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

Effect of Aspirin in patients with coronary artery disease

by Nóra Homoródi, MD

Supervisor: Prof. László Muszbek, MD, MHAS

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By: Nóra Homoródi MD

Supervisor: Prof. László Muszbek MD, MHAS

Kali Kálmán Doctoral School University of Debrecen

Head of the **Examination Committee**: Prof. Pál Soltész MD, PhD, DSc

Members of the Examination Committee: Prof.László Rudas MD, PhD, DSc

Zsolt Oláh MD, PhD

The Examination takes place at the Library of Bldg. C, Department of Internal Medicine, Faculty of Medicine, University of Debrecen

at 11.00 a.m, on 27-th of June, 2019.

Head of the **Defense Committee:** Prof. Pál Soltész MD, PhD, DSc

Reviewers: Ágnes Nagy MD, PhD

István Lőrincz MD, PhD

Members of the Defense Committee: Prof.László Rudas MD, PhD, DSc

Zsolt Oláh MD, PhD

The PhD Defense takes place at the Lecture Hall of Auguszta Building, Faculty of Medicine, University of Debrecen at 13:00 p.m., on 27-th of June, 2019.

1. INTRODUCTION AND REWIEW OF LITERATURE:

1.1 The history of Aspirin

The Aspirin is one of the most widely used and the oldest medicine. Willow bark, containing salicil acid, has been used as a traditional medicine for more than 3500 years, since its' analgetic and antipyretic effect was even mentioned in the Ebers Papyrus dated back to around 1500 BC. Hyppocrates used willow bark for pain relief in 2000 years ago, altough the active ingredient was not known until the 18-th century. The active ingredient was isolated by Felix Buchner in 1828, and named it Salicin. Later, in 1829, the process was further refined by Leroux in France, and in 1838 Piria produced a stronger compound from crystals isolated from willow bark, wich he named salicylic acid. In 1853 Charles Frederic Gerhardt firstly syntethised the acetyl salycil acid by linking acetyl group and Na salycilate. The acetyl salycil acid (ASA), named Aspirin as a drug, was developed by Felix Hoffman, Arthur Eichengrün and Heinrich Dresser in a laboratory of Bayer Company in 1897. At the beginning it was known as a „magic potion" for all diseases, and its' anti-pyretic and anti-inflammatory property became evident during the 20-th century. In 1971 Sir John Vane demonstrated the antitrombotic effect of Aspirin by inhibiting the cyclooxigenase-1 (COX-1) and hereby the synthesis of tromboxan-A_2 (TXA_2).

1.2 The effect of Aspirin

The main pharmacological effect of Aspirin is acetylating the Ser529 residue of COX-1, wich covalent modification prevents the access of arachidonic acid (AA) to the active site of COX-1. In platelets, activated by agonists, intracellular Ca release induced, wich leads to phospholipase, eminently phospholipase A_2 (PLA_2) activation. Arachidonic acid (AA) released from membrane phospholipids by PLA_2 is transformed by COX-1 to cyclic endoperoxidase, prostaglandine G2 and H2. Further on tromboxan synthase result tromboxane A_2 production (from prostaglandin H_2), a powerful platelet activating prostaglandin derivate. COX-1 is a

constitutive ensyme expressed in the tissues, and the active metabolites transformed from prostaglandin H2 by prostaglandin and or prostaciclin synthase plays role in several physiologic processes. The other isoform, cyclooxigenase 2 (COX-2) is an inductive ensyme, its expression induced in pathologic situtations (inflammation), producing prostaglandin tipes playing role in pain and fever. The amino acid sequence of COX isoensymes shows 60% identity, although the affinity of Aspirin to COX-1 is much stronger. To achieve the same degree of acetylation of COX-2 ensymes et Ser 516 site 10 to 100 fold higher acetylsalycil acid concentrations are needed. 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. This is the reason for the fact, that 75-100 mg Aspirin inhibits the COX-1 in the platelets fully, but for antipyretic and analgetic effect much higher doses are necessary. Aspirin has a half-life of 15-20 minutes in plasma, but its effect on the platelets persist during its lifetime, ie 7 to 10 days. It can explained by the fact, that the platelets lack genomic DNA, only contain some megakaryocyte derived messenger RNA and some translational machinery, wich allowes limited protein synthesis, thereby platelets are not able to resynhetize the covalently modified COX-1.

ASA is rapidly absorbed from the stomach and the upper part of small intestine. The uncoated type ASA can be detected in the plasma 20 minutes after the ingestion, and its level peaks 30 to 40 minutes after ingestion, while the enteric coated preparations reach their peak level 3 to 4 hours after administration. The systemic bioavailabilty of regular Aspirin is 40 to 50 percent, wich is independent of the efficiency of COX-1 inhibition, since the ASA firstly contant the platelets in the portal circulation, where the ASA concentration is much higher than in the systemic circulation. During chronic ASA therapy the enteric coated and plain preparations have the same effectivity on the platelet TXA2 production and inhibition of platelet aggregation.

1.3. Cardiovascular effect of Aspirin and its importance

The cardioprotective effect of Aspirin became evident at the middle of the last century. Since that recognition the low dose Aspirin has been effectivelly used in the prevention of acute atherothrombotic complications, like acut myocardial infarction and atherothrombotic ischemic stroke. It prevents vascular death and nonfatal vascular events by approximately 15 and 30%, respectivelly. Clinical trials involving high number of patients prove that small dose Aspirin therapy (75-100 mg/die) is the "Gold standard" in secundary prevention of cardio-and cerebrovascular complications. For anti-inflammatory, anti-piretic and analgetic effect much higher doses are needed.

