SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

DETECTING THE EFFECT OF ASPIRIN ON PLATELET CYCLOOXYGENASE 1

By Emese G. Kovács, MD

UNIVERSITY OF DEBRECEN

Kálmán Laki Doctoral School

DEBRECEN, 2014

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

DETECTING THE EFFECT OF ASPIRIN ON PLATELET CYCLOOXYGENASE 1

By Emese G. Kovács, MD

Supervisor:

Prof. László Muszbek, MD, MHAS

UNIVERSITY OF DEBRECEN

Kálmán Laki Doctoral School

DEBRECEN, 2014

DETECTING THE EFFECT OF ASPIRIN ON PLATELET CYCLOOXYGENASE 1

By Emese G. Kovács, MD

Supervisor: Prof. László Muszbek, MD, MHAS

Kálmán Laki Doctoral School, University of Debrecen

Head of the **Examination Committee**:
Prof. György Balla, MD, MHAS

Members of the Examination Committee:
Prof. János Kappelmayer, MD, DSc
Dániel Aradi, MD, PhD

The Examination takes place at the library of the Department of Pediatrics, Faculty of Medicine, University of Debrecen
at 11:00 AM, 7th May, 2014.

Head of the **Defense Committee**:
Prof. György Balla, MD, MHAS

Reviewers:
Prof. Pál Soltész, MD, DSc
Imre Bodó, MD, PhD

Members of the Defense Committee:
Prof. János Kappelmayer, MD, DSc
Dániel Aradi, MD, PhD

The PhD Defense takes place at the Lecture Hall of Building A, Institute of Internal Medicine, Faculty of Medicine, University of Debrecen
at 12:30 PM, 7th May, 2014.

INTRODUCTION AND REVIEW OF LITERATURE

Platelets are formed by fragmentation of bone marrow megakaryocyte cytoplasm following megakaryocyte proliferation and differentiation regulated by thrombopoietin. Inactivated human platelets are 2 to 4 μm in diameter and contain granules (α, dense and lysosomes), mitochondria, surface-connected canalicular system and dense tubular system. Platelets lack genomic DNA but contain some megakaryocyte-derived messenger RNA and the translational machinery needed for protein synthesis. This allows only limited amounts of protein synthesis. Platelets circulate in a concentration of 150 to 400 x 10^9/L and have a life span of 7 to 10 days.

Their primary role is maintaining primary hemostasis after tissue trauma or vascular injury. Prior to vascular injury, platelets are maintained in resting state by prostacyclin, nitric oxide released from endothelial cells, and CD39, an ADPase on the surface of endothelial cells. On the site of injury the development of the hemostatic platelet plug is initiated by the exposure of subendothelial matrix, mainly collagen and the local generation of thrombin. Platelets activated following an interaction with collagen and von Willebrand factor form a primary monolayer. Afterwards, via the secretion of TXA_2, ADP and other platelet agonists, most of which are ligands for G protein coupled receptors on the platelet surface, platelets are activated. During this activation through the conformational change of glycoprotein IIb/IIIa complex the fibrinogen receptor is formed on platelet surface. To the covering platelet layer further platelets are attached through fibrinogen and finally, in the growing hemostatic plug the crosslinked fibrin network produced in the course of the coagulation process stabilizes the platelet aggregate.

Pathologic platelet aggregation/activation plays an important role in the patomechanism of ischemic complications in coronary artery disease, stroke and peripheral arterial disease. Although the adhesion of platelets can be viewed as a repair-oriented response to erosion or sudden rupture of an atheromatous plaque, the following thrombus formation can lead to transient ischemia or infarction and this can cause severe organ injury.

Eicosanoids are members of the unsaturated 20-carbon fatty-acid derivative family and their synthesis plays an important role in platelet function. Thromboxane A_2 (TXA_2), a major eicosanoid always newly produced and not stored in platelet dense or α granules is a potent vasoconstrictor and platelet activating agent, induces the proliferation of vascular smooth-muscle cells and is proatherogenic. It induces irreversible platelet aggregation through its interaction with the TXA_2 receptor and amplifies the responses of platelets to different agonists.

In the first step of platelet TXA_2 synthesis arachidonic acid (AA) is released from platelet membrane phospholipids by phospholipase A_2 activated following platelet agonist induced calcium release. In the next step AA is converted to cyclic endoperoxides by prostaglandin H synthase 1. Prostaglandin H synthase 1, a hemoprotein of the myeloperoxidase family has dual enzymatic activity. Cyclooxygenase (COX) activity forms prostaglandin G_2 (PGG_2) from AA and peroxidase activity converts prostaglandin G_2 to prostaglandin H_2 (PGH_2). This process from AA release until PGH_2 formation takes place in several cell types and at the site of different tissues and cells specific isomerases convert PGH_2 to prostanoids: PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) and TXA_2. In platelets this specific isomerase, the thromboxane synthetase is responsible for the production of TXA_2.

