Determination of Aspirin Responsiveness by Use of Whole Blood Platelet Aggregometry

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Background: Insufficient platelet inhibition is associated with an increased cardiovascular risk in up to 30% of patients taking regular doses of aspirin. We describe an assay to study aspirin responsiveness.

Methods: We performed impedance aggregometry on diluted whole blood with 1 mg/L collagen and 0.5 mmol/L arachidonic acid (AA). We measured thromboxane B2 (TXB2) by RIA. We examined 66 healthy control individuals, 144 aspirin users with stable coronary artery disease (CAD), and 245 CAD patients treated with aspirin and clopidogrel. Nonresponsive samples were incubated with excess Dl-lysinmonoacetylsalicylic acid.

Results: Assay imprecision (CV) was 9.8% and 8.2% at mean (SD) 6-min impedance of 13.7 (2.8) Ω and 13.6 (2.3) Ω for collagen and AA, respectively. Collagen induced stronger aggregation (P = 0.0199) in women [n = 28, 14.6 (2.4) Ω] than in men [n = 38, 13.1 (2.9) Ω], even after sample incubation with 0.1 mmol/L acetylsalicylic acid (ASA) or 1 μmol/L terbogrel, a combined inhibitor of thromboxane synthase and receptors. The sex association persisted in aspirin users, but not if clopidogrel was also taken. A 6-min impedance >8 Ω with collagen (mean – 2 SD of the controls) was taken as evidence of nonresponsiveness, particularly if incubation with ASA did not inhibit aggregation further (>2 Ω). Compared with AA, collagen identified more nonresponsive samples among aspirin users (15%) and CAD patients who also received clopidogrel (10%). Incubation with ASA improved inhibition of aggregation in 70% of samples and consistently reduced TXB2 formation during aggregation.

Conclusions: Impedance aggregometry may prove useful to study aspirin responsiveness, and incubation with ASA may help to identify nonresponders and classify resistance.

Platelet activation and aggregation play critical roles in cardiovascular disease, triggering acute coronary syndrome (ACS) and stroke. Aspirin (acetylsalicylic acid, ASA) has become a cornerstone of the therapy of cardiovascular disease, reducing the rate of adverse clinical events in high-risk patients by ~25% (1). ASA irreversibly inhibits cyclooxygenase-1 (COX-1) activity in circulating platelets, and as a consequence, reduces the synthesis of thromboxane A2 (TXA2), a potent platelet activator (2, 3). Measurements of platelet activation and aggregation as well as of urinary TXA2 metabolite concentrations have revealed, however, that TXA2 synthesis may not be inhibited sufficiently in all ASA users. These ASA nonresponders drew attention to a phenomenon, referred to as ASA resistance, that occurs in 5% to 40% of all treated patients (3, 4). Increased platelet turnover, drug interactions, increased activity of other COX isoforms, genetic polymorphisms, and platelet hyperreactivity may all explain ASA resistance to some extent (3, 5). ASA resistance is associated with a 2- to 4-fold increased risk of recurrent cardiovascular events (6–8) and is an independent risk factor in patients presenting with ACS (9–11).

We describe a simple assay to study ASA responsiveness in diluted whole blood by use of impedance aggregometry. We incubated nonresponsive samples with excess Dl-lysinoacetylsalicylic acid to differentiate pharmacokinetic and pharmacodynamic types of ASA resistance.

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Materials and Methods

Patients

This study was approved by the Institutional Review Board in accordance with the Declaration of Helsinki. We obtained blood samples from 3 groups: healthy volunteers and blood donors (38 men and 28 women; ages 16 to 61 years, median 29 years) who denied taking any antiplatelet medication within 10 days before sampling; 144 outpatients with stable coronary artery disease (CAD) (114 men and 30 women; ages 28 to 90 years, median 66 years) who took 100 mg ASA once a day; and 245 CAD patients (180 men and 65 women; ages 29 to 90 years, median 68 years) who underwent percutaneous coronary intervention (PCI). The use of glycoprotein IIb-IIIa inhibitors was an exclusion criterion. PCI patients received 500 mg intravenous ASA and an oral loading dose of 0 to 600 mg clopidogrel 12 to 24 h before blood sampling. Therapy was continued with 100 mg ASA and 75 mg clopidogrel, both orally, once a day. We collected whole blood with a butterfly cannula (21-gauge) into 10-mL plastic syringes containing 1 mL of 0.106 mol/L trisodium citrate (Citrate Monovette; Sarstedt).