1.4. The definition of Aspirin resistance

In certain patients low dose Aspirin is not effetive in preventing acute atherothrombotic complications and for this reason the term "aspirin resistance" was introduced. The definition of Aspirin resistance is not standardised, there is still no correct and clear definition, and probably this is the reason for the fact that numerous previous studies were determined to detect the frequency of Aspirin resistance, and the results scattered wide range, 5 to 66%.

The term covers four different, but not unrelated defintions:

1. Chemical ("true") Aspirin resistance: the inbalility of Aspirin to acetylate Ser259 residue in platelet COX-1.

2. Laboratory resistance, or "non-responsiveness": diminished response to Aspirin as measured by a laboratory test.

3. In some instances the high platelet turnover results accelerated production and release into circulation of higher amount of newly formed and yet not acetylated platelets , and in these platelets the production of TXA2 is not inhibited. In this situtation we can not define Aspirin

resistance, since the Aspirin has the acetylating effect, and increased (Twice a day) dosage overcomes the diminished response to aspirin.

4. Clinical ineffectiveness: the Aspirin does not protect the patient form acut vascular event.

1.5. The Aspirin resistance „problem"

1. So far there was no available reference method wich directly measured the COX-1 acetylation by Aspirin, i.e. wich can diagnose the true Aspirin resistance. Previously we developed a new technique based on two monoclonal antibodies raised against the acetylated and non-acetylated nonapeptide that corresponded to human COX-1 525-533 residues. Using the two purified antibodies nacCOX-1 and acCOX-1 could be clearly distinguished in the lysate of washed platelets by Western blotting technique. This is the first method, wich can directly detect the acetylation of COX-1 Ser529 and valid to detect true (chemical) Aspirin resistance. Besides the direct method we developed another technique, wich detect the arachidonic acid induced TXB2 generation in platelet rich plasma. This method measures the TXB2 producing capacity of platelets and indirectly detect the inhibition of COX-1 by Aspirin, i.e.the inhibition of COX-1 acetylation. These two methods can be considered as a reference techique. In the laboratory diagnostics the reference technique is usually a relativelly sophisticated method, wich is suitable for the clear-cut decision of a problem. The reference method is hardly used in everyday practice, but able to evaluate the routinelly used techniques.

Using our former developed reference methods no real Aspirin resistance was found (the platelet COX-1 was fully acetylated) among 108 healthy volunteers taking 100 mg enteric coated ASA per day, so in heatlhy population Aspirin resistance, if it exists at all, extremly rare. However it does not mean that our findings are suitable for patienst suffering from acut atherothrombotic events, like coronary artery diseases.

2. Routinelly used laboratory tests deceting the efficacy of Aspirin show show high inter-and intraindividual variability, and there is a lack of correllation between the results. Also

no cut off results are accepted., and so far there was no accepted reference technique. Using the above mentioned two reference methods among Aspirin taking healthy volunteers we demonstrated that between the routinely used test for the detection of Aspirin effect only the detection of AA induced platelets activation (AA induced platelet aggregation, ATP release and the VerifyNow Aspirin Assay also based on AA induction) were proven to be reliable tests.

3. High platelet turnover, thought might be of clinical importance, should not be considered as Aspirin resistance. Several conditions can result high platelet turnover, with production of abnormal megacaryocytes (essential thrombochytaemia) or increassed peripherial consumption (inflammatory conditions). In these settings the acetlyation of COX-1 is not diminished, and the COX-1 in the newly formed platelets can be inhibited by elevated daily doseges. In case of essential thrombocythaemia twice a day administered 100 mg Aspirin was more effective than 1x100 mg or 1x200 mg daily Aspirin.

4. The platelet activation is a complex mechanism, and the TXA2 production is only a part of it. Platelet activation induced by high dose collagen or trombin is not inhibited by Aspirin. These mechanism have important role in acut atherothrombotic events so the Aspirin's 100 percent efficacy is not possible. The acut atherothrombotic events occuring on prophylactic Aspirin therapy should not call "aspirin resistance", should use "clinical ineffectiveness" term instead.

In patients with coronary artery disease (CAD) the situation might be different from that observed in healthy volunteers, since there could be conditions related to disease wich might influence the effect of Aspirin. As mentioned above the higher turnover of platelets, and the expression/up-regulation of COX-2 have been claimed to contribute to impaired Aspirin effect in diabetic patients and in inflammatory conditions.

The question is still occur, in these conditions real Aspirin resistance exist at all?

1.6. The clopidogrel and its effect

The other cornerstone in antihrombotic treatment is Hungary besides Aspirin is clopidogrel. The clopidogrel belongs to thienopyridine group, and irreverzibily inhibits the ADP P2Y12 receptor on the surface of the platelets. There are two different ADP receptor on the surface of the platelets. The P2Y1 and the P2Y12 receptor join to different intracellular G proteins mediating further different responses. ADP inhibits the cyclic adenosin monophophat (cAMP) production and the phosphatidil inositol trisphosphat (PIK3) pathway by coupling to Gi2 protein through P2Y2 receptor. Coupling to Gq protein through P2Y1 receptor activates the phospholipase C (PLCβ) pathway.