In vertebrates the prostaglandin H synthase 1 enzyme has a second isoform, the prostaglandin H synthase 2 or COX-2. The amino acid sequence

of COX isoenzymes shows 60% identity. Both are membrane bound homodimers of ~70 kDa subunits. COX-1 is responsible for the constitutive PGH_2 production whereas COX-2 expression is induced by various pathologic processes among which inflammation plays an important role.

Aspirin or acetylsalicylic acid is one of the first used synthetic compounds in therapeutics, it was introduced into the commercial market in June 1899 and for long it was applied for its antipyretic, analgesic and anti-inflammatory properties. Its beneficial role in the prevention of the acute complications of atherothrombotic diseases is known since the mid 20th century. Since then, the low-dose - 75-100 mg/day - aspirin therapy became the cornerstone in the prevention of myocardial infarction or stroke.

Meta-analyses based on different randomised trials involving a large number of patients show that the preventive aspirin therapy in high risk patients reduces vascular death and nonfatal vascular events by 15% and 30%, respectively. This includes the reduction of non-fatal myocardial infarction by 34%, non-fatal stroke by 25% and vascular death or death of unknown cause by one sixth. The benefits outweigh the bleeding hazards unless the absolute risk of bleeding is high.

Concerning the meta-analysis on primary prevention of acute vascular events low-dose aspirin therapy in patients without manifest vascular disease reduces the incidence of vascular events, but the absolute risk reduction is lower than in the secondary prevention.

The mechanim of protective effect exerted by low-dose aspirin therapy has been intensively studied. Today it is unequivocally acknowledged that its antithrombotic action is primarily based on the anti-platelet effect owing to COX-1 inactivation by acetylating the 529 serine (Ser529) residue. In higher doses aspirin also reduces the release of inflammatory cytokines and oxygen radicals.

COX-1 is located in the membrane of platelet dense tubular system. Aspirin diffuses through the platelet cell membrane and reaches the narrow, mainly hydrophobic COX-1 channel that leads to the enzyme's active site. In the next step, it becomes connected to the Arg120 residue, a common docking site for non-steroid anti-inflammatory drugs and then, modifies covalently (acetylates) the Ser529 sitting in the wall of the active-site pocket of the enzyme. According to in silico experiments, during the transesterification reaction the formation of the Ser529-Hγ-ASA carboxyl-group's oxigen bond occurs in paralell with the formation of the Ser529-Oγ-ASA C (acetylsalicylic acid carbonyl) bond. The acetylation prevents the acces of AA to the active site and blocks the TXA_2 production by platelet. The inhibition is irreversible and lasts throughout the lifespan of platelets.

In order to achieve the same degree of acetylation of the inducible cyclooxygenase-2 enzyme's (COX-2) at Ser516 side chain 10 to 100-fold higher acetylsalicylic acid concentrations are needed than for the acetylation of COX-1. Presumably, the main reason for this difference is that in the larger COX-2 active site cavity the relative orientation of the Ser516 and the ASA promotes the acetylation to a lesser extent.

Acetylsalicylic acid is rapidly absorbed in the stomach and the upper small intestine. 20 minutes after the ingestion uncoated aspirin can be already detected in plasma and its level peaks 30-40 minutes after ingestion. In contrast, it takes three to four hours to reach peak plasma levels after the administration of enteric-coated preparations. The systemic bioavailability of regular aspirin pills is about 40 to 50 percent which is indepedent of the efficiency of COX-1 inhibition. This can be explained by the fact that aspirin first comes into contact with platelets in the portal circulation and platelets are exposed to substantially higher drug levels than those present in the systemic circulation. Aspirin has a half-life of 15 to 20 minutes in plasma. Concerning platelet TXA_2 production and inhibition of platelet aggregation

during chronic aspirin therapy, enteric-coated and plain preparations have the same effectivity.

The daily administration of 30 mg aspirin results in complete suppression of platelet TXA_2 production after one week through a cumulative process. Regimens of 75 to 100 mg aspirin per day applied during low-dose aspirin therapy exceed the minimal effective dose required for a full pharmacodynamic effect, thus accomodating some degree of interindividual variability in drug response. Despite the rapid clearance of aspirin from the circulation, its antiplatelet effect lasts for the lifespan of a platelet owing to the permanent inactivation of COX-1. Platelets are not able to synthetise COX-1, so the effect of aspirin can be reversed only through the generation of new platelets.

The fact that aspirin prevents acute vascular events only in part of the patients suggested that significant individual variations exist in the responsiveness to the drug. For this reason the term "aspirin resistance" was introduced. Unfortunately, "aspirin resistance" has not been unequivocally defined creating difficulties in the interpretation of results and also in setting up recommendations for the treatment of patients. Previously the frequency of aspirin resistance was determined in numerous studies and the results scattered in a wide range, 5 to 66%.

