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Multifaceted effects of arachidonic acid and interaction with cyclic nucleotides in human platelets

Natalia Rukoyatkina[a], Valentina Shpakova[a], Michael Panteleev[b], Alexandra Kharazova[c], Stepan Gambaryan[a,c], Joerg Geiger[d,*]

[a] Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences St. Petersburg, Russia
[b] Center for Theoretical Problems of Physico-Chemical Pharmacology, Russian Academy of Sciences, Moscow, Russia
[c] Department of Cytology and Histology, St. Petersburg State University, St. Petersburg, Russia
[d] Interdisciplinary Bank of Biomaterials and Data, Wuerzburg, Germany

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ABSTRACT

Introduction: Arachidonic acid induced aggregation is a generally accepted test for aspirin resistance. However, doubts have been raised that arachidonic acid stimulated aggregation can be regarded as reliable testing for aspirin resistance. Arachidonic acid, in addition to platelet activation, can induce phosphatidylserine translocation on the outer surface of platelet membrane which could be mediated by apoptosis pathways or transformation of platelets to the procoagulant state.

Materials and methods: We explored effects of arachidonic acid over a vast range of concentrations and a wide range of read-outs for human platelet activation, procoagulant activity, and platelet viability. Additionally we tested whether cAMP- or cGMP-dependent protein kinase activation can inhibit procoagulant activity or platelet viability.

Results: Arachidonic acid-induced washed platelet activation was detected at low micromolar concentrations during the first 2 min of stimulation. After longer incubation and/or at higher concentrations arachidonic acid triggered platelet procoagulant activity and reduced platelet viability. At the same time, arachidonic acid stimulated adenylate cyclase mediated protein phosphorylation which correlated with reduced platelet activation. Moreover, additional stimulation of cAMP- or cGMP-dependent protein kinase inhibited only platelet activation, but did not prevent pro-coagulant activity and platelet death.

Conclusions: While arachidonic acid induces platelet activation at low concentrations and during short incubation time, higher concentrations and lasting incubation evokes adenylate cyclase activation and subsequent protein phosphorylation corresponding to reduced platelet activation, but also enhanced pro-coagulant activity and reduced viability. Our observations provide further proof for the complex fine tuning of platelet responses in a time and agonist concentration dependent manner.

1. Introduction

Platelets play an important role in propagating coagulation reactions within developing thrombi. Usually patients receive a combined therapy with aspirin, clopidogrel or ticagrelor and oral anticoagulants preventing platelet aggregation and balancing bleeding and risk for stroke. Aspirin as an inhibitor of cyclooxygenase (COX) and thromboxane A2 (TXA_2) production is the current standard of care, reducing the risk of cardiovascular diseases by about 25% [1]. However, approximately one quarter of patients with cardiovascular diseases who

Abbreviations: AA, arachidonic acid; AM, acetoxymethyl; CalDAG-GEFI, calcium- and DAG-regulated guanine nucleotide exchange factor 1; COX, cyclooxygenase; DAG, diacylglycerol; FITC, fluorescein isothiocyanate; FSC, forward scatter; G_S, stimulatory G-protein; IP_3, inositol 1,4,5 trisphosphate; LOX, lipoxygenase; NO, nitric oxide; PDE, phosphodiesterase; PE, phycoerythrin; PGD_2, prostaglandin D_2; PGE_2, prostaglandin E_2; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PRP, platelet rich plasma; PS, phosphatidylserine; ROS, reactive oxygen species; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; SSC, side scatter; TMEM16F, transmembrane protein 16F; TP, thromboxane receptor; TXA_2, thromboxane A_2; VASP, vasodilator-stimulated phosphoprotein; WP, washed platelets; Xkr8, Xk-related protein family member

* Corresponding author at: Interdisciplinary Bank of Biomaterial and Data Wuerzburg, University of Wuerzburg, Straubmuehlweg 2a/Bldg. A9, D-97080 Wuerzburg, Germany.
E-mail address: joerg.geiger@uni-wuerzburg.de (J. Geiger).

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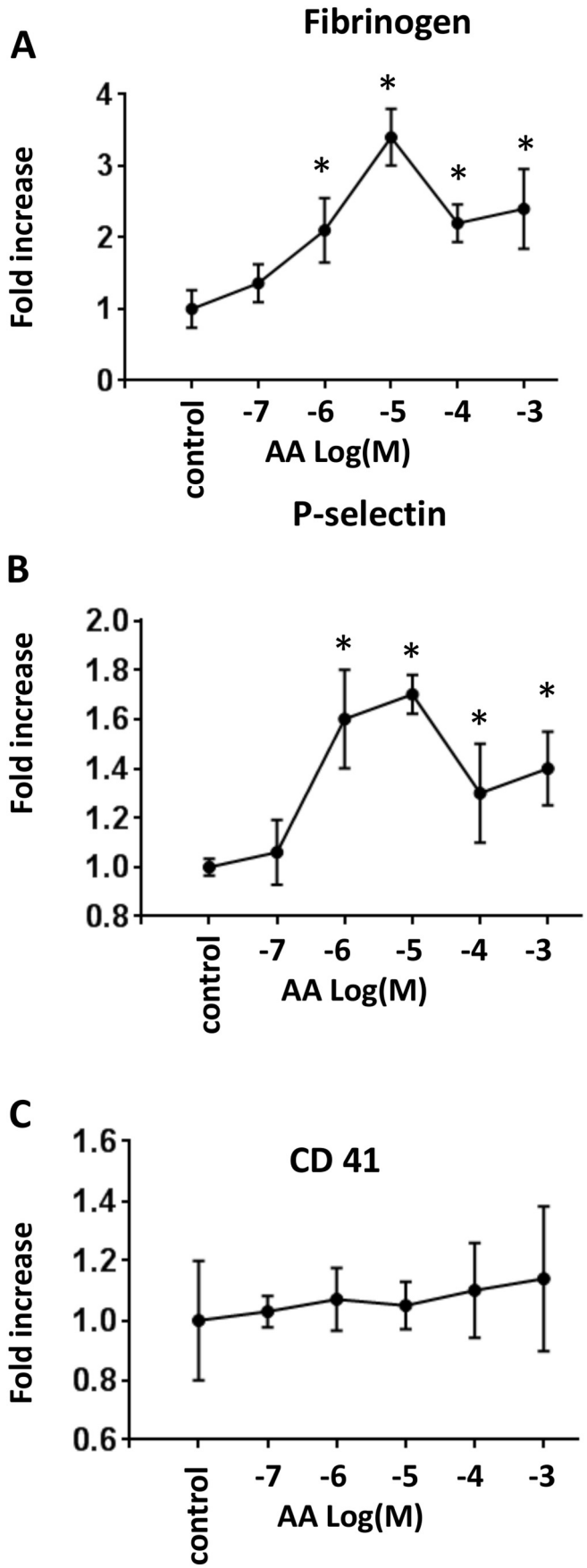

