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Effect of a single dose aspirin on platelets in humans with multiple risk factors for coronary artery disease

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Abstract

We sought to assess how one tablet of non-enteric coated aspirin (325 mg) affects human platelets in subjects with risk factors for coronary artery disease. Data from 63 individuals with multiple cardiac risk factors were analyzed. Platelets were assessed twice at baseline (pre-aspirin), and after 3–4 h (post-aspirin). We employed 5 μM epinephrine-induced conventional aggregometry, closure time with epinephrine/collagen cartridge by PFA-100® (Dade-Behring), and aspirin response units (ARU) stimulated by propyl gallat with Ultegra® (Accumetrics, San Diego, CA, USA) for measuring platelet function. In addition, the expression of platelet receptors was determined by using the following monoclonal antibodies: anti-CD31, CD41, CD42b, CD51/CD61, CD62p, CD63, CD107a, and CD151. Platelet–leukocyte formation was detected utilizing dual antibodies for a pan-platelet marker CD151, and CD14, a monocyte/macrophage marker. PAC-1 was used to measure fibrinogen–platelet binding. One pill of aspirin significantly decreased platelet-rich plasma (PRP) aggregation ($74.18 \pm 16.75\%$ vs. $24.92 \pm 8.64\%$; $p < 0.0001$) and resulted in reduction of the aspirin response units (ARU) (662.24 ± 65.65 vs. 451.05 ± 69.31; $p < 0.0001$). There was also prolongation of the closure time (194.4 ± 25.3 vs. 258.63 ± 55.61 s; $p < 0.0001$). High correlation ($r^2 = 0.73–0.86$) between platelet analyzer readings and aggregation was observed. One tablet of aspirin moderately inhibited expression of most surface platelet receptors measured, and such inhibition reached significance ($p < 0.05$) for PAC-1, CD31, CD41, CD42, CD62p, and CD151. We conclude that a single dose of aspirin affects major platelet receptors, presumably directly or indirectly through the inhibition of prostanoids via platelet cyclooxygenase-1 blockade. The Ultegra® Analyzer with a novel cartridge seems to be reliable in reflecting aspirins' effects on platelets and could be used in the future in clinical practice for monitoring aspirin therapy.

Keywords: Platelet; Aspirin; Receptor; Coronary artery disease

1. Introduction

Aspirin has been known since 1899 (Anonymous, 1899). In addition to platelet inhibition, cardioprotective effects of aspirin are well documented. Aspirin has been shown to reduce the risk of myocardial infarction in subjects with stable or unstable angina (Antiplatelet Trialists' Collaboration, 1994; Lewis et al., 1983). The antithrombotic and life saving properties of aspirin are believed to be due to inhibition of platelet function via blockade of essential eicosanoids. Aspirin acts by irreversibly acetylating enzyme cyclooxygenase-1. Aspirin selectively inhibits the cyclooxygenase-1 isoform, which converts arachidonic acid to prostaglandin H_2. Aspirin primarily exerts its antiplatelet activity through inhibition of thromboxane A_2 synthesis that is irreversible for the life of treated platelets. There are some reports suggesting that platelet-inhibiting effects of aspirin are not solely related to prostaglandin blockade. Surprisingly, the effect of aspirin on cell surface receptors remains unclear. Preliminary data from our laboratory suggest that aspirin in vitro inhibits surface glycoprotein IIb/IIIa, P-selectin, CD63, and CD107a receptor expression on human platelets (Malinin et al., 2001). To support these in vitro data, we conducted an ex vivo study questioning how one tablet of aspirin (325 mg) will affect human platelets in volunteers with multiple risk factors for cardiovascular disease. We performed a comprehensive analysis of platelet function including aggregometry, flow cytometry, and novel platelet

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analyzers before and after ingestion of one aspirin (325 mg) pill.