The term covers four different, although not unrelated definitions.

1.) Chemical ("true") aspirin resistance: platelet COX-1 Ser529 cannot be acetylated by aspirin.

2.) Laboratory resistance or preferably "non-responsiveness": 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.
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. Clinical ineffectiveness could be established only retrospectively and to evaluate the ineffectivity at a populational level statistical analysis is required.
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. Ischemic vascular events may occur not only on the basis of platelet thrombus formation, e.g. by embolic mechanism, hyalinic or microatheromic occlusion, aortic dissection or vasculitis, when the clinical effectiveness of aspirin is less likely.

Routinely used laboratory tests show high inter-, and intra-individual variability and there is 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. Furthermore, reference methods are suitable for the clear-cut decision of a problem and for the evaluation of routinely used laboratory methods. Surprisingly, no laboratory test that directly detects chemical resistance, i.e., the acetylation and the lack of acetylation of COX-1, has been described, but this would be absolutely necessary to determine the frequency of chemical ("true") aspirin resistance and to evaluate the reliability of routinely used

laboratory methods.

THE AIM OF THE STUDY

The aim of this study was to develop methods which detect the acetylation of platelet COX-1 with high certainty and using these methods as reference methods to establish the frequency of true aspirin resistance and to test the reliability of routinely used laboratory methods for the detection of aspirin effect. More specifically:

1. To develop monoclonal antibodies specific to acetylated COX-1 (acCOX-1) or to non-acetylated COX-1 (nacCOX-1) and using these monoclonal antibodies to develop a method which directly detects the presence or the lack of platelet COX-1 acetylation.

2. To develop a method which measures AA-induced production of TXB_2, the inactive metabolite of TXA_2, in platelet rich plasma. This method determines the capability of platelets to form TXA_2 from AA, and the results are to be considered as an indirect measure of COX-1 acetylation.

3. Using the aforementioned methods to establish the frequency of "true" aspirin resistance among healthy individuals.

4. Comparing the results obtained with the reference methods and the routine laboratory methods used for the detection of aspirin effect, to identify the tests that reliably measure acetylation (inactivation) of platelet COX-1 by aspirin.

MATERIALS AND METHODS

1. Study participants, inclusion and exclusion criteria

Healthy unrelated volunteers older than 18 years of age from eastern Hungary were recruited for the study. Their health status was checked by physical examination and a questionnaire.

Exclusion criteria at recruitment were contraindications of aspirin treatment (e.g., known aspirin intolerance, increased risk of bleeding, pregnancy, nursing), chronic disease, known platelet defect, and any medication in the preceding 2 weeks, with the exception of oral contraceptives. During aspirin treatment exclusion criteria were any side effect of aspirin therapy, the ingestion of any other drug, or absence at follow-up blood drawing. Six individuals out of 121 recruited volunteers were excluded on the basis of non-steroid anti-inflammatory drug ingestion in the preceding 2 weeks or during the study. In the case of three participants mild platelet function disorder was diagnosed. Further three participants did not show up at the second blood sampling. 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 strictly controlled. The results were evaluated on the remaining study population (n = 108) of healthy volunteers.

Study protocol was approved by the Regional and Institutional Ethics Committee of the University of Debrecen and written informed consent was obtained from all participants.

2. 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, 24 hours and 168 hours after the first dose of aspirin (day 0, day 1 and day 7 samples). 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). Platelet count of whole blood and PRP samples was determined with an automatic hematology analyzer (Sysmex KX-21N, Kobe, Japan).

3. Generation of monoclonal antibodies against acetylated or non-acetylated form of COX-1

Nonapeptides corresponding to the amino acid sequence of human COX-1 525–533 residues were synthesized both as unmodified peptide (H-Gly-Ala-Pro-Phe-Ser-Leu-Lys-Gly-Leu-OH) and as acetylated at Ser 529 (H-Gly-Ala-Pro-Phe-Ser(Ac)-Leu-Lys-Gly-Leu-OH). Peptides used for conjugation also possessed an N-terminal Cys residue and they were covalently linked to keyhole limpet hemocyanine (KLH) or to bovine serum albumin (BSA) through 3-maleimido-propionyl group (Bachem, Bubendorf, Switzerland). 50 μg of KLH-conjugate, emulsified in complete Freund's adjuvant, was first injected subcutaneously into Balb/c mice, then, after two weeks, 50 μg KLH-conjugate adsorbed to aluminium hydroxide gel was

administered intraperitoneally 3-times and BSA-conjugate on aluminium hydroxide gel 2-times at two-week intervals.

The following indirect ELISA using microplates coated with either of the unconjugated peptides, was used to detect peptide specific responses:

1/ coating the surface of microplates with one of the unconjugated peptides (50 μL of 2 μg/ml peptide in 0.1 M pH 9.6 sodium carbonate buffer, incubation at +4 °C for 20 hours).

