Evaluation of laboratory methods routinely used to detect the effect of aspirin against new reference methods

Emese G. Kovács a, Éva Katona a, Zsuszanna Bereczky a, Nóra Homoródi b, László Balogh b, Eszter Tóth a, Hajna Péterfy c, Róbert G. Kiss d, István Édes b, László Muszbek a,e

a Clinical Research Center, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary
b Institute of Cardiology, University of Debrecen, Medical and Health Science Center, Debrecen, Hungary
c Diagnosticum Co., Research Laboratory, Budapest, Hungary
d State Health Center, Department of Cardiology, Budapest, Hungary
e Thrombosis, Haemostasis and Vascular Biology Research Group of the Hungarian Academy of Sciences, University of Debrecen, Debrecen, Hungary

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ABSTRACT

Background: Aspirin, a commonly used antiplatelet agent, blocks platelet thromboxane A2 (TXA2) formation from arachidonic acid (AA) by acetyling platelet cyclooxygenase-1 (COX-1). Laboratory methods currently used to detect this antiplatelet effect of aspirin provide variable results. We have reported three methods that assess platelet COX-1 acetylation (inactivation) by aspirin and its direct consequences. The first and second assays use monoclonal anti-human-COX-1 antibodies that only detect acetylated (inactivated) COX-1 and active (non-acetylated) COX-1, respectively. The third method measures platelet production of TXB2 (the stable metabolite of TXA2) in vitro in response to AA. We compared the results of these three reference methods with other routinely used methods for assessing the functional consequences aspirin treatment.

Methods: 108 healthy volunteers were treated with low-dose aspirin for 7 days. On day 7 following aspirin treatment COX-1 in the platelets was fully acetylated whereas only non-acetylated COX-1 was present in the day 0 platelets. Further, TXB2 production by day 7 platelets was completely blocked. The following tests were performed on the samples obtained from study participants before and after seven days of aspirin treatment: PFA-100 closure time with collagen/epinephrine cartridge, VerifyNow® (VN) Aspirin Assay, platelet aggregation and AIPD using AA, ADP, epiinephrine and collagen as agonists.

Results: Comparing the pre-treatment and day 7 values, methods that use AA as platelet agonist (AA-induced platelet aggregation/secretion and VN Aspirin Assay) showed high discriminative power. In contrast, results of the other tests showed considerable overlap between day 7 and day 0 values.

Conclusions: Only assays that clearly distinguish between acetylated and non-acetylated platelet COX-1 are useful for establishing the antiplatelet effect of aspirin. The other tests are not suitable for this purpose.

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Introduction

Aspirin (acetylsalicylic acid) is widely used in the secondary prevention of acute atherothrombotic events [1–6]. Although aspirin is a general acetylating agent, its effect against arterial thrombosis primarily depends on the acetylation of platelet cyclooxygenase 1 (COX-1) at the side chain of Ser529 residue [2,7,8]. COX-1 converts arachidonic acid (AA), released from platelet membrane phospholipids, into prostaglandin G2 and H2, the latter of which is then transformed to thromboxane A2 (TXA2), a secondary platelet agonist. Ser529 is located in the wall of the active-site cavity of COX-1. The formation of the Ser-529-C(acetylsalicylic acid carbonyl) bond, which occurs in a single elementary step [9], prevents the access of AA to the active site and consequently blocks the production of TXA2.

As aspirin is ineffective in preventing acute vascular events in some patients the term “aspirin resistance” was introduced. Although aspirin resistance is a poorly defined term, it has been widely used and a PubMed search on July 15, 2013 resulted in 1844 citations, including 500 reviews [10–16]. Four aspects of “aspirin resistance” have been reported:

1/ Chemical ("true") aspirin resistance: inability of aspirin to acetylate platelet COX-1 at Ser529.

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2/ Aspirin “non-responsiveness” or diminished response to aspirin as measured by a laboratory test.

3/ A high platelet turnover in some instances results in an accelerated production and release into the circulation of large numbers of newly formed but not yet acetylated platelets [17–19].

4/ Clinical “aspirin resistance” where aspirin does not protect the patient from an acute vascular event.

High platelet turnover, though might be of clinical importance, should not be considered as aspirin resistance, since changing the frequency of aspirin dosage overcomes the diminished response to aspirin [20]. For clinical aspirin resistance the expression non-responsiveness should be more appropriate. The failure of aspirin to prevent acute atherothrombotic events in some patients might not be related to the lack of COX-1 acetylation. Aspirin is ineffective in inhibiting platelet activation induced by agonists more potent than AA, e.g. high dose of collagen or thrombin.