Clopidogrel is a second generation drug, a pro-drug, the active metabolite formes by the liver in a two-step process. Finally only the 15 % of clopidogrel presents in the circulation. The active metabolite compbound an SH group and covalently binds by a disulphid bridge to the P2Y12 receptor, thereby inhibits the ADP binding to its receptor and the ADP induced amplification of platelets activation. The clopidogrel monotherapy and in combination with Aspirin is widely used to prevent recurrent atherothrombotic ischemic events. The antithrombotic effect of clopidogrel does not manifest in the 10-to 50% of patients, wich has been primarily attributed to the variability in active metabolite generation due to the genetic polymorphisms of CYP isoenzymes. The relativelly high number of clopidogrel resistant patients lead to the development of the new generation oral P2Y12 inhibitors representing better metabolic profile such as prsaugrel and ticaglerol.

1.7. The resistance of clopidogrel

In case of clopidogrel therapy the problem also exists, wich method is valid for detecting clopidogrel resistance. Among the routinelly used severall laboratory methods ADP induced aggregation test is the most widely used and often considered as “gold standard” for monitoring

the clopidogrel effect. The one major drawback of this method is that is not specific to P2Y12 receptor, and Aspirin therapy influences its effect nhibits this test. The modified test A módosított PGE1 jelenlétében ADP indukálta aggregáció vizsgálata ezt a problémát kiküszöböli. The other methods wich are specific for P2Y12 receptor inhibiton such as h as the flow cytometric assay of vasodilator stimulated phosphoprotein (VASP) phophorylation and the VerifyNow P2Y12 tests are relatively expensive and require special instrumentation. The VASP is an actin- and profilin-binding protein that is expressed in platelets at high levels and plays a major role in negatively regulating secretory and adhesive events in these cells. VASP is a major substrate for cAMP- and cGMP-regulated protein kinases and it has been shown to be directly phosphorylated on Ser157, Ser 239 and Thr 278 by PKC (protein kinase C). In normal circumstances VASP is not phosphorilated, the prostaglandin E1 (PGE1) enhancing the cAMP production activates the cAMP dependent kinase and make the VASP phosphorilated, wich is inhibited by ADP through the P2Y12 mediated pathways. In the presence of PGE1 and ADP, the extent of VASP phosphorylation is proportional to the inhibition of platelets by clopidogrel, and report the result as platelet reactivity index, PRI.

The VerifyNow P2Y12 assay is a whole blood point-of-care test, which measures the ADP-induced co-agglutination of platelet and fibrinogen-coated beads. The instrument measures the change in light transmittance due to the agglutination and reports results as P2Y12 reaction units (PRU).

Aspirin and clopidogrel inhibit separate targets; however, overlapping blocked intracellular pathways suggest that Aspirin might interfere with some laboratory test used for detecting the effect of clopidogrel and vice versa.

2. THE AIM OF THE STUDY:

1. Our aim was to investigate the chemical and laboratory effectiveness of Aspirin using the previously defined reference method as a “gold standard” on cardiovascular patients being on long-term prophylactic Aspirin monotherapy. In all cases we detected the acetlation of COX-1, the COX-1 dependent and other techniques used for detection of the Aspirin’s effect in everyday practice, and also investigated the COX-2 expression in the platelets.
2. Since there are many situations when the Aspirin and clopidogrel used together as a dual antiplatelet therapy we aimed to determine wich methods, used for testing the effect of clopidogrel is infulenced by Aspirin and wich methods used for the detection of Aspirin’s effect, are affected by clopidogrel.

3. MATERIALS AND METHODS:

3.1 Study participants, inclusion and exclusion criterias

3.1.a/ Patients enrolled in the study detecting Aspirin’s effect and healthy volunteers.

Patients who had undergone percutan coronary angioplasty or coronary artery bypass grafting surgery being on aspirin monotherapy for secondary prevention were enrolled in the study. The patients were regularly admitted at the outpatient service of the Department of Cardiology, University of Debrecen, Debrecen Hungary; study participants were enrolled within a 1.5-year period. They were taking 100 mg enteric-coated aspirin daily for at least 1 month before investigation. Exclusion criteria were: acute coronary syndrome or any other major clinical event or infection in the previous 3 months, malignant disorder, hyperthyroidism, autoimmune disease and chronic renal insufficiency, intake of antiplatelet drugs other than aspirin in the previous 2 weeks, known bleeding diathesis, anemia with

hemoglobin value below 100 g/L and platelet count below 100 G/L. Finally, 144 patients were eligible for being involved in the study. The Jeffrey method was used for sample size calculation as described by Brown et al.

Apparently healthy controls (n = 108) recruited for the study have been characterized in a previous publication. Healthy unrelated volunteers older than 18 years of age were recruited for the study. Their health status was checked by questionairre and physical examination. The exclusion criterias were: chronic disease, known platelet defect, any medication in the preceding two weeks except the oral contraceptives. The results were evaluated on the remaining 108 healthy volunteers from the 122 recruited volunteers after the exclusions.