2/ blockage of the free surfaces with incubation for one hour with 100 μL dilution buffer (phosphate-buffered saline or PBS containing 0.5 % human serum albumin, 0.5 M NaCl and 0.01 M pH 7.2 phosphate buffer containing 0.05% Tween 20).

3/ addition of 50 μL antibody containing sample (diluted mouse serum or undiluted hybridoma supernatant), incubation for one hour at room temperature.

4/ addition of 50 μL HRPO-marked goat anti-mouse IgG antibody (Southern Biotechnology Associates Inc, Birmingham, USA) and incubation for one hour at room temperature.

5/ addition of 50 μL 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma Aldrich, St. Louis, MO, USA) and incubation for 30 minutes at room temperature.

Each of the above steps was followed by extensive washing.

6/ addition of 50 μL 2 M H_2SO_4.

7/ spectrophotometric detection at 450 nm.

Spleen cells were fused with Sp-2/o myeloma cells. Hybridomas producing antibodies that reacted with the non-acetylated peptide and gave no reaction with the non-acetylated peptide were selected. These antibodies reacted also with COX-1 in the lysate of platelets from non-treated volunteers. Reaction with platelet lysate was examined by Western blotting.

During the other selection process, hybridomas producing antibodies that reacted with acetylated peptide and and gave no reaction with the non-acetylated peptide were selected and cloned. In contrast, these antibodies reacted only with COX-1 in the lysate of platelets from aspirin-treated individuals. The selected hybridomas were cloned by limiting dilution method. Antibodies were purified from ascites fluid by Protein G affinity chromatography. This way 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).

All samples gave reaction with a rabbit polyclonal anti-COX-1 antibody (Rb pAb ACOX-1, Abcam, Cambridge, UK) in goat anti-rabbit IgG-HRPO (Jackson ImmunoResearch Laboratories, Inc. Baltimore Pike, USA) system. The polyclonal antibody did not discriminate between the acetylated and non-acetylated forms.

4. Western blotting of platelet lysate for acetylated and non-acetylated COX-1

Washed platelet suspension (1000 x 10^9/L) prepared from platelet rich plasma was solubilized in non-reducing SDS PAGE sample buffer by sonication at 4 °C for 3x10 sec. After centrifugation the protein content of platelet lysate supernatant was measured by BCA protein assay (Thermo Scientific, Rockford, IL). The samples were reduced by 5% mercaptoethanol and incubated in boiling water for 5 min, then subjected to SDS PAGE and analyzed by Western blotting. The blots were incubated with the monoclonal anti-nacCOX-1 or anti-acCOX-1 antibody. The immune reaction was developed by biotinylated anti-mouse IgG followed by avidin-biotinylated peroxidase complex (Vectastain ABC kit, Vector, Burlingame, CA, USA) and visualized by enhanced chemiluminescence detection (ECL Plus+,

Amersham, Little Chalfont, UK). These Western blots were also scanned by GS 800 quantitative densitometer (Bio-Rad, Hercules CA).

5. Modified TXB_2 assay for the measurement of AA-induced TXB_2 production of platelets

The platelet count of PRP samples was installed to 30 x 10^9/L by dilution with PDP. TXB_2 production was induced by the addition of 12.5 µL 5 mg/mL AA (Helena, Gateshead, UK) to 237.5 µL diluted PRP. After incubation for 5 min at 37 °C, the reaction was terminated by the addition of 12.5 µL 2 mol/L HCl. The sample was kept at 4 °C for 15 min, and then centrifuged at 12000 g (4 °C, 10 min).

Although AA cross-reacted with the antibody used in the commercial TXB_2 assay to a minimal extent (0.1%), at concentrations in a 10^4-fold molar excess to the formed TXB_2 it interfered with the assay. TXB_2 in the supernatant was separated from interfering materials, most importantly from AA, by sequential solid phase extraction on Discovery C18 columns (Sigma Aldrich, St. Louis, MO).

After application of 125 µL TXB_2 containing supernatant onto a C18 column, it was washed sequentially by 2 x 1 mL HPLC grade water, 15% ethanol, acetone:hexane 1.5:8.5 and hexane. TXB_2 was eluted in 2 ml ethyl acetate. 200 µL aliquots were dried under nitrogen and re-dissolved in 500 µL assay buffer provided as a component of the reagent kit. If the TXB_2 concentration was determined at a later date, the eluates were stored at -70°C.

TXB_2 concentration was measured by a competitive immunoassay kit (Assay Designs, Ann Arbor, MI) in 96-well goat anti-rabbit IgG covered microtiter plate according to the manufacturer's instructions. Platelet TXB_2

production was expressed as pg $TXB_2/10^6$ platelets. At 13363 pg/mL TXB_2, a value in the range of TXB_2 production by AA-induced platelets from non-treated individuals, the coefficient of variation (CV) of the method was 6.9%. At very low concentration (888 pg/mL), in the range produced by platelets obtained on day 7 of aspirin treatment, the CV was 11.7%. The recovery of added TXB_2 and transferred through the whole process showed a good reproducibility around 75%. The recovery of added TXB_2 for the samples with high endogen TXB_2 concentrations was 74.1% and 78.2% for the samples with low endogen TXB_2 concentrations.

