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Defining platelet response to acetylsalicylic acid: the relation between inhibition of serum thromboxane B_2 and agonist-induced platelet aggregation

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Abstract
Arachidonic acid (AA)-induced platelet aggregation (PA) and serum thromboxane B_2 (TxB_2) inhibition are widely used to indicate cyclooxygenase-1 activity and the antiplatelet effect of acetylsalicylic acid (ASA). Despite decades of investigations, the relation between these measurements remains unclear. We sought to evaluate the relation between AA-PA and serum TxB_2 inhibition. We serially measured AA-PA (conventional aggregation), serum TxB_2, plasma ASA and salicylic acid (SA) (liquid chromatography-mass spectrometry), and urinary 11-dehydro thromboxane B_2 (u11-dh TxB_2) (enzyme-linked immunosorbent assay) levels at 10 times over 24 hours in seventeen healthy volunteers receiving a single dose of 162 mg chewed and swallowed ASA (n = 6), 50 mg inhaled ASA (n = 6), or 100 mg inhaled ASA (n = 5) (ClinicalTrials.gov Identifier: NCT04328883, April 1, 2020). Baseline variability was more pronounced with serum TxB_2 (31–680 ng/mL) as compared to maximal AA-PA (65–81%) and u11-dh TxB_2 (1556–4440 pg/mg creatinine). The relation between serum TxB_2 inhibition and AA-PA was stepwise; after 30–40% inhibition of serum TxB_2, AA-PA fell to < 5%. By receiver operating characteristic curve analysis using AA-PA < 5% to define aspirin responsiveness, serum TxB2 inhibition > 49% and u11-dh TxB2 < 1520 pg/mg creatinine met the definition. Our study demonstrates a non-linear relation between serum TxB_2 inhibition and AA-PA. Aggregation was nil once TxB_2 inhibition reached > 49%. Moreover, these results suggest that the definition of > 95% inhibition of serum TxB_2 to indicate the level of platelet COX-1 inhibition needed for clinical efficacy may be overestimated and should be re-considered in future translational research investigations that attempt to link the clinical efficacy of ASA with a laboratory measurement cutoff.

Keywords Platelet aggregation · Acetylsalicylic acid · Thromboxane B_2 · Arachidonic acid

Highlights

- Despite decades of investigations, the relation between inhibition of arachidonic acid-induced platelet aggregation and serum thromboxane B_2 inhibition following aspirin administration remains unclear.
- Arachidonic acid-induced platelet aggregation, serum thromboxane B_2, and urinary 11-dehydro thromboxane B_2 were serially measured in healthy volunteers receiving a single dose of chewed and swallowed or inhaled aspirin.
- There was a non-linear relation between serum thromboxane B_2 inhibition and arachidonic acid- induced platelet aggregation. Aggregation was nil once thromboxane B_2 inhibition reached > 49%.
- The definition of > 95% inhibition of serum thromboxane B_2 to indicate the level of platelet cyclooxygenase-1 inhibition needed for clinical efficacy may be overestimated and should be re-considered in future translational research investigations.

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Introduction

Arachidonic acid (AA)-induced platelet aggregation (PA) and inhibition of serum thromboxane B_2 (TxB_2) have been widely used in clinical and translational investigations to indicate the level of cyclooxygenase-1 (COX-1) activity and the antiplatelet effect of acetylsalicylic acid (ASA) [1, 2]. In addition, the stable urinary metabolite of thromboxane A_2 (TxA_2), 11-dehydro thromboxane B_2 (1111-dhTxB_2) has been used as a marker of aspirin efficacy. It is widely believed that > 95% inhibition of serum TxB_2 is needed for aspirin efficacy. The latter assumption was largely based on investigations conducted decades ago demonstrating that this cut point was associated with marked inhibition of total body TxA_2 as measured by the stable urinary metabolite levels [3, 4]. This belief is also dependent upon the premise that platelets are the only source of TxA_2. However, it is known that prostaglandin intermediates produced in leukocytes are also a source of TxA_2 generated by transcellular synthesis [2, 5]. Whether lesser levels of serum TxB_2 inhibition are associated with cardioprotection from ASA is unknown. Despite decades of investigations, the relation between inhibition of serum TxB_2 and aggregation induced by AA and other agonists in subjects treated with ASA remains unclear. Therefore, the primary objective of our study was to investigate this relation. We have conducted serial laboratory assessments in a study comparing the pharmacokinetics and pharmacodynamics of orally administered ASA and inhaled ASA. Frequent sampling in our protocol allowed the opportunity to scrutinize this relation between inhibition of serum TxB_2 and AA-induced platelet aggregation.

