Determination of Clopidogrel Resistance by Whole Blood Platelet Aggregometry and Inhibitors of the P2Y₁₂ Receptor

Boris T. Ivandic,* Philipp Schlick, Peter Staritz, Kerstin Kurz, Hugo A. Katus, and Evangelos Giannitsis

Background: Inhibition of platelet aggregation by clopidogrel may be insufficient in up to 30% of users. These nonresponders carry an increased risk of cardiovascular events. We reported here a simple assay to study clopidogrel responsiveness.

Methods: Electrical impedance aggregometry was performed in diluted whole blood in the presence of 5 and 20 µmol/L ADP. Some samples were incubated with 0.1 mmol/L methyl-S-adenosine monophosphate (MeSAMP), a P2Y₁₂ receptor blocker, to maximize inhibition of aggregation before aggregometry. To validate the assay, we analyzed 6-min impedance in 21 healthy probands and 244 patients with coronary artery disease (CAD).

Results: At 5 µmol/L ADP, the imprecision of the assay was 11%. Mean (SD) impedance of the healthy cohort was 12.2 (2.2) Ω. The mean − 3 SD was used to define the cutoff for clopidogrel responsiveness: responders and nonresponders exhibited a 6-min impedance ≤5 Ω and >5 Ω, respectively. Samples from nonresponders were incubated with MeSAMP and analyzed again to distinguish pharmacokinetic and pharmacodynamic types of resistance. Sixteen percent of CAD patients were classified as nonresponders (38 and 2 cases of pharmacokinetic and pharmacodynamic resistance, respectively). Female sex was strongly associated with clopidogrel resistance (P = 0.0002, Fisher exact test). A higher clopidogrel loading dose (P = 0.0353, Mann-Whitney U-test) was given to responders (median, 450 mg) than nonresponders (median, 300 mg). Age and cardiovascular diagnosis showed no significant associations.

Conclusions: Impedance aggregometry using 5 µmol/L ADP is a useful tool for studying clopidogrel responsiveness. MeSAMP allows characterization of responsiveness “on treatment” and may be useful for optimizing clopidogrel dosing.

© 2006 American Association for Clinical Chemistry

Platelet activation and aggregation play a pivotal role in cardiovascular disease, triggering adverse events such as acute coronary syndrome and stroke. Inhibition of platelet aggregation is therefore a primary therapeutic goal. Several large trials demonstrated improved risk reduction by dual antiplatelet therapy with aspirin and clopidogrel, a thienopyridine that causes irreversible inhibition of the platelet ADP receptor P2Y₁₂ (1–3). However, evidence is accumulating that platelet inhibition by clopidogrel may not be comparable in all clopidogrel users (4–6). This phenomenon, referred to as clopidogrel resistance, is most often assessed by optical aggregometry before and after the initiation of clopidogrel therapy. The reported prevalence of clopidogrel resistance 24 h after administration of a loading dose ranged between 4% and 30% (7, 8). However, the numbers of patients identified as nonresponders were much higher than what is expected and clinically observed, e.g., as stent thrombosis after percutaneous coronary intervention (PCI).1 Different definitions of clopidogrel resistance and a lack of standardized methods may explain the still inconsistent image of this phenomenon (9).

To facilitate studies of clopidogrel resistance, we exam-
ined electrical impedance aggregometry with the aim to develop a simple assay suitable for small laboratories or for point-of-care use. We report the imprecision and the reference interval of this assay and propose a classification of clopidogrel responsiveness. The assay includes the use of methyl-5-adenosine monophosphate (MeSAMP), a specific in vitro inhibitor of the P2Y12 receptor, to measure the achievable maximum inhibition of platelet aggregation in a sample. This assay may be useful as a tool in deciding whether a nonresponder should increase the dose of clopidogrel (pharmacokinetic type of resistance) or change to a different antiplatelet drug (pharmacodynamic type of resistance).

**Materials and Methods**

The study was approved by the Institutional Review Board in accordance with the Declaration of Helsinki. After participants gave informed consent blood, samples were obtained from 21 healthy volunteers (10 men and 11 women; age range, 17–61 years) and 244 patients with coronary artery disease (CAD). CAD patients were treated with aspirin (100 mg once daily) and clopidogrel (75 mg once daily) and were examined 12–24 h after PCI. Use of a glycoprotein IIb-IIIa inhibitor was an exclusion criterion. Healthy probands were included if they denied taking any antiplatelet medication within 10 days before sampling. Whole blood was drawn via a butterfly cannula (21-gauge) into plastic syringes (9 mL) containing 0.1 volume of 38 g/L trisodium citrate. All samples were analyzed 0.5–3 h after sampling.

