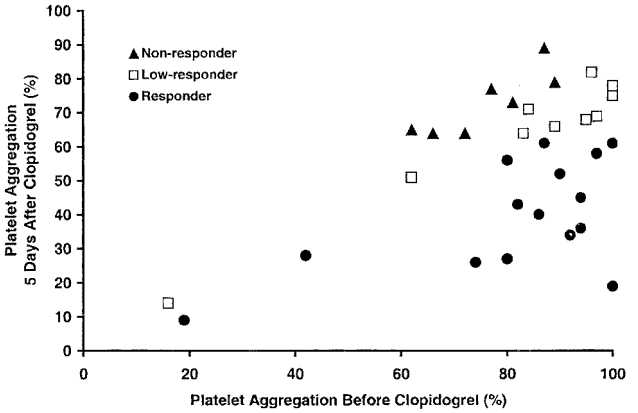

Figure 1. Percent platelet aggregation induced by 20 μmol/L ADP at baseline and 5 days after initiating clopidogrel therapy in 32 patients undergoing coronary stent implantation.

to baseline percent platelet aggregation in 25 healthy volunteers (Figure 3A). After clopidogrel (Figure 3B), there was a significant inverse correlation between platelet aggregation and CYP3A4 activity ($r=-0.6$; $P=0.003$).

CYP3A4 Induction

Clopidogrel produced interindividual variability in platelet aggregation inhibition in 10 healthy volunteers (Figure 4). Rifampin did not change platelet aggregation, but platelet aggregation inhibition after clopidogrel was enhanced by rifampin (56±20% versus 33±18%; $P=0.001$), a CYP3A4 inducer. After rifampin, the 3 initial nonresponders and 1 low responder demonstrated enhanced platelet inhibition that then met the definition for a clopidogrel responder (Figure 4).

Discussion

The variable platelet inhibition response to clopidogrel has been recognized by all who have tested clopidogrel efficacy by platelet aggregometry, and clopidogrel resistance has recently been described.[18,23,24] In a previous report, the incidence of clopidogrel nonresponders was ≈10% and the

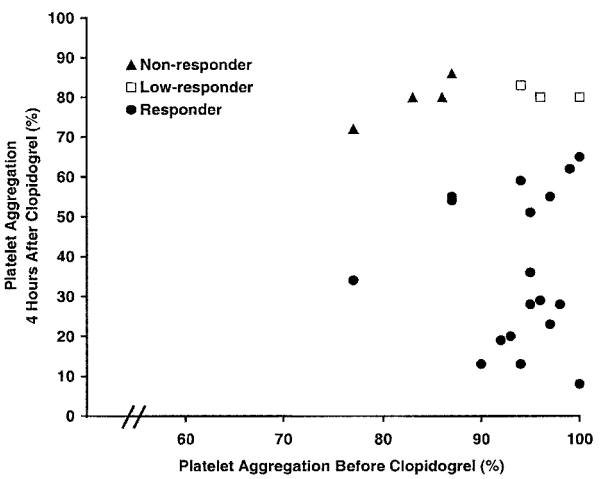

Figure 2. Percent platelet aggregation induced by 20 μmol/L ADP at baseline and 4 hours after initiating clopidogrel therapy in 25 healthy volunteers.

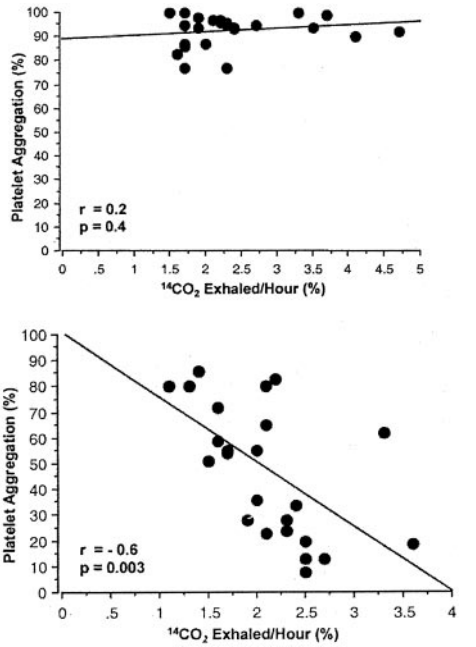

Figure 3. Percent platelet aggregation compared with CYP 3A4 activity, as measured by the erythromycin breath test before (A) and after (B) clopidogrel loading.

incidence of low responders was ≈20%.[24] These frequencies are similar to those reported for aspirin resistance using platelet aggregometry.[19] Others have demonstrated higher frequencies for clopidogrel resistance, reporting an absolute, rather than a relative, change in platelet aggregation.[18] This is an important differentiation, because it may be argued that aspirin might influence the prevalence of clopidogrel nonresponders. However, because the response to clopidogrel in this study was reported as a relative change in platelet aggregation, any change can be attributed to clopidogrel administration alone. We previously demonstrated that clopidogrel, a prodrug, was activated in humans by CYP3A4.[14,15] In the present study, we offer additional evidence in support of that observation and describe 1 mechanism to explain clopidogrel resistance: individual variability in the metabolic activity of CYP3A4.