Methods

In this pilot, open-label, single dose-escalation study conducted in healthy adult volunteers, the serial pharmacodynamics and pharmacokinetics of inhaled aspirin (I-ASA) [50 mg (n = 6) and 100 mg (n = 5)] (Otitopic, Los Angeles, CA, USA) and 162 mg chewed and swallowed (C)-ASA (Bayer Corporation, Whippany, NJ, USA) (n = 6) were determined. I-ASA was delivered as nanoparticles with a dry inhaler [6]. We measured final 1 mmol/L AA-, 4 µg/ml collagen-, and 5 µmol/L adenosine diphosphate (ADP)-induced platelet aggregation (PA) by conventional aggregometry [7]; and serum TxB_2 and plasma ASA and salicylic acid (SA) by liquid chromatography-mass spectrometry serially after drug administration (0, 2, 5, 10, 20, 30, and 40 minutes, and 1, 4, and 24 hours) [6]. U11-dh TxB_2 was measured by enzyme-linked immunosorbent assay at 4- and 24-hours post-dosing [8]. TxB_2 inhibition was defined as inhibition (%) = 100 × (baseline value − post-dose value)/baseline value [9].

Pearson correlation analysis was used to determine relation between AA-PA and TxB_2 measurements. A receiver operating characteristic (ROC) curve analysis was used to identify cut points of measurements associated with aspirin response defined as > 5% AA-induced-PA. Statistical analyses were performed using MedCalc Statistical Software version 19.2.1 (MedCalc Software Ltd, Ostend, Belgium).

Results and discussion

The study group included healthy volunteers with a normal platelet count and not on antiplatelet therapy. The average age of the study group was 27 ± 7 years. The study group included 53% Caucasians and 53% women. The original pilot study demonstrated that inhalation of ASA using a novel drug delivery device provides earlier platelet inhibition as measured by AA-induced aggregation and inhibition of serum TxB_2 than chewed and swallowed soluble ASA ([6]. We were able to scrutinize the relation between TxB_2 and platelet aggregation values by collecting a broad range of data at very early timepoints after aspirin administration and by using different formulations (inhaled vs. oral) of aspirin that we otherwise were not be able to capture for correlation purposes in prior pharmacokinetic and pharmacodynamic studies of ASA. Baseline variability was more pronounced with serum TxB_2 (31–680 ng/mL) as compared to AA-PA (65–81%) and u11-dh TxB_2 (1556–4440 pg/mg creatinine). Serum TxB_2 and u11-dh TxB_2 levels had a moderate positive correlation with AA-PA ($r = 0.57$, and $r = 0.68$, respectively, $p < 0.001$ for both). There was a strong negative correlation when AA-PA was compared to inhibition of serum TxB_2 and u11-dh TxB_2 ($r = 0.82$, and $r = 0.90$, respectively, $p < 0.001$ for both). Furthermore, there was a strong positive correlation between serum TxB_2 and u11-dh TxB_2 levels ($r = 0.70$, $p < 0.001$) and inhibition ($r = 0.91$, $p < 0.001$) (Fig. 1a).