6. The assessment of platelet function with routine laboratory tests

Collagen/epinephrine (CEPI) cartridges were used for PFA-100 closure time measurements. In the case of this point-of-care test whole blood anticoagulated with citrate is added into a disposable cartridge. The sample is aspirated continuously with the help of vacuum through a capillary and rezervoar and at last through a microscopic aperture cut into a membrane coated with platelet agonists (collagen and epinephrin or collagen and ADP). The activated platelets attach to the aperture of the membrane, aggregate forming a platelet plug and close the aperture. A vacuum sensor detects the closure of the aperture and measures this closure time in seconds.

VN Aspirin Assay was performed according to the manufacturer's instructions. This point-of-care test uses whole blood anticoagulated with citrate and AA as agonist to activate platelets. The produced TXA_2 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 TXA_2 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.

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.

7. Statistical analysis

SPSS version 16 (SPSS Inc., Chicago, Illinois) was used for statistical analyses.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, day 1 and day 7 values. Correlation analysis was performed by Spearman test. Student t test was used to analyze differences in variables between subgroups of individuals.

RESULTS

1. Characteristics of study population

After exclusions 108 participants (60 females and 48 males) remained eligible for analysis. The mean age of participants was 33.5 years (SD 9.3, range 19–59). 69.4% of them had never smoked, 9.3% of them were ex-smokers, while 21.3% were current smokers. Pretreatment mean platelet count was 271.3 x 10^9/L (SD 62.5, range 138–429); only slight variation of individual platelet counts was observed between sampling days.

2. The effect of aspirin treatment on AA-induced TXB_2 production of platelets

Whereas AA-induced TXB_2 production scattered in a relatively wide range before aspirin treatment (day 0), platelets harvested at baseline produced a significant quantity of TXB_2. Although the difference between day 0 and day 1 values was statistically highly significant ($p < 0.0001$), 24 hours after the intake of a single enteric-coated aspirin tablet TXB_2 production did not decrease below the pretreatment range in 10% of the volunteers. Furthermore, in 65% of the cases day 1 TXB_2 production did not decrease into the day 7 range, i.e. a single dose of aspirin was not sufficient to reach maximal antiplatelet effect. Most importantly, aspirin treatment for seven days decreased TXB_2 production highly effectively into a very low narrow range, without exception, i.e. aspirin intake for seven days was effective in all individuals; not even partial aspirin resistance could be detected. The median of day 7 TXB_2 production was only 1.4% of day 0 values. Day 7 values showed no correlation with the extent of TXB_2

production on day0 ($r=-0.061$, $p=0.528$), i.e., independently of pretreatment platelet reactivity aspirin uniformly decreased the capacity of platelet to form TXB_2 to a very low level.

3. The effect of aspirin treatment on the acetylation of COX-1 in platelets

The effect of aspirin was also tested by direct detection of COX-1 acetylation. In this case the extent of COX-1 acetylation by aspirin was detected by monoclonal antibodies specific for COX-1 acetylated or non-acetylated on Ser529 residue. On day 0 there was no detectable reaction with the antibody against acCOX-1, in contrast, there was highly intensive reaction with the antibody, which reacted only with nacCOX-1. In the day 1 samples the band representing COX-1 was detectable by both antibodies, demonstrating that the acetylation of COX-1 was only partial. On day 7, in direct contrast to day 0, reaction with the antibody against acCOX-1 was highly intensive, while nacCOX-1 was not detectable. Practically identical results were obtained in the case of all individuals with the exception of one "non-compliance" case. The sensitivity of the Western blotting assay was also tested on different dilutions of the lysate of platelets obtained from untreated individuals. NacCOX-1 was still detectable in 1:40 dilution of platelet lysate, i.e. as low as 2.5% of nacCOX-1 could have been detected by the assay. As no trace of nacCOX-1 was detected on any of the day 7 samples, we did not elaborate this issue. In conclusion, in complete agreement with the results on AA-induced TXB_2 production, not even a single case of aspirin resistance could be detected by this method either. On day 1 the extent of reactions with anti-acCOX-1 and anti-nacCOX-1 was highly variable. The effect of aspirin was detected in all samples, but to a variable extent.

In a single case the antibody specific to nacCOX-1 gave reaction

with the lysate of platelets obtained on day 7 and there was no reaction with the antibody specific to acCOX-1. Respectively, TXB_2 production remained in the day 0 range. As non-compliance was suspected, this person was asked to enter a second period with drug administration strictly controlled. Full platelet COX-1 acetylation was observed on day 7 of the second period and TXB_2 production was highly decreased.

