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Reciprocal regulation of human platelet function by endogenous prostanoids and through multiple prostanoid receptors ☆

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ABSTRACT

Platelets are permanently exposed to a variety of prostanoids formed by blood cells or the vessel wall. The two major prostanoids, prostacyclin and thromboxane act through well established pathways mediated by their respective G-protein coupled receptors inhibiting or promoting platelet aggregation accordingly. Yet the role of other prostanoids and prostanoid receptors for platelet function regulation has not been thoroughly investigated. We aimed at a comprehensive analysis of prostanoid effects on platelets, the receptors and pathways involved and functional consequences. We analyzed cAMP formation and phosphorylation of proteins pivotal to platelet function as well as functional platelet responses such as secretion, aggregation and phosphorylation. The types of prostanoid receptors contributing and their individual share in signaling pathways were analyzed and indicated a major role for prostanoid IP_1 and DP_1 receptors followed by prostanoid EP_4 and EP_3 receptors while prostanoid EP_2 receptors appear less relevant. We could show for the first time the reciprocal action of the endogenous prostaglandin PGE_2 on platelets by functional responses and phosphorylation events. PGE_2 evokes stimulatory as well as inhibitory effects in a concentration dependent manner in platelets via prostanoid EP_3 or EP_4 and prostanoid DP_1 receptors. A mathematical model integrating the pathway components was established which successfully reproduces the observed platelet responses. Additionally we could show that human platelets themselves produce sufficient PGE_2 to act in an autocrine or paracrine fashion. These mechanisms may provide a fine tuning of platelet responses in the circulating blood by either promoting or limiting endogenous platelet activation.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; AC, adenylyl cyclase; cAMP, adenosine 3′,5′-cyclic monophosphate; 6-Cl-PuDP, 6-chloro purine 5′ diphosphate; DMSO, dimethyl sulfoxide; ERK, extracellular signal regulated kinase; Gi, inhibitory G-protein; Gs, stimulatory G-protein; GST, glutathion-S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IGEPAL, octylphenoxypolyethoxyethanol; MAPK, mitogen activated protein kinase; OD, optical density; PBS, phosphate buffered saline; PGI2, prostaglandin I2, prostacyclin; PKA, cAMP dependent protein kinase; PKB, protein kinase B; PPP, platelet poor plasma; PRI, platelet reactivity index; PRP, platelet rich plasma; RalGDS, ral guanine nucleotide dissociation stimulator; Rap1, ras related protein 1; RBD, rap binding domain; SDS, sodium dodecyl sulfate; TBAS, tetrabutylammonium hydrogen sulfate; TMB, 3,3′,5,5′- tetramethylbenzidine; Tris, 2-amino-2-hydroxymethyl-propane-1,3-diol; TXA2, thromboxane A2; VASP, vasodilator stimulated phosphoprotein; WP, washed platelets

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1. Introduction

Platelets are regarded a major player in the development of atherothrombosis and cardiovascular diseases originating there from (Linden and Jackson, 2010). Accordingly prevention of platelet activation and aggregation has attracted significant interest as pharmacological target in the prevention and treatment of atherothrombotic events (Patrono and Rocca, 2010). Understanding the complex cross regulation of platelet stimulatory and inhibitory pathways is fundamental to the development of an efficient and specific anti-platelet treatment.

In the intact blood vessel platelets are continually exposed to platelet activating substances present in the circulating blood. However, endogenous factors and enzymes preventing platelet aggregation counterbalance the pro-aggregatory factors (Jin et al., 2005). Two prostanoids are central to this reciprocal regulation of platelet function, prostaglandin I_2 (prostacyclin, PGI_2) and thromboxane A_2 (TXA_2).

Both prostanoids are short-lived molecules which are not stored but produced and released in situ. The short lifespan entails strictly temporally and spatially confined action of the prostanoids, thus offering a fine tuning of cellular function regulation. Stimulation of the platelet prostacyclin receptor IP_1 leads to stimulatory G-protein (G_s) mediated formation of cAMP by adenylyl cyclase (AC). Elevated cAMP levels cause an inhibition of platelet aggregation via activation of cAMP dependent protein kinase (PKA) and subsequent phosphorylation of proteins essential to platelet activation (e.g. VASP) (Smolenski, 2012). In contrast, TXA_2 stimulation induces release of calcium ions from the dense tubular system into the cytoplasm via Gq-protein mediated activation of phospholipase C. By this protein kinases as well as small G-proteins are activated. This leads finally to platelet shape change, secretion, and aggregation (Begonja et al., 2007).