The relation between serum TxB_2 inhibition and AA-PA was stepwise (Fig. 1b). There was approximately 70% AA-PA observed despite the presence of up to 40% serum TxB_2 inhibition. Once 40% inhibition of serum TxB_2 was exceeded, AA-PA fell to < 5%. ROC curve analysis predicted > 49% inhibition of serum TxB_2 for < 5% AA-PA, our definition for aspirin responsiveness. This level of serum TxB_2 inhibition is much lower than the previously quoted requirement of > 95% inhibition of serum TxB_2 for the clinical efficacy of ASA [1]. Wide variability was observed in the relation between serum TxB_2 inhibition and ADP- and collagen-PA (Fig. 1c and d). There was an overall reduction in ADP-induced PA with greater levels of TxB_2 inhibition. By ROC curve analysis, u11-dh TxB_2 < 1520 pg/mg met the

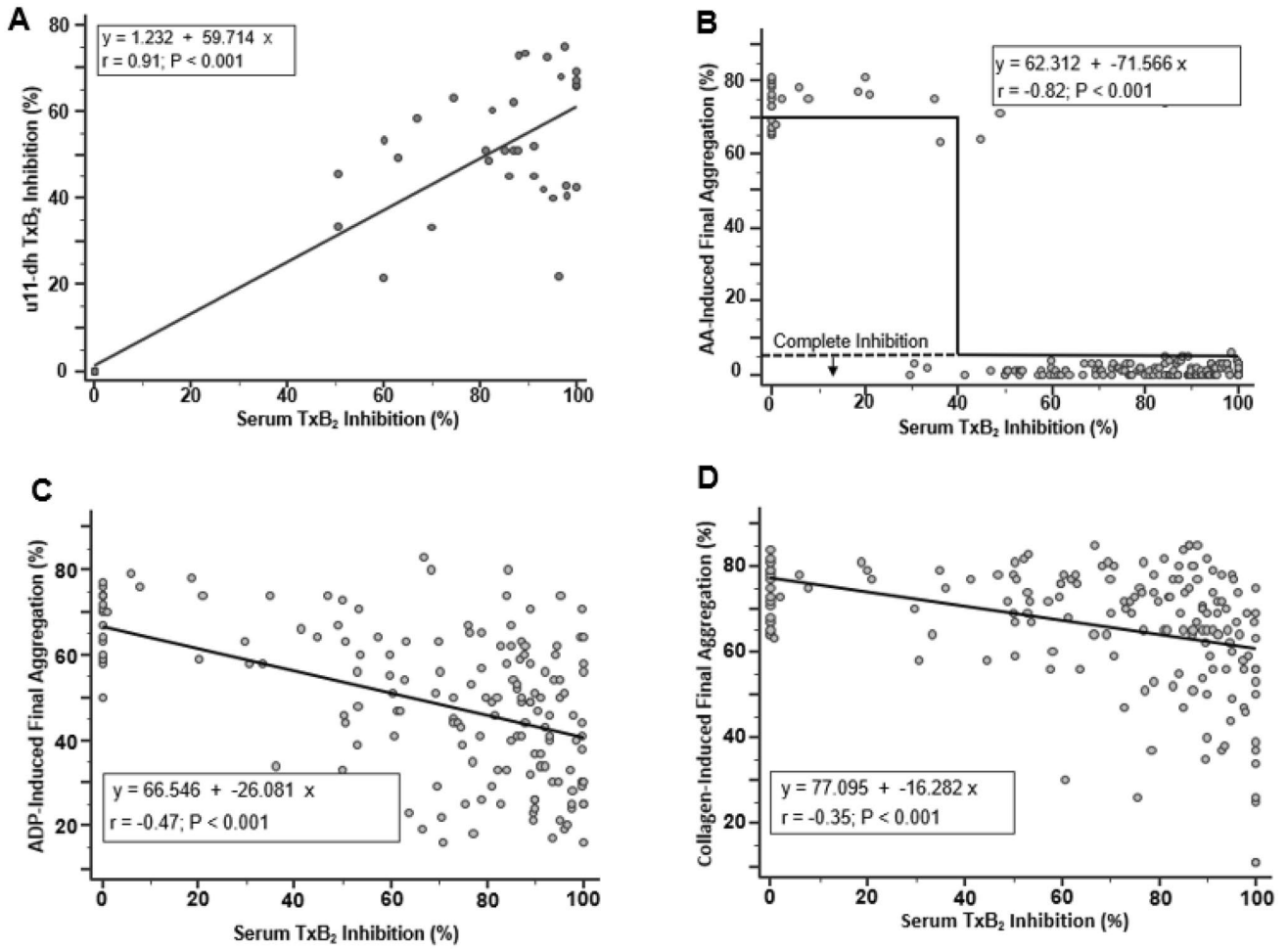

Fig. 1 **a** The relation between serum thromboxane B_2 and u11-dehydro thromboxane B_2 inhibition*, **, **b** The relation between AA-induced final platelet aggregation and serum thromboxane B_2 inhibition (**c**) adenosine diphosphate- and **d** collagen-induced final platelet aggregation and serum thromboxane B_2 inhibition *AA* arachidonic acid, *PA* platelet aggregation, *ADP* adenosine diphosphate. *Inhibition refers to inhibition of baseline (pre-treatment levels). ** Percent inhibition of urinary 11-dehyro thromboxane B_2 was determined at 4 and 24 hours post-ASA administration

definition of aspirin responsiveness (Table 1). Figure 2a and b shows the ASA and SA levels over 24 hours demonstrating greater earlier exposure with I-ASA. Complete inhibition of AA-PA was observed at plasma ASA and SA levels as low as 344 and 243 ng/mL, respectively.

