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CLINICAL STUDY

Biochemical evaluation of the antiplatelet effect of aspirin in patients at different levels of cardiovascular risk

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Abstract: *Background:* The phenomenon called aspirin resistance is being intensively discussed.
Methods: To evaluate the biochemical aspirin response, the method of urinary 11-dehydro TXB2 levels measurement was used. Quantitative detection of TXB2 in urine was determined by competitive enzyme immunoassay, using human Thromboxane B2 ELISA-kit. We investigated the urine samples from 69 patients.
Results: The mean urinary levels of 11-dehydro TXB2 were significantly lower in patients in the primary and secondary types of aspirin prevention comparing with the control group of patients not taking aspirin. The difference in thromboxane concentrations between the two groups of patients taking aspirin did not reach statistical significance. Our results did not show significant differences in the biochemically measured aspirin response when comparing diabetics with non-diabetics. Similarly, the observed tendency to higher thromboxane levels in women did not show to be significantly different from men.
Conclusion: Our pilot study did not show any significant differences among patients at different cardiovascular risk. Since there is currently no standard laboratory method to detect aspirin non-responders available, the term aspirin resistance remains controversial and requires further research. Every effort should be done to improve patients' compliance and to prevent clinically relevant interactions of aspirin with ibuprofen. The elimination of these two factors as was the case in our study may provide better efficacy of the antithrombotic prevention by aspirin *(Fig. 2, Tab. 4, Ref. 19).* Full Text (Free, PDF) *www.bmj.sk.*
Key words: aspirin resistance, urinary 11-dehydro TXB2, ELISA, aspirin non-responders.

Cardiovascular (CV) diseases account for the majority of death rates in the developed countries. Despite apparent medical advance, the incidence of acute atherothrombotic complications in high-risk patients is still high. Aspirin (acetylsalicylic acid) has been used as a basic antiplatelet agent, both in the primary and secondary types of CV prevention. It reduces the risk of acute vascular events in about 25 % (1). The main mechanism of antiplatelet activity of low-dose aspirin (75–325 mg/day) is the irreversible inhibition of platelet cyclooxygenase-1 (COX-1), thus leading to the suppression of TXA2 synthesis. TXA2 is a molecule with strong proaggregatory and vasoconstrictory properties (2). However, there is a considerable amount of patients not responding to aspirin, also referred to as aspirin non-responders. The phenomenon named aspirin resistance (AR) has been intensively discussed. The clinical definition means the occurrence of acute atherothrombotic events despite the use of low dose aspirin. On the other hand, the biochemical definition of AR is based on the results of various laboratory methods, showing inadequate aspirin effectivity to inhibit platelet aggregation (3, 4). The real prevalence of AR is still not fully clear, ranging from 5–40 %, depending on the method and criteria of definition used in different clinical studies. The possible risk factors of AR include smoking, dyslipidemia, hypertension, female gender, diabetes mellitus and advanced CV disease such as peripheral arterial occlusive disease (PAOD). Non-compliance and the pharmacodynamic interaction of aspirin with ibuprofen have been recently discussed as the most relevant causative factors of the failure of aspirin prevention (5, 6, 7). Ibuprofen as an over-the-counter drug is in our conditions the most commonly used pain-killer within the group of non-steroidal antiinflammatory drugs (NSAIDs) (8). Although there have been used many laboratory tests to detect aspirin response, none of them is specific and sensitive enough to be routinely used as a screening method to reveal aspirin non-responders in clinical practice (9). The urinary levels of 11-dehydro TXB2 have proved to correlate with CV mortality in high risk patients treated by low dose aspirin. 11-dehydro TXB2 is a stable metabolite of TXA2, well reflecting its endogenuous synthesis (10). The aim of our pilot study was the biochemical evaluation of antiplatelet effect of aspirin in patients at different level of CV risk.

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Tab. 1. Criteria for study enrollment.

Inclusion criteria	ASA 100 mg/day during ≥7 days
Exclusion criteria	MI, ischemic stroke within the period <6 weeks

MI – myocardial infarction, ASA – acetylsalicylic acid

Methods and patients

To evaluate the biochemical aspirin response, we used the method of urinary 11-dehydro TXB2 levels measurement. Quantitative detection of TXB2 in urine was determined by competitive enzyme immunoassay, using human Thromboxane B2 ELISA-kit (*EIA-3105, DRG Instruments GmbH*) according to manufacturer's instructions. All urine samples were diluted 1:10 with "Assay buffer". Seven standards were prepared with the TXB2 concentration 10.000; 3.333; 1.111; 370; 123; 41.1; 13.7 pg/ml, respectively. 100ěl of "Assay buffer" was added into every anti-TXB2 precoated microplate well. Then, 100 µl of TXB2 standard was added into each appropriate well, followed by pippeting of 100 µl of urine sample in appropriate wells. The next step included adding of 50 µl of "blue conjugate" representing the detecting TXB2 antibody into each well except the blanks. The plate was incubated for two hours on a shaker (~500rpm). After incubation, the plate was washed 3-times with "wash solution". Subsequently, 200 µl "pNpp substrate solution" was added into each well of dry microplate and then incubated for 45 minutes without shaking. The enzyme reaction was stopped by adding the 50 µl "Stop solution". The absorbance of each microwell was read by Dynex MRX 1.13 spectro-photometer (*Dynex*), using the 405nm wavelength. The 11-dehydro TXB2 concentrations were determined from standard curve prepared from seven standard dilutions. Each sample and TXB2 standard dilution was done in duplicate. The final concentrations of 11-dehydro TXB2 were expressed in units pg/ml/mmol of creatinine, taking into account individual renal functions.