Electrical aggregometry measures the increase of impedance between a pair of metal electrodes immersed in diluted whole blood (10). The increase in impedance correlates with the amount of platelet aggregates depositing on the electrodes after addition of a platelet agonist. Aggregation was analyzed by use of a CA560-CA lumi-aggregometer from Chrono-Log. Aggregometry was performed at 37 °C with a constant stir bar speed of 1000 rpm. Briefly, a 0.5-mL aliquot of citrate-anticoagulated whole blood was diluted with 1 volume of prewarmed (37 °C) NaCl (9 g/L) in a polycarbonate cuvette. The electrodes were then immersed in the diluted blood sample and incubated at 37 °C for at least 2 min. After brief electrical calibration, aggregation was started by the addition of ADP to obtain a final concentration of 5 or 20 μmol/L. The increase of impedance (Ω) was recorded for ~7 min. Only 6-min impedance values were used for the analyses. ADP (1 mmol/L; Chrono-Par® reagent no. 070212), cuvettes, and siliconized stir bars were purchased from Probe&Go GmbH. To maximize inhibition of P2Y12 receptor–mediated platelet aggregation, aliquots of citrated whole blood were also incubated with 0.1 mmol/L MeSAMP (Sigma-Aldrich; prod. no. M-1434; Mr 393.31) at room temperature for 20–30 min until analysis of aggregation.

**Results**

In native blood of a healthy, clopidogrel-naive proband, impedance increased after a short lag time in response to 5 μmol/L ADP (Fig. 1). In contrast, aggregation was suppressed (impedance of 0 Ω at 6 min) 24 h after a single 300-mg dose of clopidogrel. When we examined 21 clopidogrel-naive probands, we obtained a mean (SD) of 12.2 (2.2) Ω for 6-min impedance in the presence of 5 μmol/L ADP [13.1 (2.4) Ω for 20 μmol/L ADP]. Significant sex-related differences in 6-min impedance were not found. Samples from 9 probands were measured 4–5 times during 3–4 h to determine the imprecision (CV) of electrical aggregometry. The mean CVs were 11% and 8.7% for 5 and 20 μmol/L ADP, respectively. To define clopidogrel responsiveness in clopidogrel users, we calculated a cutoff by subtracting 3 SD from the mean 6-min impedance measured in samples from clopidogrel-naive probands in response to 5 μmol/L ADP [i.e., 12.2 − (2.2 × 3) = 5.6 Ω]. Thus, a 6-min impedance ≤ 5 Ω was considered as an adequate response to clopidogrel consistent with marked inhibition of aggregation. Conversely, a clopidogrel user with a 6-min impedance > 5 Ω was considered a nonresponder. In nonresponders, another aliquot of whole blood was first incubated with MeSAMP to maximize inhibition of ADP-mediated platelet aggregation and then analyzed again. If the result of this second analysis was ≤ 5 Ω, then a pharmacokinetic rather than a pharmacodynamic type of resistance was assumed.

Below we describe the application of this classification
for 2 clopidogrel users presenting with stent thrombosis who were not part of the CAD cohort.

The first patient, who represents an increased rate of platelet synthesis as the potential cause of clopidogrel resistance, was classified as nonresponder in the initial analysis of whole blood aggregation because his blood sample exhibited a 6-min impedance of 8 Ω. Because impedance was reduced to 0 Ω in the second aggregation analysis after incubation with MeSAMP, he was considered a case of pharmacokinetic clopidogrel resistance (Fig. 2A). This 65-year-old man was readmitted to the hospital with acute myocardial infarction caused by stent thrombosis 5 days after PCI and stent placement in the left anterior descending coronary artery. A platelet count of $747 \times 10^9/L$ suggested that a maintenance dose of 75 mg of clopidogrel once daily might not be sufficient for this patient with potentially increased platelet synthesis. The patient remained stable after an additional 600-mg loading dose and continued administration of 75 mg of clopidogrel twice daily.

The second patient was treated with clopidogrel, but ADP-induced aggregation was not inhibited in the initial analysis (19 Ω). Incubation with MeSAMP also did not achieve adequate platelet inhibition (Fig. 2B). The 6-min impedance of the second analysis was still 10 Ω. This patient was therefore classified as a nonresponder with pharmacodynamic clopidogrel resistance. This 70-year-old man had CAD and type 2 diabetes mellitus. Three days after stent placement in the left anterior descending coronary artery, stent thrombosis led to severe myocardial infarction requiring cardiopulmonary resuscitation.