Electrical aggregometry measures the impedance between a pair of electrodes immersed in diluted whole blood. The increase in impedance (Ω) is associated with the amount of platelet aggregates deposited on the electrodes after the addition of a platelet agonist (12). Details of the aggregometry method have been reported (13). We started aggregation by addition of collagen or arachidonic acid (AA), final concentrations 1 mg/L and 0.5 mmol/L, respectively. AA was provided as dried lipid and was dissolved with a bovine serum albumin buffer supplied by the manufacturer (Chrono-Log). We briefly flushed 50-μL aliquots of solubilized AA with nitrogen and stored them at −20 °C until use. We incubated aliquots of citrated whole blood with 0.1 mmol/L ASA (lysine salt; Bayer) at room temperature for 20 to 30 min and measured aggregation. We incubated additional aliquots with 1 μmol/L terbogrel (Thomae), an equipotent inhibitor of thromboxane synthase and thromboxane receptors.

Measurement of Thromboxane B2

We measured concentrations of TXB2, the stable hydrolysis product of TXA2, before and after incubation with ASA. We first analyzed diluted whole blood samples by use of collagen-induced aggregometry. We then retrieved the samples from the aggregometer and centrifuged them immediately to obtain diluted plasma, which was stored at −20 °C until measurement of TXB2 by use of a previously described RIA (14). Briefly, to prepare calibrators, we removed TXB2 from control plasma by reversed-phase C18 column (Bakerbond 7025–00) and added TXB2 (Calbiochem) to the plasma. We mixed 0.3-mL aliquots of appropriately diluted (10- to 1000-fold) samples and standard calibrators with 0.05 mL antiserum (polyclonal, rabbit) and 0.5 mL PBS (pH 7.4) containing 2 g/L gelatin and 40 μg/L [3H]TXB2. After 12-h incubation, we extracted unbound tracer with activated charcoal and measured bound radioactivity by scintillation counting. Cross-reactivity of the assay with numerous prostaglandin derivatives was <3%.

Statistical Analysis

Statistical significance was calculated by use of the Mann–Whitney U-test and Fisher exact test for continuous numerical and categorical data, respectively, allowing an

Fig. 1. Representative results of impedance aggregometry in a diluted whole blood sample from a healthy participant before (upper curve) and after (lower curve) incubation with ASA.

The addition of collagen (A) and AA (B) is indicated by an arrow. The 6-min impedance results of aggregation (dashed line) are given for each aggregation curve.
Impedance increased upon addition of the platelet agonists collagen or AA to a diluted whole blood sample from a healthy individual (Fig. 1). If citrate-anticoagulated blood was first incubated with ASA, then aggregation was markedly reduced with collagen and suppressed with AA (6-min impedance $<3\,\Omega$) (Fig. 1). We measured samples from 8 participants 4 to 5 times during a 4-h period after sampling to determine the imprecision of the assay (CV). The mean CVs were 9.8% and 8.2% for collagen and AA, respectively. The mean (SD) 6-min impedance of the healthy cohort was 13.7 (2.8) $\Omega$ for aggregometry with collagen. The reference interval, derived from the mean and 2 SD, was 8.1 to 19.3 $\Omega$. Consequently, an ASA user was considered a potential nonresponder if his or her sample exhibited a 6-min impedance $>8\,\Omega$. Mean (SD) 6-min impedance was higher in women [14.6 (2.4) $\Omega$] than in men [13.1 (2.9) $\Omega$]. This difference was significant ($P = 0.0199$), but we found no correlation between age and impedance results. We examined AA-induced aggregation in only 8 men and 13 women of the healthy cohort. The mean (SD) was 13.6 (2.3) $\Omega$, and there was no sex- or age-related difference.