We obtained the first-morning urine samples (V=10 ml) from 69 patients hospitalized at the 2nd Department of Internal Medicine in Bratislava during years 2006–2007. The samples were kept frozen under the temperature of -80 °C until analysis. The criteria for enrolment is summarized in Table 1. The first group consisted of patients with CV risk factors without documented CV disease, taking low-dose aspirin within the primary prevention (Tab. 2). The second group included patients in the secondary CV aspirin prevention, with documented history of ischemic heart disease, PAOD or ischemic stroke (Tab. 3). Finally, the control group comprised hospitalized patients not taking low-dose aspirin (Tab. 4).

Results

As we expected, the mean urinary levels of 11-dehydro TXB2 were significantly lower in patients in the primary and secondary aspirin prevention comparing with the control group of patients not taking aspirin (120.4±16.8, 145.9±15.2 vs 280.3±39.8, $p<0.05$). Although there can be seen a tendency to higher mean thromboxane concentrations in patients within the secondary aspirin prevention, the difference between the two groups of pa-

Tab. 2. Patients in the primary CV prevention (n=10).

Gender	Age	Smoking	Hypertension	Diabetes mellitus	cholesterol	homocystein	obesity
M=9 F=1	56.4±16.3	2	7	0	7	2	5

CV – cardiovascular, M – male, F – female

Tab. 3. Patients in the secondary CV prevention (n=40).

Gender	Age	Smoking	Hypertension	Ischemic heart disease	IS	PAD	DM	cholesterol	homocystein	obesity
M=20 F=20	70±12	6	34	38	5	17	23	5	19	19

DM – diabetes mellitus, CV – cardiovascular, IS – ischemic stroke, M – male, F – female, PAD – peripheral arterial disease

Tab. 4. Patients in the control group (n=19).

Gender	Age	Smoking	Hypertension	Ischemic heart disease	PAD	DM	cholesterol	homocystein	obesity
M=7 F=12	63±15.8	3	10	5	1	3	3	2	7

DM – diabetes mellitus, M – male, F – female, PAD – peripheral arterial disease

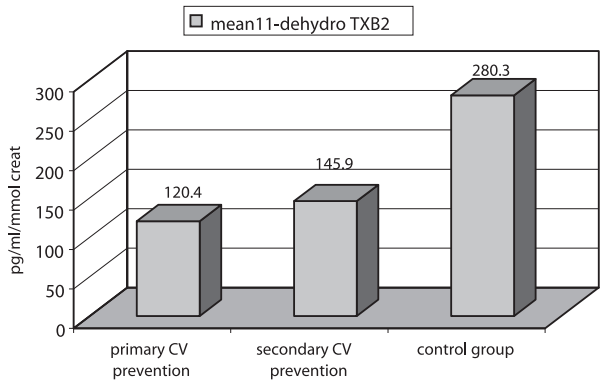

Fig. 1. Mean levels of 11-dehydro TXB2 in patients at different level of CV risk. CV – cardiovascular, creat – creatinine.

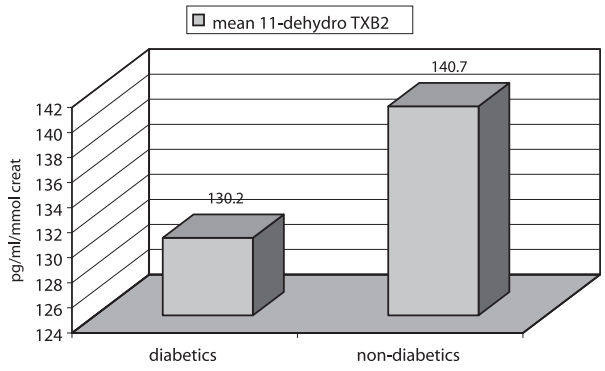

Fig. 2. Mean levels of 11-dehydro TXB2 in diabetic and non-diabetic patients. creat – creatinine.

tients taking aspirin did not reach statistical significance (280.3±39.8 vs 145.9±15.2, p>0.05) (Fig. 1). Despite we often face the problem of the failure of aspirin prevention in diabetics, our results did not show singificant differences in the biochemically measured aspirin response comparing with non-diabetics (130.2±17.04 vs 140.7±15.35, p>0.05) (Fig. 2). Within the group of patients in the secondary aspirin prevention, we did not find significant difference in thromboxane levels between gender, though there may be observed a tendency to higher levels in women (218.5±50.06 vs 131.595±18.68, p>0.05) (Fig. 3).