Using the classification described above, we examined 244 CAD patients who had undergone PCI 12–24 h previously (Table 1). An acute coronary syndrome was the reason to perform PCI in 28% of the patients analyzed. More than 90% of the patients received clopidogrel loading doses of 150–600 mg immediately after PCI. Eighty-four percent of the patients (n = 241) exhibited marked inhibition of whole blood aggregation in the presence of 5 μmol/L ADP, whereas 16% (n = 40) were classified as nonresponders. Only 2 of these were cases of pharmacodynamic resistance; both were women with arterial hypertension and stable CAD. After incubation of blood samples with MeSAMP, aggregation was markedly inhibited (median, 0 Ω) in the 38 remaining cases of pharmacokinetic resistance. Identical results were obtained with 20 μmol/L ADP as platelet agonist; the 5 and 20 μmol/L cutoffs were comparable (5.4 and 5.9 Ω, respectively). Consequently, the classification of clopidogrel responsiveness did not change when 20 μmol/L ADP was used instead of 5 μmol/L. This excluded a potential overestimation of nonresponders if the lower concentration was used. Analysis of subgroups failed to show significant associations between responsiveness and age or cardiovascular diagnosis. A higher clopidogrel loading dose was administered ($P = 0.0353$, Mann–Whitney) to responders (median, 450 mg) than nonresponders (median, 300 mg). Notably, we found a highly significant association between responsiveness and sex ($P = 0.0002$, Fisher exact test). Female sex appeared to predispose to resistance: approximately one-third of the female patients were nonresponders.
**Table 1. Characteristics of 244 CAD patients.**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Responders ((n = 204))</th>
<th>Nonresponders ((n = 40))</th>
<th>(p^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) age, years</td>
<td>65.9 (10.5)</td>
<td>69.5 (10.0)</td>
<td>NS^b</td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>159 (78)</td>
<td>19 (48)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Women</td>
<td>45 (22)</td>
<td>21 (53)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Diagnosis, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stable angina</td>
<td>148 (73)</td>
<td>28 (70)</td>
<td>NS</td>
</tr>
<tr>
<td>Unstable angina</td>
<td>8 (4)</td>
<td>3 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>NSTEMI</td>
<td>28 (14)</td>
<td>6 (15)</td>
<td>NS</td>
</tr>
<tr>
<td>STEMI</td>
<td>20 (10)</td>
<td>3 (8)</td>
<td>NS</td>
</tr>
<tr>
<td>Clopidogrel loading dose, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mg^c</td>
<td>17 (8)</td>
<td>7 (18)</td>
<td>NS</td>
</tr>
<tr>
<td>150 mg</td>
<td>2 (1)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>225 mg</td>
<td>1 (0.5)</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>300 mg</td>
<td>73 (36)</td>
<td>19 (48)</td>
<td>NS</td>
</tr>
<tr>
<td>450 mg</td>
<td>20 (10)</td>
<td>2 (5)</td>
<td>NS</td>
</tr>
<tr>
<td>600 mg</td>
<td>91 (45)</td>
<td>12 (30)</td>
<td>NS</td>
</tr>
</tbody>
</table>

^a Significance was calculated by use of the Fisher exact test for all variables except age (Mann–Whitney U test).

^b NS, not significant \((P > 0.05)\); NSTEMI, non-ST-segment elevation myocardial infarction; STEMI, ST-segment elevation myocardial infarction; NA, not applicable.

^c These patients received no loading dose because they were already being treated with a maintenance dose of 75 mg once daily when they entered the study.

**Discussion**

Widespread use of antiplatelet therapy in cardiovascular medicine and evidence suggesting a clinically relevant drug resistance have created a demand for simple assays to determine the pharmacologic effects of antiplatelet drugs. The clinical value of point-of-care technologies, such as the platelet function analyzers PFA-100 (Dade Behring) and VerifyNow (Accumetrics), remains to be shown convincingly. Optical platelet aggregometry is still accepted as a “gold standard”, but it is not a standardized method (11). Preanalytical interferences related to blood sampling and complicated sample processing add substantial technical imprecision to the already large biological variability of platelet aggregation. Optical aggregometry requires expert personnel and time-consuming centrifugation steps to obtain platelet-rich and -poor plasma. Aggregation correlates with the increase of light transmittance in platelet-rich plasma after the addition of a platelet agonist. However, platelet-rich plasma is an artificial milieu deficient in giant platelet subspecies as well as erythrocytes and leukocytes, which are regarded as critical modulators of platelet function in vivo (12).

Despite these difficulties, optical platelet aggregometry has been used for many studies of clopidogrel responsiveness. The definitions of responders and nonresponders included mostly arbitrary cutoffs based on differences in the maximum light transmittance measured before and after clopidogrel loading (13). These decision thresholds were adopted by many investigators without standardization or adaptation to local laboratory conditions. In addition, the requirement that aggregation be measured twice, before and after initiation of clopidogrel therapy, may have prevented the routine use of optical aggregometry for assessing clopidogrel resistance in the clinical laboratory setting.