2. Materials and methods

2.1. Study population

Sixty-three volunteers enrolled at the Sinai Center for Thrombosis Research into an Aspirin Response Study (Accumetrics, San Diego, CA, USA) were analyzed. This study was approved by the Sinai Hospital Institutional Review Board. Subjects were eligible if they had met all of the following inclusion criteria: males and females 21 years and older; conscious and coherent; a history of vascular disease or two out of the eight well-known risk factors for vascular disease (i.e., family history of vascular disease; sedentary lifestyle; diabetes mellitus; hypertension; morbid obesity; known history of hypercholesterolemia; post-menopausal or surgically sterile females; current smokers or recent smokers); available and willing to return for follow-up tests, and those who signed informed consent. Subjects were ineligible for the study if they met any of the following criteria: participation in another clinical trial of an investigational drug within the past 30 days; currently receiving aspirin therapy or any medications containing aspirin within the past 2 weeks; have received any antiplatelet therapy including glycoprotien IIb/IIIa inhibitors, or thienopyridines within the past 2 weeks; known history of platelet count $< 100 \times 10^9$/l; or blood dyscrasia; gastrointestinal ulcer, and bronchial asthma. Blood samples were obtained twice for this study, first at baseline (pre-aspirin), and then after 3–24 h following the first draw (post-aspirin). All subjects received one 325 mg non-enteric coated aspirin. For each time point, volunteers had blood drawn into four 1.8 ml tubes and three 4.5 ml tubes containing 3.2% citrate for the platelet studies, and one 2.0 ml tube containing tripotassium ethylene diamine tetraacetic acid (K_3EDTA) used for a complete cell count. The actual tests were performed at least 30 min after the sample collection but prior to 4 h after the draw.

2.2. Platelet aggregation

The citrate mixture was centrifuged at 1500 rpm for 10 min in order to obtain platelet-rich plasma (PRP) which was always kept at room temperature. Platelet counts were determined for each platelet-rich plasma sample with a Coulter Counter (Coulter, Hialeah, FL, USA). Platelet counts were adjusted to 3.5×10^8/ml with autologous platelet-poor plasma. Platelet aggregation was induced by 5 μM epinephrine (Chrono-log, Havertown, PA, USA). Platelet aggregation was determined by using a four-channel Chrono-log Lumi-Aggregometer and expressed as the maximum percentage of light transmittance change from the baseline at the end of the recording time. Platelet-poor plasma was used as a reference. Platelet aggregability curves were recorded for 8 min and analyzed according to internationally established standards (Ruggeri, 1994).

2.3. Cartridge-based platelet analyzers

The PFA-100™ (Dade Behring, Deerfield, IL, USA) is a cartridge-based device that simulates primary hemostasis after injury to a small vessel under flow conditions (Kundu et al., 1995). The time required to obtain occlusion of the aperture is digitally recorded as a measure of shear-induced platelet aggregation. Closure time determinations were performed in quadruplicate. A rapid platelet-function assay aspirin cartridge test (RPFA-ASA, Ultegra® Accumetrics) with propyl gallate as agonist was also used. Cationic propyl gallate is novel platelet activator that used to detect and monitor the amount of platelet inhibition induced by platelet prostaglandin antagonist, including aspirin and other non-steroid anti-inflammatory drugs (Schwartz et al., 2002). The Ultegra® System is a turbidimetric based optical detection system which measures platelet-induced aggregation as an increase in light transmittance. The test cartridge contains a lyophilized preparation of human fibrinogen-coated beads, platelet agonist, buffer, and preservative. Fibrinogen-coated microparticles are used in the Ultegra® RPFA-ASA cartridge to bind to available platelet receptors. When the activated platelets are exposed to the fibrinogen-coated microparticles, agglutination occurs in proportion to the number of available platelet receptors. To ensure consistent and uniform activation of the platelets, cationic propyl gallate is incorporated into the assay cartridge to induce platelet activation without fibrin formation. The Ultegra® Analyzer is designed to measure this agglutination as an increase in light transmittance. The whole blood citrate mixture is being added to the cartridge, and agglutination between platelets and coated beads is being recorded. Ultegra® RPFA-ASA assay results are reported as aspirin response units (ARU).