4. Assesment of the effect of aspirin with routine laboratory tests of platelet function

As the reference methods clearly distinguished between pretreatment and day 7 values and not even partial aspirin resistance could be detected, the results described above for the day 0 and day 7 platelets were suitable for the comparison with the results obtained using routine laboratory tests. If the results obtained on day 7 did not differ from the range of day 0, the given method gave false positive aspirin resistance.

4.1. Evaluation of PFA-100 CEPI closure times and the results obtained with the VN Aspirin Assay

Although the statistical analysis of PFA-100 CEPI closure times revealed significant difference between the sets of results measured on day 0 and day 7 ($p < 0.0001$), there was a considerable overlap and 38.9% of closure times of aspirin-treated individuals remained in the pretreatment "normal" range. If not compared to the individual pretreatment 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. Taking into account the outlier value, the overlap was 0.9%.

In the case of PFA-100 method pretreatment 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. In the case of VN Aspirin Assay no such correlation was observed, i.e., platelet reactivity prior to aspirin treatment, as in the case of AA induced platelet TXB_2 production, did not affect the effectivity of aspirin.

4.2. Evaluation of results obtained with agonist induced platelet aggregation and secretion

Using epinephrine as agonist, in 30% of the cases the low pretreatment 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. Even if the single low responder outlier with 39% transmission increase was eliminated, 94.4% of day 7 values overlapped the day 0 range. This result demonstrates that in the absence of pretreatment value from the same individual, ADP aggregation gives false positive aspirin resistance in a high number of cases. As the pretreatment values of ADP induced ATP release scattered in a wide range 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 and ATP release showed excellent distinctive power with no overlap between day 0 and day 7 ranges, so the test is highly suitable to detect the effect of aspirin.

The extent of ADP-, epinephrine- and collagen-induced platelet aggregation measured on day 7 significantly correlated with pretreatment values. In the case of AA-induced aggregation no such correlation was observed, similarly to AA induced TXB_2 production measured before and after 7 days of aspirin treatment.

DISCUSSION

We developed two reference methods which detect the acetylation of platelet COX-1 by aspirin with high certainty. An indirect method to assess platelet COX-1 acetylation was previously described which measures inactivation of COX-1 by aspirin 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. This rather laborious method was not validated and was not introduced in the routine laboratory practice. The methods developed in our laboratory utilizing monoclonal antibodies specific to acCOX-1 or nacCOX-1 demonstrated the state of COX-1 acetylation in platelet lysate. The antibodies were raised against a sequential epitope that is buried within the molecule, and they reacted with COX-1 only after treatment with SDS - an anionic detergent that denatures secondary and tertiary protein structures. For this reason Western blotting (SDS PAGE and immunoblotting) was used to detect the interaction of the antibodies with epitopes involving COX-1

Ser529. Besides reaction with the corresponding acetylated or not acetylated peptides, the specificity of the antibodies was demonstrated by the absence of reaction with anti-acCOX-1 in platelets of untreated individuals and by the absence of reaction with anti-nacCOX-1 in platelets from individuals treated with aspirin for seven days. To our knowledge this is the first method that detects the acetylation of COX-1 on Ser529 directly and suitable for the diagnosis of chemical aspirin resistance.

The other method aimed to measure TXA_2 production of platelets independently of in-vivo or in-vitro TXA_2 production by other cells, and avoid receptor mediated platelet activation that is influenced by factors other than the conversion of AA into TXA_2. For this purpose we used AA as agonist to generate TXB_2 in PRP diluted to a specified platelet count. This allows the determination of TXB_2 production by a single platelet and measures TXB_2 production and its inhibition independently from individual variations of platelet count. For our purpose we preferred this strategy to the determination of TXB_2 in serum or 11-dehydro-TXB_2 in urine. Although both methods might provide valuable information on the effect of aspirin, the former depends on thrombin generation during blood clotting, while the latter could be influenced by TXA_2 derived from sources other than platelets, and also by renal function. Furthermore, other potentially COX-2 containing blood cells (white blood cells) may influence the results. The method we developed determines the capability of platelets to form TXA_2 from AA, and the results are to be considered as an indirect measure of COX-1 acetylation.

Both methods are rather cumbersome and time-consuming and in their present form they are not aimed at routine determination of aspirin effect. However, they are well suitable to assess the extent of COX-1 acetylation by aspirin and establish the frequency of chemical (“true”) aspirin resistance among healthy individuals and also among patients. In

addition, they are suitable for the validation of other methods routinely used for the detection of “aspirin resistance”.