3.1.b/ Study population receiving Aspirin and clopidogrel monotherapy

A priori exclusion criteria for all individuals enrolled in the study were: non-steroid anti-inflammatory drug therapy, chronic liver disease, hemoglobin concentration <80 g/l, platelet count>500 10^9 /l or<150 10^9 /l, qualitative defects of platelet function or other types of hemorrhagic diathesis, acute infectious disease/ antibiotic treatment, major surgical procedure or major ischemic event within one month of enrollment. When recruiting individuals for one arm of the study, our aim was to select patients on effective clopidogrel monotherapy and to use their blood samples to perform laboratory methods commonly used for the detection of aspirin's effect. In this case, the study population originally included 101 patients with the history of non-cardiogenic ischemic cerebrovascular disease and being on 75 mg/day clopidogrel monotherapy for at least one month (clopidogrel group). Forty-eight patients were excluded from this group as they failed to demonstrate the effect of clopidogrel as assessed by two P2Y12-receptor-specific assays (see later). For the other arm of the study, we recruited 55 patients with coronary artery disease being on 100 mg/day aspirin monotherapy for at least one month (aspirin group). In this case, three patients had to be

excluded due to admitted non-compliance. The remaining 52 patients showed good response to the drug after testing with two different COX-1-specific laboratory tests (see later). Their samples were used to perform laboratory methods commonly used for detecting the effect of clopidogrel. For comparison, 140 healthy individuals not taking any medication that influences platelet function (control group) were also recruited and their samples were tested for all platelet function assays used in the two patient groups.

All individuals enrolled in the study gave written informed consent prior to their inclusion in the study. Ethical approval was obtained from the Ethics Committee of the Medical Faculty, University of Debrecen, Hungary and the study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

3.2. Sample preparation:

Blood samples were collected by venipuncture from an antecubital vein into Vacutainer tubes containing 0.109 mol/L trisodium citrate (Becton– Dickinson, Franklin Lakes, NJ) after overnight fasting. Anticoagulated blood was directly used for the VerifyNow® (VN) Aspirin assay (Accumetrics, San Diego, CA) and for the vasodilator-stimulated phosphoprotein phosphorilation (VASP) flow cytometric assay. Platelet rich plasma (PRP) separated by centrifugation (120g, 37 °C, 15 min) was used for AA induced platelet aggregation tests. Platelet depleted plasma (PDP), was obtained by two consecutive centrifugations (1500g, 25 °C, 20 min).

3.3. Methods used for the detection of aspirin effect

In a previous study we developed new methods for the direct and indirect detection of COX-1 acetylation by aspirin. The first method is based on two monoclonal antibodies raised against

the acetylated and non-acetylated nonapeptide that corresponded to human COX-1 525-533 residues (H-Gly-Ala-Pro-Phe-Ser-Leu-Lys-GlyLeu-OH). Using the two purified antibodies nacCOX1 and acCOX-1 could be clearly distinguished in the lysate of washed platelets by Western blotting technique. The indirect method measures 0.25 mg/mL AAinduced TXB2 generation in PRP diluted to 30 × 109/L by PDP. The produced TXB2 was separated from AA and from other interfering substances by sequential solid phase extraction. In the final eluted sample TXB2 concentration was measured by competitive immunoassay (Assay Designs, Ann Arbor, MI) and it was expressed as pg TXB2 produced by 106 platelets. As among the generally used routine laboratory tests used for the detection of aspirin effect AA-induced platelet aggregation, ATP secretion and the VN Asprin assay were proven to be the most reliable tests, we also used these assays to detect the effect of aspirin on patients with CAD being on secondary prevention. 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 in platelet rich plasma was induced by 500 μg/ mL AA (Helena, Gateshead, UK) and was monitored in Chrono-Log 700 lumiaggregometer (Chrono-Log, Havertown, PA) for 8 min. Prior to the experiments the platelet count was adjusted to 260 × 109 platelet/L. Aggregation was expressed as the percentage of maximal change in light transmission (Δtransmission %). ATP secretion was quantitated by bioluminescence method using luciferin-luciferase reagent (Biothema AB, Handen, Sweden). Maximal ATP secretion was expressed as μmol ATP/1011 platelets.

3.4. Measurement of COX1 and COX2 mRNA:

COX1 and COX2 mRNA expression was determined in the platelets of 56 patients randomly selected out of the 144 subjects with CAD. RNA was isolated from leukocyte depleted platelets

as described earlier. The integrity of RNA samples was shown by determining the GAPDH 3′:5′ signal ratio, the absence of RNA contamination from white blood cells was proven by RT-qPCR analysis of each sample for CD15 and HLA-DQβ mRNAs. Reverse transcription and RT-qPCR reaction were carried out as described by Zsóri et al. Briefly, First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) was used for reverse transcription. The reaction solution was incubated at 42 °C for 60 min, and then at 94 °C for 5 min. Notemplate control and no-reverse transcriptase controls included in each run showed negative results. The absence of contaminating DNA was also demonstrated by melting curve analysis. RT-qPCR reactions were carried out on LightCycler 480 (Roche) in duplicates using SYBR Green I Master (Roche). PCRs were set up in a final volume of 20 μL consisting 10 μL Master Mix (2× concentration), 5 μL of cDNA template derived from reverse-transcribed RNA and 300 nM primers for COX1 and 400 nM primers for COX2. The primer sequences were tccatgttggtggac tatgg (forward), gtggtggtccatgttcctg (reverse) for COX1, and cttcacgcatcagtttttcaag (forward), tcaccgtaaatatgatttaagtccac (reverse) for COX2. ACTB, GNAS and HDGF have been established as the most stable reference genes for the normalization of platelet mRNA expression in coronary artery disease; all three were amplified and used in the calculations. The amplification program was: heating for 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C and 1 s at 72 °C. Melting curve analysis was performed between 66 and 95 °C in 0.11°C/s increments with 5 acquisitions/ °C. CT values, corresponding to the number of cycles at which the fluorescence signal exceeds a threshold value, were used for the relative gene quantification based on the method of Livak and Schmittgen. ΔCT,COX1 and ΔCT,COX2 were established against each of the three reference genes and the mean ΔΔCT,COX1,COX2 values were used for the calculation of COX1 mRNA:COX2 mRNA ratios.