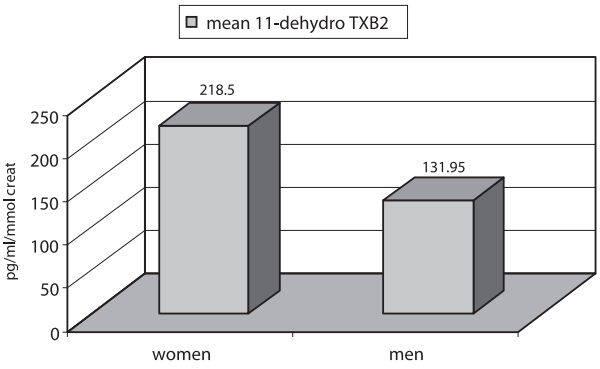

Fig. 3. Mean levels of 11-dehydro TXB2 in men and women within the secondary CV prevention. CV – cardiovascular, creat – creatinine.

Statistics

Statistical analysis was performed by using the unpaired Student's test. The probability level was set to 95 % as a limit to reject the null hypothesis. The differences between comparing values were considered statistically significant at the significant level p<0.05. Data are expressed by mean SEM.

Discussion

Our pilot study did not show any significant differences among patients at different level of CV risk, though lower aspirin efficacy could be expected in patients with documented CV disease, being at higher CV risk. Although most of the frequently discussed possible risk factors of aspirin resistance could be found in patients enrolled in our study, their real clinical relevance remains unclear. Therefore, the term failure of the aspirin prevention is currently preferred (11). The meta-analysis of clinical trials showed lower efficacy of aspirin in diabetic patients. Diabetes mellitus is associated with an increased risk of atherothrombosis and requires an effective antiplatelet therapy (12, 13). Despite this fact, we didn't prove significant differences of biochemically measured aspirin efficacy between diabetics and non-diabetics. Female gender is considered one of the possible risk factors of aspirin non-responsiveness. The results of the Women's Health Study demonstrated non-significant reduction in myocardial infarction and CV mortality in women ≥65 years old, taking low-dose aspirin (14). Despite several theories this biological issue remains unexplained and warrants further clinical investigations. Our results were not consistent with the hypothesis of the lower aspirin efficacy in women.

At present, non-compliance and interactions of low-dose aspirin with ibuprofen are considered the most relevant causes of the failure in aspirin prevention. The need for long-term daily aspirin treatment predisposes to non-compliance, which may result in higher risk of atherothrombotic events. The role of compliance in aspirin prevention evaluated by the method of questionnaire was well-documented in the prospective clinical trial of British physicians in the primary prevention, the Physicians's Health Study. A significant reduction in the risk of myocardial infarction was observed in physicians with higher rate of compliance, when comparing with individuals presenting with lower rate of compliance (15, 16). The high rate of compliance was assumed in our study as only in-patients were included.

The clinical relevance of interactions of aspirin with ibuprofen was also proved by clinical trials. The concomitant treatment with ibuprofen may lead to the failure of aspirin protection, as ibuprofen binds to the same target place on COX-1, thus preventing aspirin from its antiplatelet effect (7, 17). More-

over, the consumption of ibuprofen as an over-the-counter drug is particularly high in our country. We didn't record any concomitant use of ibuprofen with aspirin in our patients when analysing the medications from the medical documentation.

However, our pilot study has several limitations. As regards all laboratory methods used to monitor the antiplatelet activity of aspirin, the measurement of urinary levels of 11-dehydro TXB2 as well has a limited sensitivity and specificity. Although the majority of endogenous TXA2 comes from activated platelets, there are some alternative sources of thromboxane synthesis via COX-2 pathway, not affected by aspirin. These include young platelets, monocytes, macrophages and endothelial cells. Moreover, little is known about possible diurnal changes in the TXA2 production, since we analysed the thromboxane concentrations from the first morning urine samples. On the other hand, the advantages of this method include its non-invasiveness and proved correlation with the risk of acute vascular events. Further trials are needed to assess the suitability of this method for the routine detection of aspirin non-responders. Finally, the relatively small number of involved patients might also influence the statistical analysis of achieved results.

Since there is currently no standard laboratory method to detect aspirin non-responders available, the term aspirin resistance remains controversial and requires further research. For that reason, the term failure of the aspirin prevention is more preferred nowadays. The most intensively discussed causative factors of this phenomenon are non-compliance and interactions of aspirin with ibuprofen. It seems that clopidogrel as an ADP receptor antagonist could be better alternative to aspirin in certain groups of high risk patients who may present with lower efficacy of aspirin (e.g. diabetics) (18, 19). For now, every effort should be done to improve patients' compliance and to prevent clinically relevant interactions of aspirin with ibuprofen. The elimination of these two factors as was the case in our study may provide better efficacy of the antithrombotic prevention by aspirin.

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