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Can we use adenosine diphosphate (ADP) to study "aspirin resistance"? The Janus faces of ADP-triggered platelet aggregation

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Abstract:
There is a growing number of contradictory reports indicating that adenosine diphosphate (ADP) can be a useful agonist in monitoring of the antiplatelet action of acetylsalicylic acid (ASA) in humans and animals. In the current study, we aimed to determine the conditions for using ADP to trigger platelet aggregation in order to detect ASA-mediated inhibition of rat platelet reactivity.
Initially, we examined the usefulness of different ADP concentrations (0.25, 0.5, 1, 5 and 10 μM) in detecting the *in vitro* ASA-mediated platelet inhibition using whole blood aggregometry, as well as we monitored the role of ADP in generation of thromboxane A_2 (TXA_2). To study *ex vivo* ASA inhibitory potential on platelet aggregation induced by a range of ADP concentrations, animals were subjected to one or 10-day ASA administration at the dose of 50 mg/kg.
Our experiment shows that ADP in a concentration-dependent manner induces TXA_2 generation in the whole blood with hirudin as an anticoagulant. However, *in vitro* and e*x vivo* examination of ASA inhibitory potential on platelet aggregation revealed that irrespectively of administration regimen, ASA failed to block platelet aggregation induced by ADP at the concentrations higher than 0.5 μM.
Our findings suggest that the mechanism of ADP-induced platelet aggregation depends on agonist concentration. It appears that only low ADP concentrations (up to 0.5 μM) induce TXA_2-dependent rat platelet aggregation. Therefore, ADP could be considered a useful platelet agonist for monitoring of ASA-mediated platelet inhibition only if used at much lower concentrations than those commonly employed.

Key words:
platelet aggregation inhibitors, acetylsalicylic acid, rats, TXA_2 generation

Abbreviations: AA – arachidonic acid, ADP – adenosine diphosphate, A_{max} – maximal aggregation, ASA – acetylsalicylic acid, AU – arbitrary units, AUC – area under aggregation curve, TXA_2 – thromboxane A_2, TXB_2 – thromboxane B_2

Introduction

The preventive effects of acetylsalicylic acid (ASA) against atherothrombotic events, as well as rather common occurrence of the phenomenon of the so-called "aspirin resistance", are well recognized and documented [24, 29, 32]. It has also been proposed that protective efficiency of ASA could be improved if ASA dose is individually adjusted to patient's platelet reactivity. Therefore, a number of projects have been undertaken to establish sensitive and reproducible aggregation protocol for laboratory detection of ASA-mediated inhibition of blood platelet reactivity. However, the proper choice of platelet agonist capable of detecting ASA inhibitory potential appears to be a key factor in aggregation studies, being a part of protocols for the monitoring of the clinical effectiveness of ASA. Obviously, from molecular standpoint arachidonic acid (AA) seems to be the most appropriate platelet agonist used in studies detecting ASA-mediated inhibition of platelet reactivity [8, 21]. Nevertheless, in spite of this mechanistic rationale, some authors constantly employ adenosine diphosphate (ADP) at the concentrations of 5 μM [14, 22, 33] and 10 μM [12] to study ASA-mediated inhibition of platelet reactivity [31]. Although is was shown that binding of ADP to P_2Y receptors subsequently activates phospholipase A_2 (PLA_2) [9] and synthesis of thromboxane A_2 (TXA_2) [28] in calcium free environment [23], the main force of the ADP-induced platelet aggregation is driven by inside-out signaling and subsequent binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ [30]. Therefore, when using ADP to activate platelets, we detect ASA-mediated alternations in P_2Y-dependent platelet activation pathways rather than the extent of cyclooxygenase 1 (COX-1) inhibition by ASA. Such a misuse of ADP may contribute to questionable conclusions on inefficiency of ASA to inhibit platelet reactivity [3, 12, 26] or even proaggregatory action of ASA [1, 7, 16, 18]. Altogether, it raises the important questions (a) on the usefulness of ADP in the sensitive and reliable detection of ASA-mediated inhibition of platelet reactivity (b) how ASA affects the ADP-dependent [27] signaling pathways.