The data mirror turbidometric platelet aggregation and reflect the degree of platelet prostaglandin blockade (Smith et al., 1999). Ultegra® assays were performed in duplicate. An electronic quality control test was performed on each instrument every day prior to performing any subject samples.

2.4. Whole blood flow cytometry

Blood for flow cytometry was obtained from 22 out of 63 study subjects. The expression of platelet receptors was determined by using the following monoclonal antibodies: anti-CD41 (glycoprotein [GP] IIb/IIIa, α IIb β_3), CD42b (GP Ib), CD62p (P-selectin), CD51/CD61 (α_v β_3, or vitronectin receptor), CD31 (platelet endothelial cell adhesion molecule (PECAM-1), CD107a (lysomal associated membrane protein-1, LAMP-1), CD63 (lysomal associated membrane protein-3, LAMP-3), and CD151 (platelet endothelial cell tetraspan antigen-3, PETA-3) and PAC-1 for fibrinogen–

Table 1
Demographic and risk factors

Parameter	
Age, years	43.16 ± 9.5
Male	19 (30%)
Female	44 (70%)
Ethnic origin	
White	39 (62%)
African–American	23 (37%)
Other	1 (1.6%)
Smoking status	
Current	18 (29%)
Quit <6 months	1 (1.6%)
Quit >6 months	16 (25%)
Never smoked	28 (44%)
Vascular risk factors	
Family history of CAD	43 (68%)
Sedentary lifestyle	17 (27%)
Diabetes mellitus	10 (16%)
Hypertension	17 (27%)
Morbid obesity	2 (3.2%)
Hypercholesterolemia	24 (38%)
Post-menopausal female	13 (21%)

platelet binding determination (PharMingen, San Diego, CA). Platelet–leukocyte interactions were assessed by using dual antibodies for a pan-platelet marker (CD151), together with CD14, a monocyte/macrophage marker. The blood–citrate mixture (50 μl) was diluted with 450 μl Tris buffered saline (10 mmol/l Tris, 0.15 mol/l sodium chloride) and mixed by gently inverting an Eppendorf tube two times. Five microliters of the different antibodies were then added to each solution and samples were incubated for 30 min. After incubation, 400 μl of 2% buffered paraformaldehyde was added for fixation. The samples were analyzed on a Becton Dickinson FACScan flow cytometer set up to measure fluorescent light scatter, as previously described (Gurbel et al., 1998). The data were collected in list mode files and then analyzed. P-selectin was expressed as percent positive cells as previously described (Gurbel et al., 2000). Other antigens were expressed as *log* mean fluorescence intensity.

2.5. Statistical analysis

All comparisons were calculated by *t*-test to identify specific differences in platelet aggregation, results of Ultegra®, Dade-PFA 100™, and receptor expression between baseline and post-aspirin values. The Mann–Whitney *U*-test was used to analyze non-parametric data. Normally distributed data were expressed as mean ± S.E., and skewed data as median (range). Probability values of $p < 0.05$ were regarded as statistically significant. Linear regression analysis was applied to normally distributed data for all study participants, using the SPSS v 9.0 software (SPSS, Chicago, IL).

3. Results

3.1. Volunteers

Sixty-three participants without a history of bleeding disorders, who had at least two risk factors of vascular disease, and were at least free of pharmacologic agent use for 2 weeks, were enrolled in the study. From among them, 19 were current smokers, 16 had quit smoking more than 6 months ago, and 28 have never smoked. Demographic data are presented in Table 1.

Baseline blood samples were drawn between 8 and 12 a.m. to avoid diurnal influences. The post-aspirin blood was collected 3–6 h after aspirin administration in the vast majority of subjects. In three study participants, the follow-up samples were collected at 24 h post-aspirin. To avoid possible observer bias, blood samples were coded and blinded. Individuals unaware of the protocol performed sampling procedures and platelet studies.

3.2. Platelet characteristics

Combined data of the cartridge-based platelet analyzers, platelet aggregation, and flow cytometry data are presented in Table 2.

