Thrombosis Research 133 (2014) 811–816

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

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

Emese G. Kovács [a], Éva Katona [a], Zsuzsanna 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

ARTICLE INFO

Article history:
Received 19 July 2013
Received in revised form 1 October 2013
Accepted 1 October 2013
Available online 16 October 2013

Keywords:
aspirin
aspirin resistance
platelet aggregation
platelet secretion
reference method
thromboxane

ABSTRACT

Background: Aspirin, a commonly used antiplatelet agent, blocks platelet thromboxane A_2 (TXA_2) formation from arachidonic acid (AA) by acetylating 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 TXB_2 (the stable metabolite of TXA_2) 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, TXB_2 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 ATP secretion using AA, ADP, epinephrine 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.

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 G_2 and H_2, the latter of which is then transformed to thromboxane A_2 (TXA_2), a secondary platelet agonist. Ser529 is located in the wall of the active-site cavity of COX-1. The formation of the Ser-Oγ-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 TXA_2.

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.

Abbreviations: COX-1, cyclooxygenase-1; AA, arachidonic acid; TXA_2, thromboxane A_2; acCOX-1, acetylated COX-1; nacCOX-1, non-acetylated COX-1; TXB_2, thromboxane B_2; PRP, Platelet rich plasma; VN Aspirin Assay, VerifyNow® Aspirin assay; PDP, Platelet depleted plasma; anti-nacCOX-1, antibody specific to non-acetylated COX-1; anti-acCOX-1, antibody specific to acetylated COX-1; CEPI, PFA-100 collagen/epinephrine cartridge; ARU, Aspirin Reaction Unit; IQR, interquartile range.

* Corresponding author at: University of Debrecen, Medical and Health Science Center, Clinical Research Center, 98 Nagyerdei krt., 4032 Debrecen, Hungary. Tel.: +36 52 431956; fax: +36 52 340011.
E-mail address: muszbek@med.unideb.hu (L. Muszbek).

http://dx.doi.org/10.1016/j.thromres.2013.10.008

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 first developed in the seventies, in which inactivation of COX-1 by aspirin was measured as a reduction in the ability of [^{3}H-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 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 B_2 (TXB_2), the inactive metabolite of TXA_2, in platelet rich plasma (PRP) [23]. This method determines the TXB_2 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 TXB_2 in PRP [23]. The method is based on the extraction of generated TXB_2 from PRP and its separation from AA that would interfere with the assay, by solid phase extraction. The extracted TXB_2 is then, measured by the competitive immunoassay kit of Assay Designs (Ann Arbor, MI). Its production was expressed as pg $TXB_2/10^6$ 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 10^9/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 (Biothema AB, Handen, Sweden). Maximal ATP secretion was expressed as μmol ATP/10^{11} platelets.