3.5. Assays used for detecting the response to clopidogrel

Methods used for testing the effect of clopidogrel were performed on the aspirin group. These methods included: the flow cytometric analysis of VASP phosphorylation, ADP-induced platelet aggregation and a newly developed P2Y12 receptorspecific aggregation method in which ADP-induced aggregation is performed on prostaglandin E1 (PGE1)-treated platelets (ADP[PGE1] test). The VASP phosphorylation assay was performed according to the manufacturer's (Biocytex, Marseille, France) instructions within 24 h after blood sampling. The test is based on the fact that in the presence of PGE1 and ADP, the extent of VASP phosphorylation is proportional to the inhibition of platelets by clopidogrel. Results were expressed as platelet reactivity index (PRI). ADP-induced platelet aggregation and secretion in PRP were monitored using a Chrono-Log model 700 lumiaggregometer (Chrono-Log Corporation, Havertown, PA). Platelet count was set to 250 109 /l in PRP by the addition of PPP. All aggregation studies were performed within 4 h after blood sampling. Aggregation induced by 5 and 20 μM ADP (Helena Laboratories, Beaumont, TX) was monitored for 6 min. Luciferin–luciferase reagent (Biothema AB, Handen, Sweden) was added to each sample for the measurement of ATP release from platelet delta granules. Maximal increase in light transmission (Δtransmission %) and ATP release (μmol ATP/10^{11} platelets) were recorded for each sample. The ADP[PGE1] platelet aggregation test was carried out as described previously. In this assay conventional ADP-induced platelet aggregation is modified in order to obtain P2Y12 receptor-specific platelet aggregation. Briefly, in order to suppress the undesirable contribution of P2Y1 receptors PRP was pre-incubated with 0.31 μM PGE1 (Sigma-Aldrich, St. Louis, MO) for 3 min at 37 °C prior to platelet aggregation induced by 40 μM ADP. Results were expressed as Δtransmission %. For each method the reference interval corresponding to the 99% central interval was determined according to the C28-A3 guideline of Clinical Laboratory Standards Institute (CLSI; Wane, PA) using the samples of the control group

(n=140). The lowest value of the reference interval was used to decide which patients had to be excluded from the clopidogrel group. Those who were on clopidogrel therapy and had results of VASP phosphorylation and ADP[PGE1] tests above the lower limit of reference interval (72% PRI and 9.1% Δtransmission, respectively) were considered to demonstrate inadequate antiplatelet effect and were excluded from the study.

3.6. Assays used for detecting the effect of aspirin

Methods used for testing the effect of aspirin were AA-induced platelet aggregation and secretion, AA-induced generation of TXB2 in PRP and the VN Aspirin assay. Platelet aggregation and secretion were induced by 500 μg/ml (1.53 mM) AA (Helena, Gateshead, UK). Results were expressed as percentage of maximal change in light transmission and ATP secretion of platelets as described above. The measurement of AA-induced TXB2 production of platelets was performed from PRP samples as described earlier. 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 was measured by the competitive immunoassay kit of Assay Designs (Ann Arbor, MI); the results were expressed as pg TXB2/10^6 platelets. The VerifyNow Aspirin assay was carried out according to the manufacturer's (Accumetrics, San Diego, CA) instructions; results were expressed as Aspirin Reaction Units (ARU). For each method the reference interval corresponding to the 99% central interval was determined as described above. With the exception of the three patients excluded from the study due to non-compliance, all aspirin-treated patients' results were well below the lower limit of reference intervals, none of them demonstrated impaired response to aspirin.

3.7. Statistical analysis

Variables were expressed as mean and SD or median, interquartile range (IQR) and total range according to their distribution, established by the Kolmogorov–Smirnov test. Data obtained by different methods were correlated according to Spearman's rank correlation method. Differences between the patients and controls were analyzed using ANOVA/Kruskal–Wallis analysis depending on their distribution or Student's t-test/Mann–Whitney U-test whenever the two groups were compared. $p<0.05$ was considered as significant. Statistical analysis was performed using GraphPad Prism Software (La Jolla, CA).

4. RESULTS:

4.1.The acetylation of COX-1 by aspirin in patients with CAD

In the platelet lysate of 138 patients only acetylated COX1 was detected by Western blotting. Previously it has been shown that the method is highly sensitive and could detect as low as 2.5 % of the total platelet COX1 in non-acetylated form, i.e. more than 97.5 % of platelet COX-1 was acetylated as the consequence of long term aspirin treatment. It is to be noted that in platelets from these treated patients no nacCOX1 could be detected, which contradicts an earlier finding showing that only one monomer of the active dimeric enzyme is acetylated by aspirin. In the case of six patients different amounts of non-acetylated COX1 remained in the platelet lysate. As noncompliance was assumed, these patients were contacted and their attention was drawn to the danger of not taking or irregularly taking the drug. After an additional two weeks the tests were repeated and full effectiveness of aspirin was demonstrated by the absence of non-acetylated COX1 and the presence of acetylated COX1 in the platelet lysate . According to sample size calculations the lack of aspirin resistance in all of the 144 patients indicated that aspirin is effective in more than 98 % of patients with stable coronary artery disease (confidence

interval 95 %). It is to be noted that aspirin was fully effective in diabetic patients and also in smokers.