The single dose of aspirin significantly reduced platelet-rich plasma aggregation induced by 5 μM epinephrine, and resulted in reduction of the aspirin response units (ARU) assessed by the Ultegra® Platelet Analyzer and prolongation of the closure time with the PFA-100® instrument. Correlation between Ultegra®, PFA-100, and platelet aggregation are presented in Fig. 1A–C. A statistically significant change of GP IIb/IIIa, Ib, P-selectin, PECAM-1, CD151 and PAC-1 expression was observed between the baseline and the post-aspirin samples. Other markers of platelet activation (CD 51/61, CD 63, CD 107a and CD151 + CD14) also were dimin-

Table 2
Effects of a single dose aspirin on human platelets

Variable	Baseline	Post-aspirin	*p*-value
Platelet aggregation 5 μM epinephrine (%)	74.18 ± 16.75	24.92 ± 8.64	$p < 0.00005$
PFA-100 cartridge (s)	119.40 ± 25.30	258.63 ± 55.61	$p < 0.00005$
RPFA-ASA (ARU)	662.24 ± 65.65	451.05 ± 69.31	$p < 0.00005$
CD31 (MFI)	46.96 ± 10.77	39.35 ± 10.32*	0.0277
CD41a (MFI)	125.12 ± 25.18	105.46 ± 21.08*	0.027
CD42b (MFI)	108.72 ± 25.7	95.9 ± 24.87*	0.046
CD62p (%+)	3.5 ± 0.72	2.8 ± 0.76*	0.046
CD63 (MFI)	2.11 ± 0.56	1.84 ± 0.53	0.172
CD107a (MFI)	3.04 ± 0.78	2.63 ± 1.028	0.64
CD151 (MFI)	50.36 ± 13.17	44.13 ± 14.43*	0.027
CD151 + CD14 (MFI)	52.86 ± 18.01	48.56 ± 17.98	0.079
PAC-1 (MFI)	3.24 ± 0.87	2.37 ± 0.96*	0.02775
Unstained cells (MFI)	0.98 ± 0.34		

Data presented as mean ± S.D.
Abbreviations are as follows: ARU = aspirin reaction units; MFI = mean fluorescence intensity; % + = percent positivity.
* Significant difference between the baseline and post-aspirin level.