Routinely used laboratory tests show high inter-, and intra-individual variability and there is a considerable discrepancy between results obtained by different tests. To assess these tests one needs comparisons to reference methods, which might be too sophisticated for everyday clinical use, but detect the acetylation of platelet COX-1 with high certainty. Such a method was developed in the seventies, in which inactivation of COX-1 by aspirin was measured as a reduction in the ability of [²¹H-acetyl]-aspirin to bind to a 85 kDa protein, COX-1, in the 180,000 g supernatant of platelet lysate [21,22]. Most recently we developed two methods that directly detect the acetylation or the lack of acetylation of COX-1 in platelets by Western blotting [23]. The methods utilize two monoclonal antibodies, one of which reacts only with acetylated COX-1 (acCOX-1), while the only the non-acetylated form (nacCOX-1) is recognized by the other one. In addition we designed a third method that measures the AA-induced formation of thromboxane B₂ (TXB₂), the inactive metabolite of TXA₂, in platelet rich plasma (PRP) [23]. This method determines the TXB₂ producing capability of platelets and indirectly detects the inhibition of COX-1 by aspirin. Although these methods are too laborious for a routine laboratory, they are well suitable for establishing the frequency of chemical aspirin resistance in a population, and also for the evaluation of laboratory methods routinely used for the detection of aspirin effect. Using the aforementioned methods we demonstrated the lack of aspirin resistance among 108 healthy volunteers taking 100 mg enteric-coated aspirin daily for seven days [23]. This population was utilized to evaluate several laboratory methods widely used for the detection of aspirin effect, i.e., to reveal if these methods diagnose false positive aspirin resistance in individuals with platelet COX-1 fully acetylated by aspirin.

Methods

Study Participants, Inclusion and Exclusion Criteria

Healthy individuals recruited for the study have been characterized in a previous publication [23]. Ten individuals out of 121 recruited volunteers were excluded on the basis of apriori exclusion criteria (contraindications of aspirin treatment, chronic disease, known platelet defect, and any medication in the preceding 2 weeks or during the study, with the exception of oral contraceptives). Three participants did not show up at the second blood sampling. The remaining study population (n = 108) had the following characteristics: 60 women and 48 men, mean age: 33.5 years (SD 9.3, range 19–59), 69.4% of them had never smoked, 21.3% were current smokers. Study protocol was approved by the National Ethics Committee and written informed consent was obtained from all participants.

Protocol of Aspirin Treatment and Blood Sample Collection

Study participants received 100 mg enteric-coated aspirin (Aspirin protect, Bayer) once daily between 8 and 9 a.m. for 7 days. Compliance was checked by pill count. In a single case, when non-compliance was suspected, the seven-day period of aspirin treatment was repeated and the intake of aspirin tablets was controlled by the study nurse. Blood samples were collected into Vacutainer tubes containing 0.109 mol/L trisodium citrate (Becton-Dickinson, Franklin Lakes, NJ) after overnight fasting before and 168 hours after the first dose of aspirin (day 0 and day 7 samples). PFA-100 closure time assay (Siemens, Marburg, Germany) and VerifyNow® (VN) Aspirin assay (Accumetrics, San Diego, CA) were performed on anti-coagulated whole blood. PRP was separated by centrifugation (120 g, 37 °C, 15 min). Platelet depleted plasma (PDP), was obtained by two consecutive centrifugations (1500 g, 25 °C, 20 min).

Reference Methods for the Detection of COX-1 Acetylation in Platelets

We developed reference methods for the direct and indirect detection of COX-1 acetylation, which have been described in details in a separate publication [23]. The first two methods are based on monoclonal antibodies generated against acetylated or non-acetylated form of COX-1. The antibodies were raised against protein-linked nonapeptides with the amino acid sequence that corresponded to human COX-1 525–533 residues. Both unmodified peptide (H-Gly-Ala-Pro-Phe-Ser-Leu-Lys-Gly-Leu-OH) and peptide acetylated at Ser 529 (H-Gly-Ala-Pro-Phe-Ser(Ac)-Leu-Lys-Gly-Leu-OH) were used for the generation of monoclonal antibodies. By selecting the appropriate clones two types of antibodies were produced; one specific to non-acetylated COX-1 (anti-nacCOX-1) and another one that reacted only with acetylated COX-1 (anti-acCOX-1). Antibodies purified from ascites fluid by Protein G affinity chromatography were used to detect the two forms of COX-1 by Western blotting of platelet lysate. To our knowledge this is the first method that directly detects the state of acetylation of COX-1 in platelets.