4.2.The effect of aspirin on arachidonic acid induced TXB2 production

Aspirin drastically decreased the TXB2 generation, the median TXB2 production was only 1.2 % of that measured in the control group. Here six patients also had higher TXB2 generation in the range of 43–835 pg TXB2/10^6 platelets, but at the second occasion their value returned to the range of the remaining 138 patients.

4.3.The effect of aspirin on arachidonic acid induced platelet activation

In accordance with the considerably decreased TXB2 production, the extent of AA induced aggregation of platelets from aspirin treated patients was uniformly very low and no overlap between controls and patients was observed. The median value of AA induced ATP release in PRP was 1.1 μmol ATP/10^{11} platelets (IQR: 0.9–1.4) in the control group, while in the PRP of all aspirin treated patients the amount of released ATP was below the limit of detection. In the case of VerifyNow Aspirin assay four ARU values from the patients group (2.8 %) overlapped with the total range of ARU values of the control group. For this assay the manufacturer recommends 550 ARU as the cut off value for aspirin effect, however according to the results of the control group a cut off value of 585 ARU seems to be more appropriate. The reason for the slight difference between the other assays and the VerifyNow Aspirin Assay, the latter also uses AA as agonist, is not clear. Maybe the detection system used in the VerifyNow Aspirin Assay (aggregation of fibrinogen coated beads) is responsible for the few outliers in the patient's group. It is to be noted that 24 % of the study group were diabetic patients, the CRP level was above 5 mg/L in 21 % of the patients and in spite of the clinical condition 10 % of

them was unable to quit smoking. In none of these patients was impaired aspirin effect measured by any of the methods used in the study.

4.4. The effect of aspirin on platelets with COX2 mRNA expression

Out of the 56 patients investigated for the expression of COX2 mRNA only in 22 cases was the amount above the limit of detection. Even in these cases the relative amount of COX2 mRNA was less than 0.4 % of COX1 mRNA and with the exception of three patients this value was less than 0.05 %. None of the patients expressing COX2 mRNA in platelets showed aspirin resistance.

4.5. The effect of clopidogrel therapy on the methods used for detecting the effect of aspirin

Of the methods used for detecting the effect of aspirin, clopidogrel monotherapy significantly ($p<0.001$) inhibited AA induced platelet aggregation and secretion. As compared to the control population, clopidogrel treatment reduced AA-induced platelet aggregation in 41.5% of the patients, AA-induced platelet secretion was even more markedly reduced in all the patients and 79.2% of them had a result below the lower limit of reference interval. Similar results were found in the case of AA-induced TXB2 production measured in the PRP of clopidogrel-treated patients. 81.1% of the patients on clopidogrel monotherapy demonstrated markedly reduced AAstimulated TXB2 production by platelets. Results of VASP phosphorylation assay (e.g. the extent of P2Y12 receptor inhibition by clopidogrel) and AA-induced platelet aggregation showed a fair correlation in patients on clopidogrel monotherapy (Spearman $r=0.49$, 95% CI 0.23–0.67, $p<0.001$). This finding further supports that pathways leading to AAinduced platelet aggregation are influenced by clopidogrel. Although clopidogrel clearly inhibited AA-induced platelet aggregation and secretion, the VerifyNow Aspirin test, also based on AA, was

practically uninfluenced by the drug. In this AA-induced agglutination based assay, the results of the controls (median: 657 ARU, IQR: 650–661 ARU) and those of the patients on clopidogrel monotherapy (median: 661 ARU, IQR: 652–666 ARU) were similar. Among patients on clopidogrel monotherapy only three outliers had results below 550 ARU, the cut-off for effective response to aspirin therapy.

4.6. The effect of aspirin therapy on the methods used for detecting the effect of clopidogrel

As it was expected, of the methods used for detecting the effect of clopidogrel, aspirin monotherapy significantly inhibited ADPinduced aggregation and secretion. Although the extent of inhibition by clopidogrel monotherapy was considerably higher than the inhibition exerted by aspirin monotherapy, the results clearly indicate that the effect of aspirin could influence results of ADP-induced platelet aggregation and obscure the effect of clopidogrel in patients on dual antiplatelet therapy. Aspirin as monotherapy did not have an effect on the ADP[PGE1] platelet aggregation test, where ADP aggregation is performed on PGE1-treated platelets. Although this method is also based on ADP-induced platelet aggregation, the main difference between the two methods is that the ADP[PGE1] test is specific to the P2Y12 receptor inhibition by clopidogrel. Another P2Y12 receptor specific assay, the VASP phosphorylation test was also unaffected by aspirin monotherapy. These results suggest that among the methods used for detecting the effect of clopidogrel, aspirin does not influence P2Y12 receptor specific ones.