Both reference methods developed in our laboratory demonstrated unequivocally that among 108 healthy individuals there was not a single case of aspirin resistance. Our finding contradicts with the results of several previous investigations in the case of which for the determination of aspirin resistance frequency (5 to 66%) inadequate methods were used. In all volunteers participating in our study, AA-induced TXB_2 production of platelets uniformly decreased to a very low level. Practically full acetylation of platelet COX-1 was observed after seven days of daily low-dose aspirin intake, again in all volunteers. This also means that frequent genetic polymorphisms in the gene coding for COX-1 do not play a significant role in the responsiveness to aspirin, and the extent of pretreatment TXB_2 production ("platelet reactivity”) does not influence the extent of acetylation and the blockade of TXA_2 generation. The study was conducted on a group of healthy volunteers not taking any medication and the results suggest that in healthy individuals aspirin resistance - as defined by the lack of acetylation of COX-1 by aspirin - does not exist, or if it does, it must be a rarity. It is to be noted that in accordance with our results, serum TXA_2 level was also uniformly suppressed by aspirin in 47 healthy volunteers.

The effect of a single enteric-coated aspirin tablet varied considerably, which is probably due to individual differences in its rate of absorbance and metabolism. Such individual variability should be kept in mind when aspirin therapy is initiated.

A number of laboratory methods are used to detect the effect of aspirin on platelet function. This study compared the results of several routine platelet tests and the two reference methods with the goal of identifying tests that specifically measure acetylation (inactivation) of platelet COX-1.

A significant overlap between PFA-100 CEPI closure times of non-treated and treated patients has also been reported by other authors. We also tested the PFA-100 closure time with collagen/ADP cartridge, but the day 0 and day 7 results were fully overlapping. 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 only in some of the individuals. It is interesting that the closure times measured after aspirin treatment showed a highly significant correlation with the pretreatment closure times. This finding suggests that high pretreatment platelet reactivity considerably influences the results and could overcome the effect of aspirin in this assay. PFA-100 closure time is also influenced by von Willebrand factor, hematocrit value and platelet count. 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. In our study population, with a single exception (586 ARU), all pretreatment (day 0) values were above 600 ARU. 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, i.e., the pretreatment platelet reactivity did not influence the effect of aspirin.

As expected, AA that utilizes COX-1-dependent pathway for aggregation/secretion had a high power in distinguishing platelets with acCOX-1 and nacCOX-1. 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 of non-acetylated platelets did not influence the effect of aspirin.

Due to the low pretreatment aggregation and secretory responses to epinephrine in about a quarter of individuals, this agonist is hardly suitable for the detection of aspirin effect. 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, in absence of individual pretreatment values, excludes the correct evaluation of aspirin effect. Even if individual pretreatment values are available, the slight decrease in light transmission as a result of aspirin effect makes uncertain the assessment of COX-1 inhibition efficiency. 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. 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 pretreatment 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, pretreatment 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 TXA_2 production was blocked.

Taking into account that serum TXA_2 and urinary 11-dehydro-TXB_2 determination are not generally used in routine laboratory diagnostics, these methods were not included in the study for comparison. Previous results suggest that in platelets activated by thrombin formed in the course of the coagulation process, TXA_2 generation reflected by serum TXB_2 is a good indicator of the effect of aspirin. At the same time results of individuals not treated with aspirin depend on the rate of thrombin generation which varies individually and can be influenced by anticoagulant therapy. Furthermore,

other blood cells like COX-2 containing white blood cells may contribute to TXB_2 generation and this is not inhibited by preventive doses of aspirin. The contribution of white blood cells to serum TXB_2 levels is currently investigated in our laboratory.

The investigations detailed in the thesis proved the lack of "aspirin resistance" on a group of healthy volunteers. In the case of patients receiving preventive aspirin therapy further viewpoints have to be considered. 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 TXA_2 production by aspirin, although the significance of such a mechanism has been debated. Our preliminary studies on patient populations with cardiovascular disease suggest that the results are similar to those obtained on healthy volunteers. In our opinion, the main reasons for the lack of aspirin effect on COX-1 acetylation and platelet TXA_2 production is non-compliance, so the main indication for testing the effect of aspirin is the laboratory detection of non-compliance. In our study we confirmed that the new reference methods and the corresponding specific tests are suitable for the detection of non-compliance also. Further field of indication is the screening of interfering non-steroid anti-inflammatory drugs that prevent the access of aspirin to Ser529 in the active-site cavity of COX-1.

In summary, true aspirin resistance, if it exists, must be a rarity in the population. The main reasons for testing the effect of aspirin in the laboratory is the exclusion of non-compliance and the use of interfering non-steroid anti-inflammatory drugs. Not counting our reference methods, AA-induced platelet aggregation/secretion, VN Aspirin Assay and very likely also serum TXB_2 determination are the assays that can be used successfully for the detection of aspirin effect. PFA-100 CEPI closure time and aggregation/secretion induced by agonists other than AA do not seem to be

reliable for such a purpose. These assays, testing platelet functions that also involve COX-1-independent pathways, are not suitable to detect the effect of aspirin, but they might be useful in the detection of highly reactive platelets besides and also independently of acetylated COX-1. However, this possibility should be supported by large-scale clinical studies.