The third reference method measures AA-induced generation of TXB₂ in PRP [23]. The method is based on the extraction of generated TXB₂ from PRP and its separation from AA that would interfere with the assay, by solid phase extraction. The extracted TXB₂ is then, measured by the competitive immunoassay kit of Assay Designs (Ann Arbor, MI). Its production was expressed as pg TXB₂/10⁶ platelets.

Routine Laboratory Tests of Platelet Function

Collagen/epinephrine (CEPI) cartridges were used for PFA-100 closure time measurements. VN Aspirin Assay was performed according to the manufacturer’s instructions and the results were expressed as Aspirin Reaction Units (ARU). Platelet aggregation and secretion were followed in Chrono-Log 700 lumiaggregometer (Chrono-Log, Havertown, PA) on PRP adjusted to 260 x 10⁹/L platelet count by PDP. Platelets were activated by either of the following agonists: 500 μg/mL (1.53 mmol/L) AA (Helena, Gateshead, UK), 1 μg/mL fibrillar collagen (Nycomed, Zurich, Switzerland), 10 μmol/L ADP (Sigma Aldrich, St. Louis, MO), 10 μg/mL (54.6 μmol/L) epinephrine (Gedeon Richter, Budapest, Hungary). Aggregation was recorded for 6 min (ADP) or 8 min (AA), or 10 min (epinephrine and collagen) and the results were expressed as percentage maximal change in light transmission. ATP secretion of activated platelets was quantitated by bioluminescence method using luciferin-luciferase reagent (Biothera AB, Handen, Sweden). Maximal ATP secretion was expressed as μmol ATP/10¹⁰ platelets.
Variables were expressed as mean and SD or median, interquartile range (IQR) and total range according to their distribution, established by Kolmogorov-Smirnov test. Wilcoxon signed rank test was used to analyze differences between day 0 and day 7 values. Correlation analysis was performed by Spearman test. Student’s t test was used to analyze differences in variables between subgroups of individuals. SPSS version 16 (SPSS Inc., Chicago, Illinois) was used for statistical analyses.

Results

As demonstrated with two representative volunteers, only native (non-acetylated) COX-1 was detected in day 0 platelet lysates whereas only inactive (fully acetylated) COX-1 was detectable in the day 7 platelet lysates (Fig. 1). Based on our observation that the methods used readily detects 2.5% of the platelet COX-1 content, no true aspirin resistance was observed in this group of volunteers studied [23]. In full agreement with the results above the AA-induced TXB2 production measured on day 0 (median: 1140 pg TXB2/10⁶ platelets, IQR: 854-1396 pg TXB2/10⁶ platelets, total range: 4-35 pg TXB2/10⁶ platelets, total range: 4-35 pg TXB2/10⁶ platelets) decreased to its 1.4% (median: 16 pg TXB2/10⁶ platelets, IQR: 12-19 pg TXB2/10⁶ platelets, total range: 4-35 pg TXB2/10⁶ platelets) by day 7. It is to be noted that platelet count did not change during the one-week aspirin treatment: at entry the mean platelet count was 271.3 (SD 62.5) x 10⁹/L, on day 7 it was 278.5 (SD, 63.0) x 10⁹/L.

As the reference methods clearly distinguished between pre-treatment and day 7 values, and not even partial aspirin resistance could be detected, we compared the results above for the day 0 and day 7 platelets with the results obtained using routine laboratory tests to evaluate the effects of aspirin on platelets. Although the statistical analysis of PFA-100 CEPI closure time revealed significant difference between the two sets of results (p < 0.0001), there was a considerable overlap and 41% of closure times of aspirin-treated individuals remained in the pre-treatment “normal” range (Fig. 2, Table 1). If not compared to the individual pre-treatment value, but to a reference interval established for non-treated persons, in a high number of cases, falsely, aspirin resistance would be diagnosed. In contrast, with the exception of a single outlier, no overlap was observed between day 0 and day 7 ranges by VN Aspirin Assay (Fig. 2). In the case of PFA-100 method pre-treatment values correlated significantly with day 7 values, i.e., individuals with highly reactive platelets in the absence of aspirin also showed relatively high platelet reactivity following aspirin treatment (Table 2). In the case of VN Aspirin Assay no such correlation was observed.