The limitations of optical aggregometry led us to explore impedance aggregometry as an alternative method to study platelet aggregation. Impedance aggregometry offers a higher sensitivity for antiplatelet drug effects and platelet hyperactivity than does optical aggregometry (14). Since its introduction by Cardinal and Flower in 1980, impedance aggregometry has not gained much popularity, probably because semiautomated equipment was not available until very recently (10). Nevertheless, several investigators evaluated impedance aggregometry and suggested standardization to normalize for hematocrit, platelet count, and sample age (15–20). We describe here a simple assay of whole blood aggregation to study clopidogrel responsiveness “on treatment”. This assay can be performed on commercially available, multichannel equipment and is suitable for routine use, even in small laboratories located in coronary care units or catheterization laboratories. Small stand-alone Chronolog aggregometers and disposable electrodes are available for point-of-care use. The turnaround time for detecting potential nonresponders is 10 min and could be extended by another 30 min if a nonresponder is characterized further by use of MeSAMP (including a 20-min incubation). When we used 5 \(\mu\)mol/L ADP as the platelet agonist, the CV of the assay was slightly higher than when we used 20 \(\mu\)mol/L ADP. We recommend, however, the use of 5 \(\mu\)mol/L ADP because the distribution of impedance results for clopidogrel-naïve probands was symmetric about the mean (skewness was 0.032 for 5 \(\mu\)mol/L ADP and 0.718 for 20 \(\mu\)mol/L). Moreover, a concentration of 5 \(\mu\)mol/L is closer to estimated physiologic ADP concentrations, which are typically \(\leq 1 \mu\)mol/L.

We used impedance aggregometry to study clopidogrel responsiveness in 244 CAD patients and identified that 16% were potential nonresponders. This prevalence of clopidogrel resistance was well within the range of 4%–30% reported by others (7, 8). We conclude that our assay does not detect more potential nonresponders than does optical aggregometry, but it does offer superior handling and throughput. Analysis of subgroups suggested that female sex predisposed to clopidogrel resistance (15–20). This marked sex-related difference in clopidogrel responsiveness has not been reported previously and needs to be confirmed in a larger number of patients matched for other potential confounders. The influence of the loading dose on clopidogrel responsiveness is well recognized...
(23, 24). Responders received a slightly higher clopidogrel loading dose than did nonresponders. There was no significant association between responsiveness and age or cardiovascular diagnoses, including stable and unstable angina and non-ST-segment elevation and ST-segment elevation myocardial infarction. The lack of association may be explained by the small sizes of the subgroups. Another limitation is the lack of clinical follow-up information about our CAD cohort. To date, only 2 authors have reported follow-up data from nonresponders suggesting an increased risk of cardiovascular events (13, 25). A prospective, clinical endpoint study is certainly needed now to confirm the clinical benefit of electrical aggregometry. It must be emphasized, however, that the clinical significance of clopidogrel resistance often remains unclear. Although patients with stent thrombosis frequently present with insufficient inhibition of platelet aggregation (26, 27), the risk of stent thrombosis also depends on the quality of stent expansion and other procedural variables related to PCI (28).

Careful interpretation of test results should take into account potential limitations applying to all aggregation tests. Two conditions should be recognized and distinguished from true drug resistance: (a) failure of antiplatelet therapy to prevent a cardiovascular event, which may be triggered by mechanisms unrelated to platelet function; and (b) noncompliance, i.e., patients do not follow the advised therapeutic regimen. In the former case, aggregation is inhibited adequately and the patient is classified correctly as a responder. In the latter case, noncompliance, aggregation is not inhibited in native blood, but inhibition can be achieved by use of MeSAMP. This case cannot be distinguished from true pharmacokinetic resistance attributable to diminished bioavailability for other reasons. It must be pointed out that ADP-induced platelet aggregation is determined largely, but not exclusively, by P2Y12 receptor-mediated platelet activation. In certain conditions, alternative pathways lead to marked platelet activation, which outweighs inhibition by clopidogrel or even MeSAMP and mimics pharmacodynamic resistance in our assay. Analysis of P2Y12 receptor-mediated signaling events, such as phosphorylation of the vasodilator-stimulated phosphoprotein, would certainly be more specific for assessing the pharmacologic effects of clopidogrel, but it may also fail to detect strong platelet activation by alternative pathways (29–31). Ultimately, differentiation of platelet hyperreactivity may not be crucial from a clinical point of view. A useful screening test for clopidogrel resistance should answer the question of whether inhibition of platelet aggregation by clopidogrel is already maximal or could be maximized by increasing the clopidogrel dose. The issue of maximum inhibition of the P2Y12 receptor is critical because clopidogrel may require >95% inhibition of its pharmacologic target for optimal efficacy. The individual dose of clopidogrel should be optimized accordingly.

We thank Christa Dewald and Barbara Calvo for excellent technical assistance.

References