This encouraged us to study the usefulness of different ADP concentrations in detecting the ASA-mediated platelet inhibition using whole blood aggregometry, and to evaluate the role of ADP in generation of TXA_2 in the presence of platelet COX-1 inhibitor, ASA. Deliberately we have chosen whole blood aggregometry, which mimics the physiological conditions much better than tubridimetric aggregometry, since it allows blood platelets to interact with other blood cells and components in their natural *milieu*. In the initial phase of the experiment, we aimed to elucidate the usefulness of different ADP concentrations (0.25, 0.5, 1, 5 and 10 μM) in detecting *in vitro* impact of ASA on rat platelet aggregation. To clarify the role of ADP in the platelet TXA_2 synthesis, we performed the ADP-stimulated thromboxane B_2 (TXB_2) generation assay in whole blood collected to hirudin-containing tubes (in the presence of Ca^{2+}). Finally, animals were subjected to one or 10-day administration of ASA at dose of 50 mg/kg to study platelet aggregation within the range of ADP concentrations. At all stages of the experiment, AA-triggered aggregation was used to confirm effective inactivation of COX-1 by ASA.

Our experiment shows that the mechanism of ADP-triggered platelet aggregation strongly depends on agonist concentration. We revealed that low ADP concentrations (up to 0.5 μM) induce predominantly TXA_2-dependent platelet aggregation which is sensitive to ASA. Therefore, only low ADP concentrations could be used for reliable monitoring of ASA-mediated platelet inhibition.

Materials and Methods

Animals

Fifty adult male Wistar rats with a body weight of 250–350 g were used in the study.

Throughout the experiment animals, housed in cages in the groups of 5 animals each, remained in unrestrained state and received tap water and a standard laboratory pellet chow *ad libitum*.

All rats received care in compliance with the guidelines of the International Guiding Principles for Bio-

medical Research Involving Animals of the Council for International Organizations of Medical Sciences (CIOMS 1983), which concurred the principles of respect for life.

All experiments were performed in the Department of Toxicology, Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985), as well as with the guidelines of the European Community for the Use of Experimental Animals (L358-86/609/EEC).

Chemicals

Hirudin [Refludan® (lepirudin (rDNA) for injection] was purchased from Aventis (Aventis Pharma Deutschland GmbH, Bad Soden a. Ts., Germany) and Laspal® (lysine acetylsalicylate) was obtained from Synthelabo (Synthelabo Groupe, Quetigny, France). Platelet agonists: ADP and AA were provided by Chrono-Log (Havertown, PA USA) and anesthetics: xylazine (Sedazin) and ketamine •HCl (Ketanet) were purchased from Biowet (Puławy, Poland). Unless otherwise stated, all other chemicals were from Polish Chemical Reagents (Gliwice, Poland). Water used for solution preparation and glassware washing was purified using Easy Pure UF unit (Thermolyne Barnstead, USA).

Blood collection technique

Animals were anesthetized with i.m. injection of ketamine ·HCl (100 mg//kg) and xylazine (10 mg/kg). For the *in vitro* and *ex vivo* experiments, blood was collected from abdominal aorta of anesthetized rats. Briefly, once deep anesthesia was achieved, skin and muscles in the midline of the abdomen were cut exposing abdominal cavity. Aorta was carefully dissected to enable catheterization using venous cannula Venacat-G24 (Polfa, Bolesławiec, Poland). After canulation, blood was collected under free outflow conditions into a tube containing the saline solution of hirudin (final hirudin: blood ratio was 1:9 v/v and final hirudin concentration was 50 μg/ml of whole blood). Throughout the experiment, all blood preparations were handled with a special caution to avoid undesirable platelet activation. Noteworthy, blood collection and platelet aggregation tests were performed 60 min after the last injection of Laspal®.