We also investigated the effect of aspirin on agonist-induced platelet aggregation (Fig. 3, Table 1) and ATP release (Table 3) in the study population. Using epinephrine as agonist, in 30% of the cases the low pre-treatment values fell into the range of day 7. Similarly, in 26% of individuals epinephrine-induced ATP release on day 0 was below the limit of quantitation. Due to the significant number of low responders to epinephrine, this agonist is hardly suitable to estimate the effect of aspirin. In the case of ADP aggregation, in spite of the statistically significant difference, the sets of day 0 and day 7 results were highly overlapping (Fig. 3, Table 1). Even if the single low responder outlier with 39% transmission increase (the lowest value on Fig. 2) was eliminated, 94.4% of day 7 values overlapped the day 0 range. This result demonstrates that in the absence of pre-treatment value from the same individual, ADP aggregation gives false positive aspirin resistance in a high number of cases. As the pre-treatment values of ADP induced ATP release scattered in a wide range (Table 3) this parameter cannot be used for testing the effect of aspirin, either. In the case of collagen-induced aggregation and ATP secretion, 5% and 25% of the day 7 values were above the lower limit of day 0 values, respectively. AA-induced platelet aggregation (Fig. 3, Table 1) and ATP release (Table 3) showed excellent distinctive power with no overlap between day 0 and day 7 ranges. The extent of ADP-, epinephrine- and collagen-induced platelet aggregation measured on day 7 significantly correlated with pre-treatment values (Table 2). In the case of AA-induced aggregation no such correlation was observed. It is to be noted that in a previous study there was no correlation between AA induced TXB2 production measured before and after 7 days of aspirin treatment [23].

Discussion

A number of laboratory methods are used to detect the effect of aspirin on platelet function (see reviewed in references [14,15,24]). This study compared the results of several routine platelet tests and
the three specific tests with a goal of identifying tests that specifically measure acetylation (inactivation) of platelet COX-1. As detailed in Figs. 1, 2 and 3, only tests using AA as agonist clearly and without equivocation differentiated platelets with native COX-1 from platelets with inactive COX-1.

A significant overlap between PFA-100 CEPI closure times of non-treated and treated patients has also been reported by other authors [25,26]. We also tested the PFA-100 closure time with collagen/ADP cartridge, but the day 0 and day 7 results were fully overlapping and they were not included in the manuscript. COX-1 independent pathways, not influenced by aspirin, play an important role in the formation of platelet plug at the aperture and the closure time reflects the effect of aspirin measured after aspirin treatment showed a highly significant correlation with the pretreatment closure times (Table 2). This finding suggests that high pretreatment platelet reactivity considerably influences the results and could overestimate the effect of aspirin in this assay. PFA-100 closure time is also influenced by von Willebrand factor, hematocrit value and platelet count [24,27–29]. The one-week aspirin treatment did not change the platelet count and it is assumed that the two other variables did not change significantly, either.

The VN Aspirin Assay performed much better than the PFA-100 closure time with CEPI cartridge. This test uses AA as agonist to activate platelets. The produced TXA2 initiates the biochemical signaling pathway that ultimately transforms platelet glycoprotein IIb/IIIa complex into a fibrinogen binding receptor. Activated platelets then bind to fibrinogen-coated beads present in the cartridge, agglutinate them resulting in increased light transmission. The blockade of TXA2 formation by aspirin prevents platelet activation and, consequently the elevation of light transmission. The results are expressed as Aspirin Reaction Units (ARU). According to the manufacturer’s instruction a cut-off of <550 ARU is consistent with efficient aspirin effect. In our study population, with a single exception (586 ARU), all pre-treatment (day 0) values were above 600 ARU (Fig. 2). Seven days of aspirin treatment brought down the response to AA in all, but one (590 ARU) case, below 550 ARU. Thus, the VN Aspirin assay in 99% of the cases reliably reflected the state of COX-1 acetylation in healthy volunteers. There was no correlation between the day 0 and day 7 values (Table 2), i.e., the pre-treatment platelet reactivity did not influence the effect of aspirin.

Light transmission aggregometry is a conventional technique to detect platelet aggregation and, if a lumiaggregometer is available, it can be supplemented by the determination of agonist induced ATP secretion. This technique has been adapted to the detection of aspirin effect and using various agonists it still enjoys considerable popularity. As expected, AA that utilizes COX-1-dependent pathway for aggregation/secretion had a high power in distinguishing platelet samples with acCOX-1 and naCOX-1 (Fig. 3). There was no overlap between day 0 and day 7 results of AA-induced aggregation/secretion i.e., the assay reliably reflected the state of COX-1 acetylation in healthy volunteers. Exactly like in the case of VN Aspirin Assay the aggregability