The main focus of this work was to examine the suitability of collagen-induced whole blood aggregation to study the pharmacologic effects of ASA. To determine the extent to which COX-1 inhibition and TXA$_2$ receptor blockade inhibit whole blood aggregation, we analyzed diluted whole blood samples from 18 blood donors (12 men and 6 women; ages 22 to 58 years, median 34 years) before and after incubation with ASA and terbogrel (Fig. 2). We also examined CAD patients who exhibited platelet aggregation within the reference interval in spite of treatment with ASA alone (11 men and 5 women; ages 52 to 83 years, median 67 years) or ASA and clopidogrel (23 men and 5 women; ages 39 to 91 years, median 65 years). Incubation with ASA reduced 6-min impedance considerably ($>2\,\Omega$) in all groups. Terbogrel caused even more inhibition, suggesting incomplete COX-1 inhibition by ASA or COX-1–independent TXA$_2$ formation. Notably, the sex-related difference found in the healthy cohort persisted after incubation with ASA and terbogrel (Fig. 2A). This difference was also present in CAD patients treated with ASA (Fig. 2B).

Next, we examined whole blood aggregation in 144 outpatients with stable CAD who were treated with 100 mg of ASA daily. If collagen was used as a platelet agonist, mean (SD) of 6-min impedance was 8.7 (4.6) $\Omega$ in all patients and 8.4 (4.6) $\Omega$ and 10.0 (4.3) $\Omega$ in male and female patients, respectively. The distribution of impedance results in men differed from the relatively symmetric distribution in women by a small additional peak at around 2 to 4 $\Omega$ (Fig. 3). This peak may have accounted for the sex-related difference, which was not significant ($P = 0.0660$). If we used AA as a platelet agonist, aggregation was completely suppressed ($<3\,\Omega$) in 126 (88%) patients. Regression plots of 6-min impedance results obtained with AA and collagen showed 2 different groups of nonresponders (Fig. 4A): 1 group was characterized by increased impedance with collagen and suppressed aggregation with AA; in the other group, impedance results of AA and collagen appeared to correlate. Thus, collagen-induced aggregometry identified more nonresponders than AA-induced aggregometry. Any sample exhibiting a 6-min impedance $>8\,\Omega$ was analyzed again after incubation with ASA. A measurable reduction ($>2\,\Omega$) was achieved in 40 of 56 men and 5 of 19 women, suggesting that the full inhibitory potential of ASA was not achieved in about one-third of the male patients and three-quarters of the female patients (pharmacokinetic type of resistance). Conversely, 21 CAD patients (15%, 16 men and 5
women) maintained an aggregation with collagen >8 Ω despite incubation with ASA (pharmacodynamic type of resistance).

Even in the presence of double antiplatelet therapy, it may be desirable to test for ASA resistance. We examined whole blood aggregation in 245 CAD patients who received ASA and clopidogrel. This double antiplatelet therapy inhibited collagen-mediated aggregation more than ASA alone. Impedance results ranged from 0 to 18 Ω and exhibited a skewed distribution [skewness 1.7; median 2 Ω; mean (SD) 3.4 (3.6) Ω]. If we used AA as a platelet agonist, aggregation was suppressed in 218 of 225 patients tested (97%). Again, the nonresponders detected by both agonists were only partly identical (Fig. 4B). In collagen-induced aggregometry, 24 of 245 samples (10%) exhibited a 6-min impedance >8 Ω and were considered nonresponsive. Incubation with ASA reduced impedance to <8 Ω in 17 samples (pharmacokinetic resistance), whereas 7 samples exhibited a pharmacodynamic type of resistance. ASA resistance was independent of age, diagnosis (stable angina, unstable angina, non-ST-segment elevation myocardial infarction, or ST-segment elevation myocardial infarction), and clopidogrel loading dose, but was associated with female sex (P = 0.0301). Other factors previously implicated in ASA resistance showed no significant association: a familial history of myocardial infarction, ACS while on ASA therapy, diabetes mellitus, or concomitant therapy with angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, or statins.

We hypothesized that the formation of TXB₂ during whole blood aggregometry correlated with ASA responsiveness. Therefore, we examined TXB₂ concentrations in samples from 155 of 245 CAD patients on double antiplatelet therapy (Fig. 5). TXB₂ concentrations were significantly higher in nonresponsive samples (n = 15, 1 patient with pharmacodynamic resistance) than in responsive samples (n = 140) before (P <0.0001) and also after (P = 0.0142) incubation with ASA.

Discussion
Widespread use of antiplatelet drugs has created a demand for simple assays to determine their pharmacological effects. Many investigators have suggested arbitrary cutoffs for nonresponsiveness, which are usually derived
from the difference in maximal light transmittance of optical platelet aggregometry measured before and after the start of ASA therapy. Optical aggregometry requires expert personnel and time-consuming centrifugation steps to obtain platelet-rich and -poor plasma. 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 (15). Erythrocytes may increase the release of AA and the formation of activating eicosanoids (16–18). Technical difficulties and the requirement to perform aggregometry twice—before and after initiation of ASA therapy—may have prevented the widespread clinical use of optical aggregometry.