The presence of additional prostanoid receptors on platelets has been investigated by means of functional and binding assays with authentic and synthetic prostaglandins (Fabre et al., 2001; Wise et al., 2002; Heptinstall et al., 2008; Singh et al., 2009) and, in some cases on the molecular level as well (Dovlatova et al., 2008; Kuriyama et al., 2010). To date 9 prostanoid receptors are known in humans: the prostaglandin D receptors DP_1 and DP_2 (CRTH2 receptor), the prostaglandin E receptors EP_1, EP_2, EP_3 and EP_4, the prostaglandin I_2 (prostacyclin) receptor IP_1, the prostaglandin F receptor FP, and the thromboxane A_2 receptor TP (Tsuboi et al., 2002). The expression and function of prostaglandin receptors on blood platelets has been discussed controversially (Armstrong, 1996; Schober et al., 2011). Yet the mode of action, interaction and quantitative contribution of endogenous prostanoids and prostanoid receptors to platelet activation and inhibition has not been shown conclusively. Reasons for the difficulties analyzing platelet prostanoid action originate particularly from the low specificity of prostanoid receptors and the susceptibility of transcription and expression analysis of platelets to contamination by other blood cells or cell debris.

We aimed at a comprehensive analysis of human platelet expression of prostanoid receptors and platelet regulation by prostanoids. By a combination of molecular, biochemical, pharmacological, and mathematical techniques we approached this objective and collected sufficient data of adequate quality to generate a functional model representing platelet prostanoid regulation.

2. Materials and methods

2.1. Reagents and materials

The murine tissues used as positive controls in immunoblots were kind gifts from Cora Reiß (Center for Thrombosis & Haemostasis, Mainz, Germany). The oligonucleotides were commercially synthesized from eurofins nwg operon (Ebersberg, Germany). Human normal tissues cDNA was purchased from BioChain (AMS Biotechnology, Abington, UK). Superscript II reverse transcriptase was obtained from Invitrogen (Darmstadt, Germany). The prostanoid EP_2, EP_3, EP_4 and DP_1 receptor polyclonal antibodies as well as the prostanoid DP_1 receptor agonists Prostaglandin D_2 and 15(R)-15-methyl-PGD_2, the prostanoid DP_1 antagonist BW A868C, the prostanoid EP_2 receptor agonist (R)-butaprost, the prostanoid IP_1 receptor antagonist CAY 10441, the prostanoid EP_3 receptor agonist sulprostone, Prostaglandin A_1, and the PGE_2 and PGE_2M EIA kits were purchased from Cayman Chemicals (Ann Arbor, MI, USA). MAPK, phospho-T180/Y182 MAPK, ERK, phospho-T202/Y204 ERK, PKB, and phospho-S473 PKB antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). CD62P-FITC and Rap1 antibody were from BD Biosciences (Heidelberg, Germany). The phospho-VASP antibody 5C6 (phospho-Ser157) and the total VASP antibody IE273 were gifts from vasopharm GmbH (Würzburg, Germany). The polyclonal actin antibody was from Santa Cruz Biotechnology (Heidelberg, Germany), the goat anti-mouse IgG was from Biorad (Hercules CA, USA). The stable thromboxane A_2 receptor agonist U-46619, the prostanoid DP_1 receptor agonist BW 245c and the prostaglandins PGE_1, and PGE_2 were from Sigma-Aldrich (Schnelldorf, Germany). The prostanoid EP_3 receptor antagonist L798,106 and the prostanoid EP_4 receptor antagonist L161,982 were from Tocris bioscience (Bristol, UK). The prostanoid IP_1 receptor agonist Iloprost (Ilomedin) was from Schering (Berlin, Germany). 6-Cl-PuDP (6-Chloro purine 5′ diphosphate) was obtained from Biolog (Bremen, Germany). The P2Y12 antagonist cangrelor was from Medicines Company (Parsippany, NJ, USA). Fibrinogen, ADP and Fura-2/AM were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and mini complete® EDTA-free (protease inhibitor cocktail tablets) were obtained from Roche (Mannheim, Germany). The cyclic AMP Enzyme Immunoassay Kit was from Assay designs (Ann Arbor, MI, USA). All other chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) at the highest purity available. Nitrocellulose membranes were obtained from Schleicher & Schuell (Dassel, Germany).

2.2. Platelet preparation

Platelets were used as washed platelets (WP) resuspended in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH = 7.4), depending on the assay applied. WP were prepared from whole human blood as described elsewhere (Geiger et al., 2010) maintaining the platelet functionally intact (Schweigel et al., 2013) with modifications to avoid contamination by other cells (Burkhart et al., 2012). Whole human blood was obtained from healthy volunteers who had not taken any medication affecting platelet function within 2 weeks prior to the experiment after informed consent according to the declaration of Helsinki and our institutional guidelines and as approved by the local ethics committee. The blood was drawn by venipuncture and collected in 1/5 volume of HEPES/citrate buffer (120 mM NaCl, 20 mM sodium citrate, 4 mM KCl, 1.5 mM citric acid, 30 mM D-glucose, 8 mM HEPES, pH = 6.5) and centrifuged at 300*g* for 20 min at 20 °C to obtain platelet rich plasma (PRP). For the preparation of washed platelets the PRP was diluted 1:1 with HEPES/citrate buffer, apyrase (1 U/ml) added and centrifuged again at 100*g* for 10 min at 20 °C. The pellet was discarded and the supernatant was centrifuged at 380*g* for 10 min. The resulting pellet was resuspended in HEPES/citrate, left resting for 5 min and centrifuged again at 380*g* for 10 min. The platelet pellet was resuspended in PBS buffer to a cell density of 3×10^8 platelets/ml