Recently, the international journal Thrombosis Research appreciated the published results in an editorial article.

SUMMARY

Low dose aspirin therapy is widely used in the prevention of acute atherothrombotic complications. It irreversibly acetylates the Ser529 residue in cyclooxygenase-1 and prevents thromboxane A_2 formation from arachidonic acid in platelets.

Laboratory methods currently used to detect this antiplatelet effect of aspirin provide variable results. Comparison with reference method(s) is required to validate these routinely used methods. As aspirin is ineffective in preventing acute vascular events in some patients, and some of the routine laboratory methods give inconsequent results the term "aspirin resistance" was introduced. This term has not been unequivocally defined creating difficulties in the interpretation of results and also in setting up recommendations for the treatment of patients.

We developed two novel reference methods that directly and indirectly assess the acetylation of platelet COX-1 by aspirin. The first direct method uses two monoclonal anti-human-cyclooxygenase-1 antibodies one of which only detects acetylated (inactivated) cyclooxygenase-1, while the other only reacts with active (non-acetylated) cyclooxygenase-1. The antibodies were raised against acetylated and non-acetylated nonapeptides corresponding to the amino acid sequence of human cyclooxygenase-1 525-

533 residues. Using Western blotting technique the antibodies clearly distinguished between acetylated and non-acetylated cyclooxygenase-1 in platelet lysate.

The second method measures the arachidonic acid-induced formation of thromboxane B_2, the inactive, stable metabolite of thromboxane A_2 in vitro in platelet rich plasma. This method determines the thromboxane B_2 producing capability of platelets and indirectly detects the inhibition of cyclooxygenase-1 by aspirin.

Using the aforementioned methods we analized the effect of aspirin on 108 healthy volunteers. The volunteers involved in the study received 100 mg enteric-coated aspirin daily for one week. Blood samples were collected before, 24 hours and 168 hours after the first dose of aspirin (day 0, day 1 and day 7 samples). In all samples obtained on day 7 following the initiation of aspirin treatment cyclooxygenase-1 in the platelets was fully acetylated whereas only non-acetylated cyclooxygenase-1 was present in the day 0 platelets. Further, thromboxane B_2 production by day 7 of platelets was completely blocked, i.e. none of the 108 healthy individuals was resistant to the effect of aspirin.

The results obtained with the reference methods on the healthy individuals were compared to the results of the following routine laboratory tests generally used for the determination of aspirin's effect: PFA-100 closure time with collagen/epinephrine cartridge, VerifyNow Aspirin Assay, platelet aggregation and ATP secretion using arachidonic acid, ADP, epinephrine and collagen as agonists.

Comparing the pretreatment and day 7 values with the results obtained with the reference methods, laboratory tests that use arachidonic acid as platelet agonist (arachidonic acid-induced platelet aggregation/secretion and VerifyNow Aspirin Assay) showed high discriminative power. In contrast, results obtained on day 7 and day 0 by the

other tests showed considerable overlap, i.e., they demonstrated false positive aspirin resistance in a part of the cases.

Our results obtained with the newly developed reference methods prove that chemical ("true") aspirin resistance, if it exists, must be a rarity among healthy individuals. In our opinion, only assays that use arachidonic acid as agonist are useful for establishing the antiplatelet effect of aspirin.

Register Number: DEENKÉTK/392/2013.
Item Number:
Subject: Ph.D. List of Publications

Candidate: Emese Gyöngyvér Kovács
Neptun ID: BMUED0
Doctoral School: Kálmán Laki Doctoral School
Mtmt ID: 10037210

List of publications related to the dissertation

1. **Kovács, E.G.**, Katona, É., Bereczky, Z., Homoródi, N., Balogh, L., Tóth, E., Péterfy, H., Kiss, R.G., Édes, I., Muszbek, L.: Evaluation of laboratory methods routinely used to detect the effect of aspirin against new reference methods.
Thromb. Res. Epub ahead of print (2013)
DOI: http://dx.doi.org/10.1016/j.thromres.2013.10.008
IF:3.133 (2012)

2. **Kovács, E.G.**, Katona, É., Bereczky, Z., Homoródi, N., Balogh, L., Tóth, E., Péterfy, H., Kiss, R.G., Édes, I., Muszbek, L.: New direct and indirect methods for the detection of cyclooxygenase 1 acetylation by aspirin; the lack of aspirin resistance among healthy individuals.
Thromb. Res. 131 (4), 320-324, 2013.
DOI: http://dx.doi.org/10.1016/j.thromres.2013.01.033
IF:3.133 (2012)

Total IF of journals (all publications): 6.266
Total IF of journals (publications related to the dissertation): 6.266

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

03 December, 2013