It is generally accepted that AA activates platelets by generating TxA_2 by COX-1 and thromboxane synthase activity. AA-PA is potently inhibited by COX-1 blockade from ASA. Platelet activation and aggregation induced by TxA_2 and ADP play synergistic and important roles during arterial thrombus formation in the presence of high shear [10]. In addition, collagen-induced platelet activation has been shown to induce AA release and TxA2 generation by COX-1 activity in platelets [7]. Similarly, in the current study, serum TxB_2 inhibition was inversely but weakly associated with ADP- and collagen-induced platelet aggregation.

Debate exists in the literature regarding the extent of serum TxB_2 inhibition needed for arterial thrombosis prevention, with high levels of serum TxB_2 inhibition (> 95%) proposed to have clinical efficacy for aspirin [3, 4]. Earlier, a linear relation between AA-PA and serum TxB_2 generation was demonstrated in an *in vitro* assay using a 96 well plate platelet aggregation assay [4]. Our current *ex vivo* investigation evaluating the relation of serum TxB_2 to platelet function may elucidate potential ASA efficacy and safety at lower levels of TxB_2 inhibition. Finally, here we show that only modest inhibition of serum TxB_2 (> 49%) is sufficient before profound inhibition of AA-PA occurs.

An optimal laboratory assay that correctly reflects an *in vivo* pharmacodynamic (antiplatelet) effect of aspirin and its relation to clinical outcome (anti-ischemic effect) is still being debated. The precise role of thromboxane A_2 generated by exogenous AA during platelet aggregation in the

Table 1 Receiver operating characteristic curve analysis

	Criterion	Area under the curve	Sensitivity (%)	Specificity (%)	p-value
Serum thromboxane B_2 Inhibition (%)	> 49	0.82	96.4	64.3	< 0.001
Serum thromboxane B_2 (ng/mL)	< 100	0.76	86.2	67.7	< 0.001
Urinary 11-dehydro thromboxane B_2 (pg/mg creatinine)	< 1520	0.92	76.0	100	< 0.001