Fig. 1. Dose-dependent changes of AA-induced integrin $\alpha_{IIb}\beta_3$ activation and P-selectin exposure.
(A) Flow cytometric analysis of activated $\alpha_{IIb}\beta_3$ integrin determined by binding of fluorophore-labelled fibrinogen, (B) P-selectin surface exposure, and (C) total $\alpha_{IIb}\beta_3$ integrin content (detected with CD41 antibody) of AA-stimulated platelets. Washed platelets (WP 1×10^8/ml) were incubated with fluorophore-labelled fibrinogen (A), P-selectin-PE (B) or CD41-FITC (C) antibodies, treated by different AA concentrations during 2 min and then fixed, washed, and analyzed by flow cytometry. Maximum platelet activation was observed with 10 μM AA. Platelet activation started to decrease at AA concentration $\geq 10^{-4}$ M. Data are presented as means ± SD, n = 7,*, $p < 0.05$ compared to controls taken as 1.

receive aspirin appear to have an inadequate response to aspirin [2]. Insufficient inhibition of platelet aggregation due to aspirin non-responsiveness accounts for a significantly increased risk of major cardiovascular events [3]. Arachidonic acid (AA)-induced aggregation is the widely accepted standard test to control for aspirin resistance. In platelet rich plasma (PRP), binding to serum proteins can diminish the effective concentration of arachidonic acid [4]. Moreover, arachidonic acid induces aspirin-insensitive platelet activation at high concentrations [5,6]. Accordingly, there are doubts whether AA-induced platelet activation can be used as a reliable diagnostic test [7]. In platelets, AA is released by activation of soluble phospholipase A_2 and metabolized by cyclooxygenases (COX) and lipoxygenases (LOX) finally leading to the formation of thromboxane A_2 (TXA_2), prostaglandin D_2 (PGD_2) and E_2 (PGE_2) which have opposite effects on platelet function [8–12]. TXA_2 is a well-established stimulant of platelet aggregation while PGD_2 triggers the formation of cAMP [8,10,13], thus stimulating one of the major platelet inhibitory pathways. PGE_2 concentration dependently stimulates either a G_i- or G_s-protein thus either amplifying or preventing platelet activation in a concentration dependent manner [8,14]. Similarly, LOX metabolites were shown to have anti- and pro-thrombotic properties however, the molecular mechanisms of 12-HETE action on platelet functions are still controversial and remain incompletely understood (reviewed in [15,16]). Moreover, AA can bring platelets to a coagulant state and induce exposure of phosphatidylserine (PS) on their surface [17,18], hence initiating the blood coagulation system [19]. PS externalization is also a marker for platelet apoptosis and death. Apoptotic platelets show impairment of activation despite PS exposure and thrombin generation [20,21]. Yet it is not clear whether AA-induced PS surface exposure is mediated by caspase-dependent platelet apoptosis, or transformation of platelets to a pro-coagulant state, and whether AA affects platelet viability.

Cyclic nucleotides (cAMP and cGMP) and their corresponding protein kinases cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG) represent the major platelet inhibitory mechanisms affecting almost any agonist-induced platelet activating pathway including calcium signaling, integrin activation, degranulation, shape change, adhesion, aggregation, etc. [22,23]. The inhibition is mediated by phosphorylation of several proteins including the inositol 1,4,5 trisphosphate (IP_3) receptor, vasodilator-stimulated phosphoprotein (VASP), thromboxane receptor, and calcium- and DAG-regulated guanine nucleotide exchange factor 1 (CalDAG-GEFI, RASGRP2) by PKA and/or PKG [24–26]. Consequently a number of pharmacologic agents modulating cyclic nucleotide regulation have emerged for the treatment of cardiovascular diseases [27–30]. Recently, activation of PKA has been shown to protect platelets from apoptosis and clearance induced by different pathological stimuli [31]. However, it is not known whether PKA/PKG activation can inhibit platelet pro-coagulant activity and influence platelet viability induced by AA. Here we show that AA-induced washed platelet activation occurs at low micromolar concentrations during the first 2 min of stimulation. Prolonged incubation and/or higher concentrations of AA trigger platelet pro-coagulant activity and reduce platelet viability. At the same time, AA stimulates adenylate cyclase and induces VASP phosphorylation, both of which are correlated with diminished platelet