Experimental protocol

In vitro protocol for monitoring of platelet function

In the initial phase of the experiment, we aimed to examine usefulness of different ADP concentrations in detection of the *in vitro* inhibitory effect of ASA on rat blood platelets in absence of vascular system. After collection of blood from 10 male rats according to procedure described above, two samples of blood per each ADP concentration were prepared and mixed with either saline or ASA to final concentration of 30 μg/ml [11]. After 10 min of incubation at 37°C, the ADP-induced platelet aggregation was measured using the protocol described below. Additionally, AA-triggered aggregation was tested to confirm the impact of the inhibition of TXA_2 synthesis on platelet function. Inhibitory effect of ASA was expressed as a percent of control aggregation.

Ex vivo protocol for monitoring of platelet function

To assess usefulness of ADP for monitoring of the inhibitory effects of immediate and short-term ASA administration on blood platelet aggregation, 30 rats were randomly assigned into three groups and treated with lysine acetylsalicylate (dissolved in deionized water) at dose corresponding to 50 mg/kg of pure ASA. ASA was given either as a single dose or administered daily for 10 consecutive days in the intramuscular injections according to recommendations of the manufacturer. Administration of a vehicle served as a control. At the end of experiment blood was collected from each animal at 1 h after ASA or vehicle injection and tested for ADP- and AA-induced platelet aggregation in accordance with the protocol described below.

In vitro TXB_2 generation assay

Whole blood TXB_2 generation was used to measure the ADP-dependent synthesis of TXA_2 in either absence or presence of ASA. The reaction was monitored with the employment of a novel setup described in detail in our earlier paper [4]. Briefly, two series of six equal samples of blood collected from each of 10 animals were immediately supplemented with either

saline (blank) or ASA to give the final concentration of 30 µg/ml [11]. Afterwards, ADP was added at a final concentration of 0, 0.25, 0.5, 1, 5 or 10 µM to each series of samples, and vials were subjected to 15 min incubation at 37°C in a rotary mixer (referred to as the "dynamic" model of TXB_2 generation) [4]. TXA_2 generation was terminated by the addition of 100 µg/ml ASA. Finally, samples were centrifuged at 4°C (10000 × g for 3 min) and plasma TXB_2 concentration was measured using enzyme-linked immunoassay kit with a sensitivity of 0.004 mg/l (Cayman Chemical Company, Ann Arbor, MI, USA). The results of TXB_2 generation assay were presented in relation to TXB_2 formed in the course of spontaneous generation.

Platelet aggregation

Aggregation was studied in whole blood using Multiplate® (Multiple Platelet Function Analyzer, Dynabyte Medical, Germany [34]), the five-channel aggregometer based on measurements of electric impedance [5]. The measurements were performed according to modified Dynabyte Medical protocol. Briefly, whole blood samples (0.3 ml), diluted 1:1 with 0.85% saline, were preincubated for 10 min at 37°C and then supplemented with ADP to give the final concentrations of 0.25, 0.5, 1, 5 or 10 µM. AA at a final concentration of 0.5 mM was used to confirm the effectiveness of ASA administration and the subsequent inhibition of TXA_2 generation. Aggregation curves were recorded and analyzed using Dynabyte Medical software enabling the calculation of the following two parameters of platelet aggregation:

1. A_{max}, the maximal value of platelet aggregation expressed in arbitrary units (AU) of aggregation.
2. AUC, total area under the aggregation curve.

Statistical analysis

In vitro protocol

The values A_{max} and AUC of ADP-induced aggregation were expressed as the mean and standard error of the mean. To estimate significance of differences, one-way ANOVA was used. If null hypothesis had been rejected, the *post-hoc* Student-Newman-Keuls test was employed. The results of percentage inhibition of AA-induced maximal aggregation were expressed as median and interquartile ranges (Me, IQ: lower quartile, LQ, to upper quartile, UQ), as they showed departures from normality (according to Shapiro-Wilk's W test). The significance of differences was estimated using Mann-Whitney U test.