<table>
<thead>
<tr>
<th>Assay method</th>
<th>Correlation coefficient (r)</th>
<th>Significance</th>
</tr>
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<tbody>
<tr>
<td>PFA-100 CEPI closure time</td>
<td>0.577</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>VerifyNow Aspirin Assay</td>
<td>0.039</td>
<td>p = 0.686</td>
</tr>
<tr>
<td>Arachidonic acid-induced aggregation</td>
<td>0.019</td>
<td>p = 0.844</td>
</tr>
<tr>
<td>ADP-induced aggregation</td>
<td>0.581</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>Epinephrine-induced aggregation</td>
<td>0.483</td>
<td>p = 0.0001</td>
</tr>
<tr>
<td>Collagen-induced aggregation</td>
<td>0.259</td>
<td>p = 0.007</td>
</tr>
</tbody>
</table>

Table 2: Correlation between pre-treatment values and values obtained after 7 days of aspirin treatment.
of non-acetylated platelets did not influence the effect of aspirin (Table 2).

Due to the low pre-treatment aggregation and secretory responses to epinephrine in about a quarter of individuals [30,31], this agonist is hardly suitable for the detection of aspirin effect [Fig 3, Table 3]. In the case of ADP induced aggregation/secretion the median day 7 values were lower than the day 0 median values, however the considerable overlap of the two sets of results excludes the correct evaluation of aspirin effect. Changing the ADP concentration in the range of 2.5-20 μM did not improve the power of this test to detect the effect of aspirin (data not shown). Low dose (1 μg/mL) of collagen as agonist had better distinguishing power than ADP. Using this agonist the overlap of day 7 values with the range of pre-treatment values was lower, but was not eliminated. Aggregation and ATP release induced by ADP and collagen involve COX-1-independent mechanisms, which may obscure the effect of aspirin. In these cases, just like in the case of PFA-100 CEPI closure time, pre-treatment values were significant determinants of day 7 values, i.e. non-acetylated platelets demonstrating high reactivity to the agonists also showed comparably high reactivity while their COX-1 was fully acetylated and TXA2 production was blocked (Table 2).

The main reasons for the lack of aspirin effect on COX-1 acetylation and platelet TXA2 production in patients, and probably also the main reason for testing the effect of aspirin in the laboratory, is non-compliance and the use of interfering non-steroid anti-inflammatory drugs that prevent the access of aspirin to Ser529 in the active-site cavity of COX-1 [32–35]. The expression of COX-2, which is not inhibited by low dose aspirin, in certain pathological conditions like inflammatory diseases, might also be responsible for a diminished suppression of platelet TXA2 production by aspirin [36–38], although the significance of such a mechanism has been debated [39]. The above possibilities should not be considered as aspirin resistance and should be specified, if COX-1 dependent diagnostic tests suggest the lack of aspirin effect. Not counting our reference methods, AA-induced platelet aggregation/secretion, VN Aspirin Assay and very likely also serum TXB2 determination [40] are the assays that can be used successfully for the detection of such conditions. PFA-100 CEPI closure time and aggregation/secretion induced by agonists other than AA do not seem to be reliable for such a purpose. However, these assays, testing platelet functions that also involve COX-1-independent pathways, might be useful in the detection of highly reactive COX-1-acetylated platelets and might serve as predictors of adverse clinical outcome [11,41]. However, this possibility should be supported by large-scale clinical studies.

### Conflict of Interest Statement

None of the authors have any conflict of interest to declare.

### Acknowledgements

Support from the National Office of Research and Technology (Jedlik Ányos grant, NKFP-07-A1-2008-0127), from the Hungarian Academy of Sciences (MTA11003, TK1227) and from the National Development Agency (TÁMOP projects 4.2.2.B-10/1-2010-0042, 4.2.2.A-11/1-KONV-2012-0045) is acknowledged. Zsuzsanna Bereczky is a recipient of János Bólyai fellowship from the Hungarian Academy of Sciences. The authors are indebted to Judith Csápi BSc, Gizella Haramura BSc and Éva Molnár BSc for the skilled technical assistance.

### References


### Table 3

<table>
<thead>
<tr>
<th>Agonist</th>
<th>μmol ATP/10^12 platelets (day 0 median, IQR, total range)</th>
<th>overlap of day 7 values with the day 0 range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>1.1 (0.9-1.4; 0.4-2.6)</td>
<td>0%</td>
</tr>
<tr>
<td>ADP</td>
<td>1.0 (0.7-1.2; 0.2-2.2)</td>
<td>100%</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>1.2 (0.2-1.5; 0.2-8)</td>
<td>0%</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.0 (0.8-1.3; 0.3-2.2)</td>
<td>25.0%</td>
</tr>
</tbody>
</table>