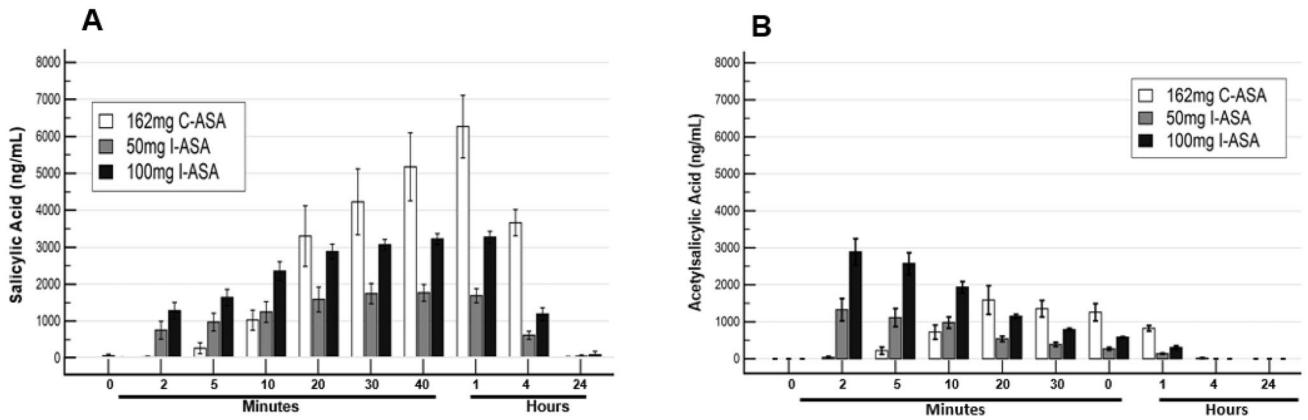

Fig. 2 Acetylsalicylic acid (**a**) and salicylic acid (**b**) levels over 24-hr time period by treatment. Data presented as mean ± SEM. *AA* arachidonic acid, *ADP* adenosine diphosphate, *C-ASA* chewed and swallowed acetyl salicylic acid, *I-ASA* inhaled acetyl salicylic acid

presence of physiologic calcium (Ca^{++}) concentrations is also controversial. The endogenously generated TxA_2 may amplify platelet activation by stimulating Ca^{++} release in the presence of weak agonists such as shear-stress or low-dose collagen rather than playing a significant role in platelet aggregation [11]. Furthermore, serum thromboxane is a capacity term that is used to describe the pharmacological potency of aspirin to block platelet COX-1-mediated thromboxane formation. Although it determines the time-independent thromboxane-forming capacity of platelets when blood clots in a test tube, it has no natural correlate *in vivo*. U 11-dh TxB_2, in addition to platelet COX-1 activity, also reflects a low-grade inflammatory process of atherosclerosis and activation of inflammatory white cells [1].

Conclusions

Our study demonstrates a non-linear relation between inhibition of serum TxB_2 and AA-PA, where aggregation was nil once TxB_2 inhibition reached > 49%. This observation is important for translational research that attempts to link the antithrombotic efficacy of ASA with a laboratory measurement cutoff. Moreover, these results suggest that the definition of > 95% inhibition of serum TxB_2 to indicate the level of platelet COX-1 inhibition needed for clinical efficacy may be overestimated and should be re-considered in future investigations.

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Author contributions PAG contributed to concept and design, interpretation of data; critical writing or revising the intellectual content; and final approval of the version to be published. KPB and UST contributed to concept and design, collection and interpretation of data; critical writing or revising the intellectual content.

Compliance with ethical standards

Conflict of Interest Dr P. A. Gurbel reports grants and personal fees from Bayer and grants and personal fees from Otitopic during the conduct of the study; grants from Instrumentation Labs, Haemonetics, Amgen, Medicure, Janssen, Idorsia, and Hikari Dx and personal fees from Amgen, Janssen, and UpToDate outside the submitted work; in addition, Dr Gurbel has a patent to Detection of restenosis risk in patients issued and a patent to Assessment of cardiac health and thrombotic risk in a patient pending. Dr. U. S. Tantry reports personal fees from UpToDate. Mr. K. P. Bliden reports no conflict of interest.

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