Ex vivo protocol

The values A_{max} and AUC of ADP-induced aggregation were expressed as the mean and standard error of the mean. To estimate significance of differences between groups and ADP concentrations, one-way ANOVA was used. If null hypothesis had been rejected the *post-hoc* Student-Newman-Keuls test was employed.

Due to extremely bimodal distributions of data representing AA-dependent aggregation parameters, all these data were shown as the fractions of the total number of animals in each group, in which the values of A_{max} or AUC were laying beyond the arbitrarily accepted cut-off threshold values calculated for control group and equal to a lower quartile (LQ). Significance of differences in AA-induced aggregation was estimated by the exact Fisher's test.

In vitro TXB_2 generation assay

The data were expressed as the mean and standard error of the mean of the relative values estimated in relation to spontaneous TXB_2 generation (without agonist) and presented in percentages. To estimate significance of differences between corresponding control and ASA samples, *t*-test was used. Significance of differences between different ADP concentrations was determined with one-way ANOVA. If null hypothesis had been rejected, the *post-hoc* Student-Newman-Keuls test was employed. Baselines TXB_2 values were expressed in mg/ml. For the analysis, the data were log transformed in order to improve data conformity with a characteristics of normal distribution.

Results

In vitro examinations of platelet function

The *in vitro* analysis of the ASA-mediated platelet inhibition showed that monitoring of aggregation induced by ADP at the concentrations higher than

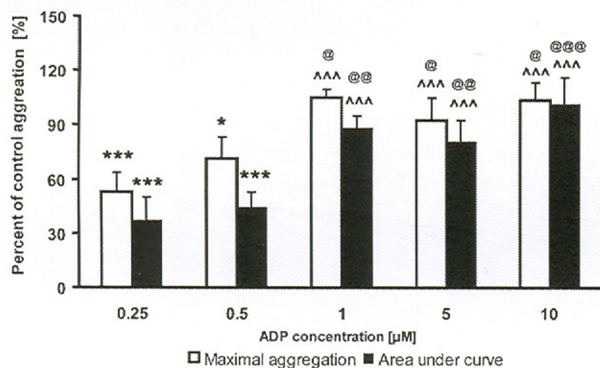


Fig. 1. The *in vitro* effects of ASA on platelet aggregation. ADP-induced (0.25, 0.5, 1, 5, 10 µM) platelet aggregation was performed in the absence or presence of 30 µg/ml of ASA. The extent of aggregation was expressed as maximal value of aggregation and area under curve. The values were estimated as relative measures with respect to control aggregation (without ASA) and expressed in percentages. * $p < 0.05$, with respect to control (no ASA); *** $p < 0.001$ vs. control (no ASA); ^^^ $p < 0.001$, *vs.* 0.25 µM ADP; @ $p < 0.05$, *vs.* 0.5 µM ADP; @@ $p < 0.01$, *vs.* 0.5 µM ADP; @@@ $p < 0.001$, *vs.* 0.5 µM ADP

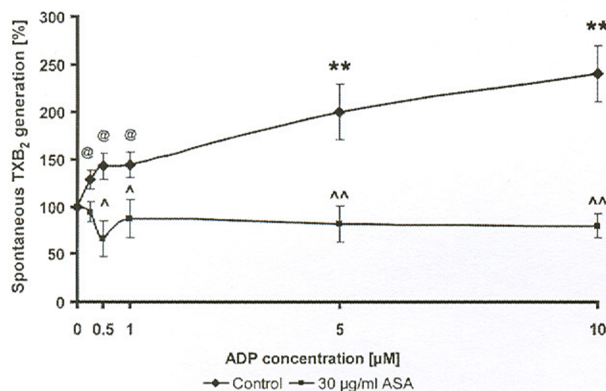
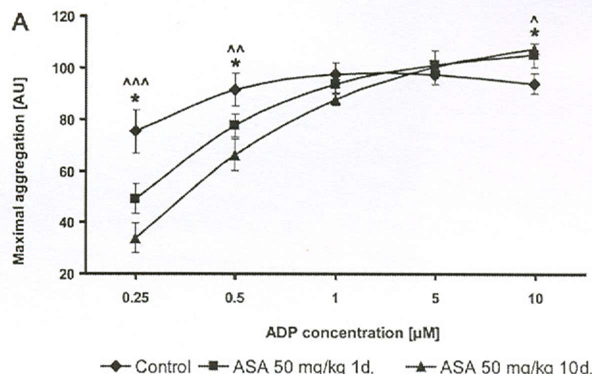