5. DISCUSSION:

So far, the direct measurement of COX1 acetylation in healthy volunteers taking 100 mg aspirin has been carried out in two studies using different methodologies. Our former study measured nacCOX1 and acCOX1 by Western blotting technique using specific monoclonal antibodies, in another study platelet proteins were separated by nuPAGE then, in-gel enzymatic digestion was carried out and the COX1 peptide carrying the Ser529 residue was determined by mass spectrometry. One hundred and eight and twenty-four individuals were enrolled in the studies, respectively and in none of the cases was true aspirin resistance revealed. It is to be noted that using AA-induced platelet aggregation and serum TXB2 measurement Grosser et al. also failed to detect specific phenotype of true aspirin resistance among 400 healthy volunteers. As in patients with CAD the occurrence of aspirin resistance has not been explored by detecting the acetylation of COX1 in platelets, our method developed as part of basic research was translated to address this question in clinical setting. In this study none of the 144 patients enrolled in the study showed impaired acetylation of platelet COX1, TXB2 generation, AA-induced platelet aggregation and ATP release were profoundly inhibited by aspirin in all patients. Neither diabetes nor smoking influenced the effectiveness of aspirin and inflammatory condition shown by elevated CRP also failed to influence COX1 acetylation. This finding suggests that in patients with coronary artery disease, just like in healthy individuals aspirin resistance, if it exists at all, is a rarity. Several factors have been suggested to influence the effect of aspirin in pathological conditions. Increased platelet turnover was presumed to release newly formed platelets with nacCOX1 into the circulation and produce enough TXA2 to activate platelets, even platelets with acCOX1. This problem was thought particularly important in the case of enteric-coated aspirin administered in a single daily dose. However, important component of the inhibitory effect of aspirin occurs in the presystemic circulation. Furthermore, COX1 of megakaryocytes is acetylated by aspirin and platelets released from megakaryocytes contain

acCOX1. Furthermore, the finding of a persistent level of acCOX1 throughout a 7 days interval of treatment with once daily 100 mg enteric-coated aspirin provides direct evidence that acetylated platelets entered into the circulation during this time-frame. Our present finding demonstrating that patients on long-term aspirin contain more than 97.5 % COX1 in acetylated form also support the cumulative nature of platelet COX1 inhibition upon repeated daily dosing. The reports on the increased level of COX1 acetylation 24 h after the sixth daily dose of aspirin as compared to the level measured 24 h after a single aspirin dose also indicate cumulative saturable acetylation of platelet COX1. COX2, an isoenzyme of COX1, is also sensitive to the inhibitory effect of aspirin. However a much higher dose of aspirin, well above the range reached during the prophylactic low dose aspirin therapy, is needed to acetylate the respective serine residue (in this case Ser516). This enzyme is present in megakaryocytes, it is expressed in young platelets and its up-regulation has been described in diabetes mellitus and inflammatory conditions. It has been proposed that COX2 expression in platelets might result in the suppression of aspirin effect. However, Riondino et al. demonstrated on 100 patients being on chronic aspirin treatment by immunoblot analysis that COX2 could be detected only in 46 % of patients and its amount was markedly lower than that of COX1. By using COX2 inhibitor CAY10404 and aspirin they also demonstrated that COX2 dependent TXA2 production is less than 2 %. Our results are in line with the latter observations. We found detectable amount of platelet COX2 mRNA only in 39 % of patients with stable coronary artery disease. Even in those cases in which COX2 mRNA was detected, its amount was incomparably lower than that of COX1 mRNA and platelets from these patients produced a very low amount of TXB2 comparable to the rest of the aspirin treated patients. These findings do not support the role of COX2 expressed in platelets in the diminished response to low dose aspirin therapy. In the light of the above findings it is a question if aspirin therapy should be monitored at all. In theory, mutations in the COX1 gene may result in a situation in which aspirin is unable to

acetylate the enzyme, however such a situation has not been reported. The results suggest that the effect of aspirin does not need to be controlled in patients with coronary artery disease, unless non-compliance or drug interference is suspected. The main reason for the ineffectiveness of aspirin as detected by laboratory tests is non-compliance, testing of patients on aspirin therapy could provide information on their compliance. Non-steroid anti-inflammatory drugs may interfere with the effect of aspirin and insufficient aspirin effect measured by adequate laboratory tests could draw the attention to such drug interference. It is to be emphasized that for the above purposes only methods specific to the effect of COX1 acetylation should be used. Among the routinely used methods these include AA induced platelet aggregation and secretion, the VerifyNow Aspirin Assay and the measurement of serum TXB2. This statement is valid only for aspirin monotherapy, its combination with P2Y12 receptor inhibitors (dual antiplatelet therapy) might influence the test results.

The most popular P2Y12 receptor inhibitor thienoprydine drug is the clopidogrel. This is a second generation drug, and the active metabolite is formed by the liver in a multi step process. The active compbound binds covalently to the P2Y12 receptor by disulfid bridge. Different reduction of antithrombotic effect of clopidogrel occurs in some cases, wich has been primarily attributed to the variability in active metabolite generation due to the genetic polymorphisms of CYP isoenzymes. The fact of the clopidogrel resistance lead to the development of the new generation oral P2Y12 inhibitors representing better metabolic profile, such as prasugrel and ticagrelor]. Similarly to Aspirin in case of clopidogrel therapy the lack of the specific laboratory method to determine the clopidogrel resistance is still problem. Altough the most widely used, “standard” method is the ADP induced aggretion test, its not specific and the Aspirin medication influences the result.