In our earlier study,[14] we showed that the pharmacological manipulation of CYP3A4 metabolic activity affected the ability of clopidogrel to inhibit platelet aggregation, as measured by platelet aggregometry. Erythromycin and troleandomycin, CYP3A4 inhibitors, decreased clopidogrel efficacy, as did atorvastatin lactone, a competitive CYP3A4 substrate. In contrast, rifampin, a CYP3A4 inducer, increased clopidogrel efficacy.

There exists substantial interindividual variation in CYP3A4 expression that cannot be accounted for by known inducers and inhibitors.[31] A genetic basis for this variation has not been identified, but it is possible to use the erythromycin breath test to determine an individual's CYP3A4 activity. In our second study performed in healthy volunteers, the baseline distribution of individual CYP3A4 metabolic activity was randomly distributed (Figure 3A). CYP3A4 activity levels correlated with platelet aggregation values

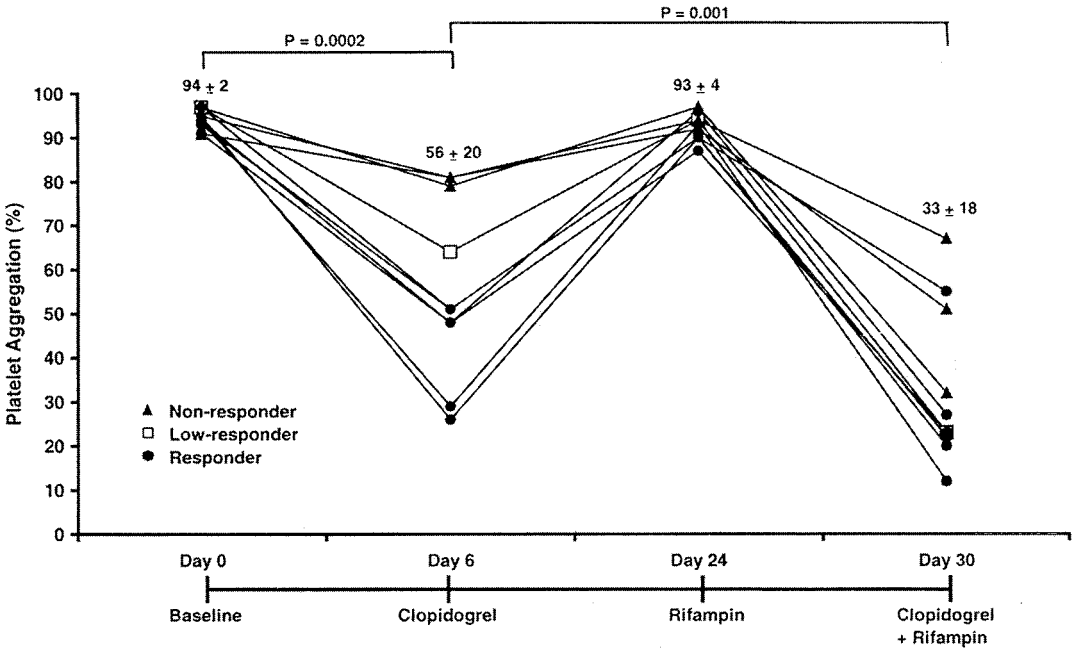

Figure 4. Percent platelet aggregation after clopidogrel, rifampin, and clopidogrel plus rifampin.

after clopidogrel, and the correlation was inverse, as expected (ie, the lower the CYP3A4 activity, the less clopidogrel was activated [Figure 3B]). Variation in hepatic CYP3A4 activity accounted for approximately one third ($R=0.36$) of the interindividual variability in response. In a patient population receiving potential inducers and inhibitors of CYP3A4 and who may have varying degrees of liver dysfunction because of disease, variation in CYP3A4 activity would be significantly greater than in our healthy volunteers. A logical hypothesis is that variation in CYP3A4 activity would account for significantly more than one third of the variability in clopidogrel response in patients.

Our data also show that the role of CYP3A4 in clopidogrel activation can be exploited to convert nonresponders. In Figure 4, it can be seen that clopidogrel efficacy in 3 nonresponders and 1 low responder was improved by coadministration of rifampin. This suggests that agents that induce the expression of CYP3A4 metabolic activity can decrease the incidence of clopidogrel resistance.