Fig. 2. The in *vitro* TXA_2 generation assay. Whole blood was activated at 37°C with ADP (0.25, 0.5, 1, 5, 10 µM) in the presence or absence of 30 µg/ml of ASA. The values were estimated as relative measures with respect to spontaneous thromboxane generation (without agonist) and expressed as percentages. The baseline values for control (no ASA) and ASA-treated samples were 2.44 (95%CI: 0.55–6.25) mg/l and 1.31 (95%CI: 0.66–2.63) mg/l, respectively. ** $p < 0.01$, *vs.* generation in the absence of agonist; ^ $p < 0.05$, with respect to control (no ASA); ^^ $p < 0.01$ *vs.* control (no ASA); @ $p < 0.05$, *vs.* 10 µM ADP

0.5 µM failed to detect inhibitory effect of ASA (Fig. 1). Complete lack of aggregation upon stimulation with AA at 0.5 mM (0%, p = 0.003) confirms the effective blockade of COX-1.



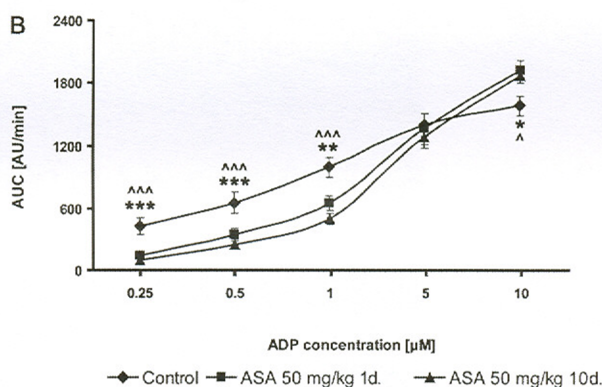


Fig. 3. The *ex vivo* effects of ASA on platelet aggregation. ADP-induced (0.25, 0.5, 1, 5, 10 µM) platelet aggregation was studied in blood obtained form animals treated with a single dose of 50 mg/kg ASA or given 50 mg/kg ASA per day for 10 consecutive days. The extent of aggregation was expressed as maximal value of aggregation **(A)** and area under curve **(B)**. * $p < 0.05$, *vs.* single dose; ** $p < 0.01$, *vs.* single dose; *** $p < 0.001$, vs. single dose; ^^ $p < 0.05$, *vs.* 10 day treatment; ^^^ $p < 0.01$, *vs.* 10 day treatment, A_{max}; ^^^ $p < 0.001$, *vs.* 10 day treatment, AUC

In vitro ADP-induced TXB_2 generation

In control samples, ADP significantly induced TXB_2 generation in a concentration-dependent manner. In the presence of ASA, synthesis of TXB_2 was completely abolished at all ADP concentrations (Fig. 2).

Ex vivo examinations of platelet function

Platelet response to the activation with ADP

Fluctuations in A_{max} revealed that ADP induced platelet aggregation in a concentration-dependent man-

ner only in ASA-treated animals. In control animals, maximal aggregation was achieved at 1 μM of ADP (Fig. 3A). When the extent of aggregation was expressed as AUC, the dependence on ADP concentration was observed in all groups (Fig. 3B). There was no difference in ADP-induced aggregation between animals treated with ASA either once or for 10 consecutive days (Fig. 3A, B). Similarly to the *in vitro* experiment, ASA failed to inhibit platelet aggregation induced by ADP at the concentrations higher than 0.5 μM (Fig. 3A, B). Interestingly, aggregation induced by ADP at 10 μM revealed relative hyperreactivity of platelets from ASA-treated animals (Fig. 3A, B).