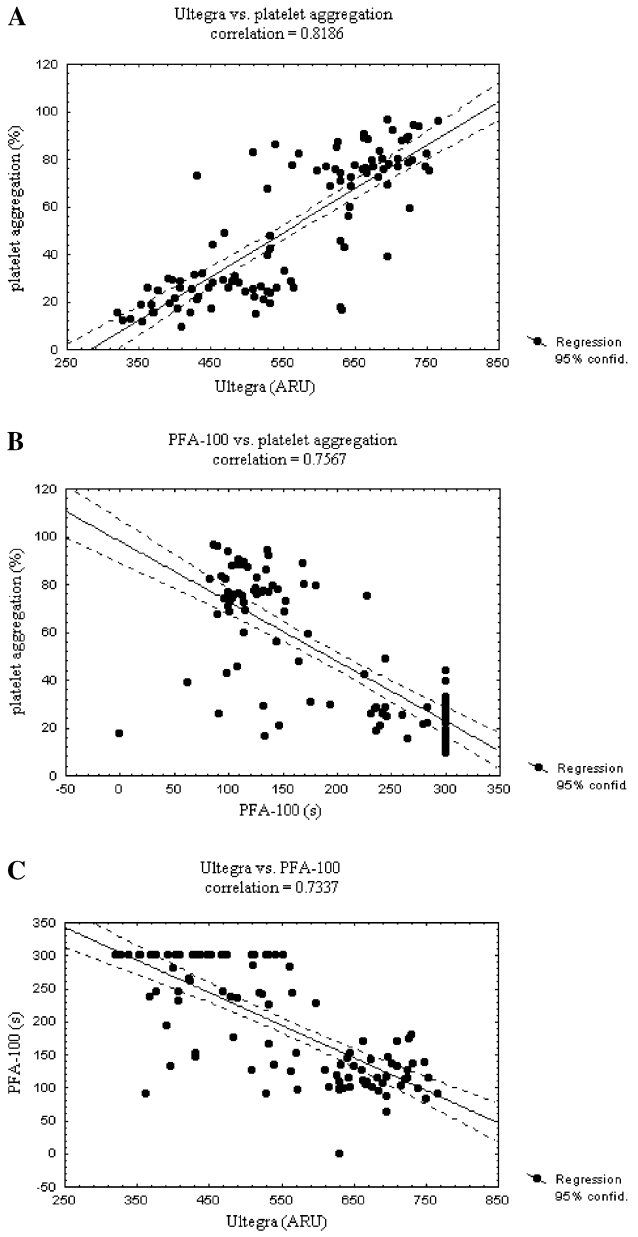

Fig. 1. (A–C) Graphs showing the correlation between Ultegra, PFA, and platelet aggregation.

ished between baseline and post-aspirin samples, however, these changes did not reach statistical significance.

4. Discussion

This study revealed substantial platelet inhibition after one 325 mg aspirin tablet in subjects with existing multiple risk factors for vascular disease. All applied techniques for platelet assessment were consistent supporting these changes. Indeed, platelet aggregation decreased almost 75% as compared with the baseline values, the closure time more than doubled (from 119 to 258 s) and frequently exceeded 300 s, the maximal reference range in the post-aspirin samples. We also observed the decrease of aspirin response units (ARU) in each and every subject (from 666 to 451 ARU) between the baseline and post-aspirin readings. Strong correlation between the above-mentioned techniques, including platelet aggregometry, allows us to suggest that the Ultegra® instrument could be successfully used for monitoring of aspirin therapy, as well as PFA-100. Both of above-mentioned methods have been approved by the United Stated Food and Drug Administration. Our study has also revealed that just one tablet of aspirin resulted in inhibition of GP IIb/IIIa and PAC-1, GPIb, P-selectin, PECAM-1 and CD151. Despite the fact that changes between the baseline and post-aspirin samples for flow cytometry were not so dramatically altered as the aggregometry data, there was enough change to produce statistical differences. It is interesting to note that expression of other known platelet receptors (vitronectin, CD63, CD107a, formation of platelet–leukocyte microparticles) was also diminished after therapy with aspirin. Our findings can help in understanding the nature of aspirins' high efficacy. Platelet receptor expression could be decreased via the direct aspirin action targeting certain receptors—or indirectly by inhibiting protein synthesis, and/or cell metabolism in general. It has recently been reported that aspirin inhibits P-selectin expression via thromboxane synthesis (Valles et al., 2002), while its effect on the expression of GP IIb/IIIa could be directly related to tyrosine phosphorylation of platelet proteins that is cyclooxygenase-1 independent (Santos et al., 2000). Taken together, it is becoming increasingly obvious that aspirin is producing universal antiplatelet properties which are probably multi-factorial.

In conclusion, aspirin, the oldest antiplatelet agent in use, affects key platelet characteristics involved in thrombogenesis and development of vascular pathology. In subjects with risk factors for vascular disease, just one tablet of aspirin decreases aggregation, analyzer readings, and receptor expression on human platelets. The unique properties of aspirin provide additional synergism in combination with other antiplatelet agents (Diener et al., 1996; CAPRIE Steering Committee, 1996; Yusuf et al., 2001). We do not know how exactly the effect of aspirin on platelet surface receptors relates to the exclusive inhibition of prostanoids via platelet cyclooxygenase-1 blockade. It is conceivable that apparently independent additional mechanisms of aspirins' action are involved. Recognizing the unique and universal properties as well as fast efficacy of just one pill of aspirin, the notion of an effective and safe antiplatelet protection by aspirin in patients with coronary artery disease is confirmed. For monitoring purposes during aspirin therapy, a new modification of the Ultegra® Analyzer as well as PFA-100 is promising regarding a possible future implementation in clinical practice.

5. Limitations

There was no control group with normal subjects without any risk factors of coronary artery disease in out observation,

even we suppose that aspirin will produce the same effect on this population

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