The different definitions of antiplatelet drug resistance are empiric. Muller et al[24] defined clopidogrel nonresponse as a relative inhibition of ADP-induced platelet aggregation of <10%. Low responders were identified by an inhibition of 10% to 29%. Whereas platelet receptor P2 Y_{12} genetic polymorphisms[25] or defects in signaling pathways downstream from the receptor would represent durable mechanisms for clopidogrel resistance, the observation that some patients with initial clopidogrel resistance become more responsive to clopidogrel over time[23] could be explained either by subsequent induction of CYP3A4 expression and increased metabolism of the prodrug or decreasing platelet reactivity after stenting. Increased loading (>300 mg) or maintenance (>75 mg) doses would be expected to decrease clopidogrel resistance because of baseline platelet activation.[17,18] The Intracoronary Stenting and Antithrombotic Regimen–Rapid Early Action for Coronary Treatment (ISAR-REACT) study[7] demonstrated no difference between glycoprotein IIb/IIIa receptor inhibitors and placebo in low-risk patients undergoing coronary stent implantation treated with a 600-mg loading dose of clopidogrel.

Aspirin resistance has been defined either as the failure to prevent individuals from clinical thrombotic complications or as the failure to produce an expected response on a laboratory measurement of platelet activation or aggregation.[22] Unfortunately, the incidence varies from 5% to 50%, depending on the definition used, the test used, and methodological differences among laboratories. Nevertheless, the phenomenon appears real, however defined, and 4 reports suggest that patients with aspirin resistance are at increased risk for thrombotic complications.[20,21,32,33] Additionally, the concomitant administration of ibuprofen seems to antagonize the irreversible platelet inhibition induced by aspirin[34] and may also be associated with increased thrombotic complications.[35,36]

Clopidogrel offers added antiplatelet efficacy to aspirin in patients with acute coronary syndromes[3] or after stent implantation.[6] It is possible, however, that the clinical benefit of clopidogrel is more complementary than additive. In the CAPRIE study,[2] clopidogrel reduced the annual absolute risk of vascular death, myocardial infarction, and stroke by 0.5% (5.3% versus 5.8%) compared with aspirin. In the CURE trial,[3] although the relative risk reduction for 30-day death or myocardial infarction with aspirin/clopidogrel versus aspirin alone was 21%, the absolute reduction was <1% (3.9% versus 4.8%), an effect that easily could be accounted for by clopidogrel efficacy in patients who were aspirin resistant or taking concomitant ibuprofen with aspirin. If aspirin and clopidogrel were proven to be complementary rather than additive agents, the potential of limiting clopidogrel use to patients who do not respond to aspirin would offer major

cost-effectiveness advantages[37] that would justify the cost of measuring platelet function in patients receiving antiplatelet therapy.

Subacute stent thrombosis continues to occur in 1% to 3% of patients despite dual antiplatelet therapy.[38,39] Future investigations need to determine whether these patients are aspirin resistant, clopidogrel resistant, or both. Additionally, the possibility that drug–drug interactions between aspirin and ibuprofen[34] or clopidogrel and atorvastatin[14] contribute to these events needs to be evaluated. In view of the recent concern that the Cypher sirolimus-eluting coronary stent (Cordis Corporation) may be associated with an increased risk of subacute stent thrombosis, it should also be noted that sirolimus promotes platelet aggregation.[40] Therefore, the efficacy of clopidogrel therapy in patients receiving these stents needs to be defined.

Our study has several potential limitations. First, it could be argued that measuring platelet aggregation is instrument dependent and laboratory dependent. However, the same interindividual variability was seen in this study with light transmission aggregometry in platelet-rich plasma as with point-of-care aggregometry in whole blood, and other investigators using the same instruments have produced similar results.[17,18,24] Second, one measure of platelet function may not be sufficient to diagnose clopidogrel resistance. Nevertheless, resistance was defined by only 1 measurement in each of the studies correlating aspirin resistance with increased thrombotic complications.[20,21,32,33] Third, the clinical importance of low responders can be disputed. However, the AU–Assessing Ultegra (GOLD) study[41] suggested that suboptimal platelet function inhibition with a glycoprotein IIb/IIIa antagonist, as measured by a point-of-care assay, was associated with increased thrombotic complications after percutaneous coronary intervention.

In conclusion, interindividual variations in platelet inhibition by both clopidogrel and aspirin exist, and some patients are resistant to these antiplatelet agents because of biological variability or drug–drug interactions. With clopidogrel, interindividual variation in platelet inhibition in part reflects variation in CYP3A4 activity, and we have shown that this is true even in healthy volunteers not treated with known CYP3A4 inducers or inhibitors. Consistent definitions for aspirin resistance and clopidogrel resistance are needed that can be documented by reliable laboratory testing and associated with increased risk for thrombotic complications. In the future, measurement of antiplatelet drug efficacy with a point-of-care device and alternative antithrombotic strategies for nonresponders or low responders could reduce the incidence of thrombotic events that continue to occur despite oral antiplatelet therapy.

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