Alternatively, we explored the use of impedance aggregometry, because it reportedly offers a higher sensitivity for antiplatelet drug effects and platelet hyperactivity than optical aggregometry (19). Since its introduction by Cardinal and Flower in 1980 (12), impedance aggregometry has been thoroughly evaluated and improved (20–25). We describe here a simple assay to study ASA responsiveness by a single measurement of whole blood aggregation, when a patient is already taking ASA. This assay is potentially suitable for point-of-care use by nursing staff in coronary care units or catheterization laboratories. In contrast to light transmission aggregometry, this assay does not involve centrifugation steps. Thus, the turnaround time for detecting potential nonresponders is 10 min and is extended by another 30 min only if a nonresponsive sample requires further characterization by in vitro incubation.

Incubation with ASA or terbogrel revealed that collagen-mediated aggregation depended largely, but not exclusively, on TXA2 synthesis by COX-1 activity. Other commercially available COX-independent inhibitors (such as BM 567) may be used instead of terbogrel. Interestingly, inhibition of COX-1—by ASA either in vitro or in vivo—revealed that collagen-induced aggregation was stronger in women than in men, although this difference was not statistically significant in the small number of studied samples. The difference was not dependent on TXA2 and disappeared if patients were also treated with clopidogrel. This sex difference may have contributed to the failure of ASA to lower the risk of myocardial infarction or cardiovascular death in nearly 40 000 women in the Women’s Health Study (26).

Many authors have employed AA instead of collagen as an agonist for aggregometry. Although AA is a substrate of COX-1, significant aggregation with AA in ASA users does not specifically indicate a failure of ASA to inhibit COX-1 activity, because TXA2 may have other origins (5). The use of collagen as an alternative platelet agonist may offer certain advantages over AA. Preparation, handling, storage, and costs favor collagen. In the presence of low concentrations of collagen, aggregation is sensitive to inhibition by ASA (27). In whole blood, collagen stimulates TXA2 synthesis from blood cell membrane–derived AA in addition to binding to known collagen receptors, including the glycoprotein GpVI/Fc receptor γ-chain complex. Collagen receptor-dependent platelet activation may be influenced by sex and may also explain the differences in aggregation response obtained with collagen and arachidonic acid. This hypothesis awaits further investigation by the use of more specific receptor ligands such as collagen-related peptides. Our preferred approach to use collagen as a platelet agonist integrates all relevant sources of TXA2 formation and may also detect TXA2-independent platelet hyperreactivity, an important cause of ASA failure (28).

A limitation of whole blood aggregometry was the specific assessment of ASA responsiveness in patients with double antiplatelet therapy. This was a particular challenge, because clopidogrel also affected collagen-induced aggregation to some extent. However, the results of TXB2 analysis suggested a relatively high specificity of collagen-induced aggregometry. We defined nonresponsiveness as a 6-min impedance >8 Ω (mean ± 2 SD of the healthy cohort) for patients with single or double antiplatelet therapy. We understand, however, that a classification based on results of impedance aggregometry in ASA-naive individuals may be inappropriate for ASA users. Receiver-operating-curve analysis of whole blood aggregometry and clinical endpoints is required to define appropriate response thresholds after examination of var-
ious larger patient cohorts on single or double antiplatelet therapy. Until future studies can provide these data, we suggest incubating samples from potentially nonresponsive patients with 0.1 mmol/L ASA to determine the maximal inhibition that can theoretically be achieved. For comparison, 2 min after intravenous administration of a 500-mg dose of ASA, the plasma peak concentration reaches 0.15 mmol/L (drug prescription information). The issue of maximal inhibition is critical, because ASA reaches 0.15 mmol/L (drug prescription information). For sive patients with 0.1 mmol/L ASA to determine the maximal inhibition that can theoretically be achieved. For comparison, 2 min after intravenous administration of a 500-mg dose of ASA, the plasma peak concentration reaches 0.15 mmol/L (drug prescription information). The issue of maximal inhibition is critical, because ASA reaches 0.15 mmol/L (drug prescription information).

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