When the patient is on dual antiplatelet therapy choosing the right, specific laboratory test for monitoring the effect of individual antiplatelet agent is a highly important issue. Due to the

overlapping intracellular pathways inhibited by these drugs, it is reasonable to assume that some laboratory tests, otherwise appropriate for monitoring single antiplatelet therapy, cannot be used for patients on dual antiplatelet therapy. In fact, a number of studies on clopidogrel resistance include patients on combined antiplatelet therapy (aspirin + clopidogrel) and some of them use laboratory methods, which are influenced by aspirin. It is to be emphasized that although ADP-induced platelet aggregation is often considered as ‘‘gold standard’’ for monitoring the effect of clopidogrel, aspirin inhibits this test and in dual antiplatelet therapy one can hardly establish to which extent were platelets inhibited by aspirin and by clopidogrel (or by another P2Y12 receptor inhibitor). In this study, we show that only those methods, which are specific to the P2Y12 receptor inhibition by clopidogrel, are not influenced by aspirin therapy and could be recommended for testing the effect of clopidogrel in patients on dual antiplatelet therapy. However, it should be noted that the effect of aspirin on the results of these tests in patients showing moderate or weak response to clopidogrel was not investigated in this study. Although AA-induced platelet function tests are generally thought to be specific to the effect of aspirin, here we provide proof that clopidogrel monotherapy inhibits AA-induced platelet aggregation. The inhibition of AA-induced platelet secretion and AA-induced TXB2 generation by clopidogrel was even more pronounced than the inhibition of platelet aggregation. These results could be explained by the elegant in vitro experiments demonstrating that TXA2 generation is potentiated by the P2Y12 receptor mediated signaling pathway. Blocking this potentiating effect by clopidogrel could result in diminished TXA2 formation and impaired platelet secretion observed in our study. Surprisingly, clopidogrel monotherapy failed to influence the VerifyNow Aspirin test. In the latter assay, AA-induced agglutination between the fibrinogen receptor of activated platelets and fibrinogen-coated beads is the predominant feature. The test depends only on the surface availability of fibrinogen receptor and not on subsequent events, like platelet secretion and aggregation. It might be that the diminished amount of TXA2 formed in platelets

from clopidogrel-treated patients is sufficient to bring about the inside-out signaling that leads to the transformation of GPIIb/IIIa into fibrinogen receptor, but insufficient to elicit full strength platelet secretion and aggregation. Obviously, this hypothesis should be confirmed by experimental evidence. In conclusion, when testing patients on dual antiplatelet therapy one should be aware that laboratory methods used for detecting the effect of aspirin or the effect of P2Y12 receptor inhibitors are not necessarily specific to the respective antiplatelet agent. Laboratory tests that are generally thought to measure the pharmacological effect of a certain antiplatelet drug could be affected by the other antiplatelet drug to a various extent due to overlapping intracellular pathways. According to our study, clopidogrel did not inhibit the VerifyNow Aspirin test significantly, which means that the latter test reflects the effect of aspirin even in case of dual antiplatelet therapy. When testing the effect of clopidogrel in patients on dual antiplatelet therapy, P2Y12 receptor specific methods not influenced by aspirin monotherapy (e.g. VASP phosphorylation assay, ADP[PGE1] test), are recommended.

SUMMARY

Salicylic acid derivatives have been known for more than 3 500 years, and belong to the oldest medicines, but their effectiveness in inhibiting platelet aggregation inhibition became evident 50 years ago. Clinical trials involving high number of patients prove that small dose Aspirin effectively decreases the acute complications of atherosclerotic diseases. At the same time it also became evident that Aspirin is not always protective against such events, which led to the concept of Aspirin resistance. The thienopyridine group represents the other cornerstone for antithrombotic treatment by irreversibly inhibiting the P2Y12 ADP receptors on the surface of the platelets. Clopidogrel is the most frequently used drug for such a purpose.

Our aim was to investigate the chemical and laboratory effectiveness of Aspirin using the previously defined reference methods on cardiovascular patients being on long-term prophylactic Aspirin monotherapy. Another aim was to determine which methods, used for testing the effect of clopidogrel, are influenced by Aspirin and which methods used for the detection of Aspirin's effect are affected by clopidogrel in patients with double antiplatelet therapy.

Platelet COX-1 became fully acetylated by Aspirin, along with drastic decrease of TXB2 synthesis. Consequently, arachidonic acid induced platelet aggregation was uniformly low. Part of the patients expressed COX-2 mRNS, but no Aspirin resistance could be proven in this patient group, either. Clopidogrel monotherapy significantly inhibited arachidonic acid induced platelet aggregation and secretion, along with significant decrease in TXB2 production. The vasodilator stimulated phosphoprotein (VASP) phosphorylation assay and the

arachidonic acid induced platelet aggregation showed good correlation, whereas clopidogrel monotherapy had no effect on the result of the VerifyNow test. Aspirin monotherapy significantly inhibited the ADP induced platelet aggregation and secretion, but had no effect on the VASP phosphorylation assay results and on the ADP aggregation tests of prostaglandin E pretreated platelets.

Our results clearly suggest that Aspirin resistance if it exist at all, is extreme rare. It was observed that arachidonic acid induced platelet aggregation and secretion tests, that specifically demonstrate the effect of Aspirin monotherapy, were influenced by the effect of clopidogrel. Clopidogrel therapy did not influence the results of VerifyNow Assay, i.e. it shows the effect of Aspirin even in the case of dual antiplatelet therapy. P2Y12 specific tests (e.g. VASP phosphorylation or ADP aggregation in the presence of PGE1) are recommended for testing the effect of clopidogrel in patients on dual antiplatelet therapy, since the results of these tests are not influenced by Aspirin.

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DEBRECENI EGYETEM
EGYETEMI ÉS NEMZETI KÖNYVTÁR
H-4002 Debrecen, Egyetem tér 1, Pf.: 400
Tel.: 52/410-443, e-mail: publikaciok@lib.unideb.hu

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