Platelet response to the activation with AA

The results were in line with the *in vitro* data and showed that both ASA administration regimens led to complete inhibition of AA-dependent platelet aggregation expressed as either decreased A_{max} (8/10 *vs*. 2/8, $p < 0.0001$) or AUC (8/10 *vs*. 2/8, $p < 0.0001$) of platelet aggregation curves.

Discussion

Over the recent years, we have witnessed a disturbing tendency to employ ADP in detection of ASA effectiveness [12, 14, 15, 36]. So far, the principal argument against use of ADP for detection of ASA-depended platelet inhibition was based on findings showing that ADP-induced platelet aggregation is TXA_2-independent. Unexpectedly, in the current study we revealed that ADP at a broad range of concentrations induced the TXA_2 generation in whole blood in rats under conditions of physiological Ca^{2+} level. These results are contradictory to previous findings showing that generation of TXA_2 in ADP-stimulated human platelets may be merely elicited in citrated plasma [19, 20, 23] Although there is a lack of published evidence showing ADP-mediated TXA_2 generation in whole blood in either absence or presence of calcium, in our opinion this approach mimics physiological conditions of platelet interactions with other cells better than the suspensions of isolated platelets and takes into account leukocyte-derived pool of generated thromboxane A_2, which may also contribute to platelet stimulation. Overall, our results provide an important evidence on molecular basis of ASA-mediated inhibition of ADP-induced blood platelet aggregation. Further, *in vitro* and *in vivo* experiments showed, however, that the inhibitory effect of ASA on ADP-induced blood platelet aggregation depended on the agonist concentration. Both approaches provided an evidence that ASA failed to block platelet aggregation induced by ADP at the concentrations higher than 0.5 μM. Altogether, it suggests that ADP at low concentrations (0.5 μM) triggers predominately TXA_2-dependent rat platelet aggregation consequent to indirect $\alpha_{IIb}\beta_3$ activation [25]. These results are in the line with findings reported by Jin et al. [13] who showed that triggering of inside-out and outside-in signaling pathways following the $P2Y_{12}$ activation were responsible for liberation of AA by PLA_2. The authors demonstrated that blockade of $P2Y_{12}$ and subsequent activation of $\alpha_{IIb}\beta_3$ with LIBS6 resulted in cessation of TXB_2 generation, thus pointing to a cross-talk between $P2Y_{12}$ and $\alpha_{IIb}\beta_3$-dependent pathways in the activation of PLA_2. Hence, one may speculate that high ADP concentrations (exceeding 0.5 μM) directly induce massive activation of $\alpha_{IIb}\beta_3$, which predominates over the concomitant TXA_2-dependent activation of $\alpha_{IIb}\beta_3$ [13, 19].

Finally, platelets from animals treated with ASA revealed augmented reactivity to 10 μM of ADP in respect to control rats. Interestingly, such an effect has not been observed after *in vitro* incubation of platelets with ASA. This suggests the enhanced inside-out signaling *via* $\alpha_{IIb}\beta_3$ in response to high concentrations of ADP following ASA treatment. Futhermore, this observation implies the rearrangements at the level of transcriptional activity, which is absent in platelets [2, 10]. Therefore, *in vitro* incubation of platelets with ASA failed to show ASA-mediated platelet hypersensitivity to ADP. Probably, only *in vivo* administration of ASA may induce some changes in gene expression at the level of megakaryocytes and alter response of platelets to ADP [6, 35]. This facts might be of clinical importance since Mattiasson and co-workers have shown that ASA treatment enhances ADP-triggered platelet aggregation and may be associated with increased incidence of stroke recurrence [17].

The current study is an important contribution to ongoing discussion on the use of ADP in monitoring the efficiency of ASA treatment. We definitely do not recommend the use of high ADP concentrations in detection of ASA inhibitory effect. Importantly, we

showed for the first time that ADP at concentrations much lower than those currently employed (below 0.5 μM) appeared to be promising in monitoring of laboratory effectiveness of ASA in rats.

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