Thrombosis Research 126 (2010) e23–e29

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

PGE_2 decreases reactivity of human platelets by activating EP2 and EP4☆

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ARTICLE INFO

Article history:
Received 16 November 2009
Received in revised form 17 February 2010
Accepted 7 April 2010
Available online 8 May 2010

Keywords:
EP receptors
Platelet aggregation
Platelet reactivity
Prostaglandin E_2
Thromboxane

ABSTRACT

Introduction: Platelet hyperreactivity associates with cardiovascular events in humans. Studies in mice and humans suggest that prostaglandin E_2 (PGE_2) regulates platelet activation. In mice, activation of the PGE_2 receptor subtype 3 (EP3) promotes thrombosis, but the significance of EP3 in humans is less well understood.
Objectives: To characterize the regulation of thromboxane-dependent human platelet activation by PGE_2.
Patients/Methods: Platelets collected from nineteen healthy adults were studied using an agonist of the thromboxane receptor (U46,619), PGE_2, and selective agonists and/or antagonists of the EP receptor subtypes. Platelet activation was assayed by (1) optical aggregometry, (2) measurement of dense granule release, and (3) single-platelet counting.
Results: Healthy volunteers demonstrated significant interindividual variation in platelet response to PGE_2. PGE_2 completely inhibited U46,619-induced platelet aggregation and ATP release in 26% of subjects; the remaining 74% had partial or no response to PGE_2. Antagonism of EP4 abolished the inhibitory effect of PGE_2. In all volunteers, a selective EP2 agonist inhibited U46,619-induced aggregation. Furthermore, the selective EP3 antagonist DG-041 converted all PGE_2 nonresponders to full responders.
Conclusions: There is significant interindividual variation of platelet response to PGE_2 in humans. The balance between EP2, EP3, and EP4 activation determines its net effect. PGE_2 can prevent thromboxane-induced platelet aggregation in an EP4-dependent manner. EP3 antagonism converts platelets of nonresponders to a PGE_2-responsive phenotype. These data suggest that therapeutic targeting of EP pathways may have cardiovascular benefit by decreasing platelet reactivity.

Platelet hyperreactivity contributes to the pathophysiology of occlusive thrombi that cause cardiovascular events. Patients with increased platelet reactivity are at increased risk for complications after percutaneous coronary interventions (PCI) and cardiovascular mortality.[1–5] Endothelium-derived mediators, including several prostaglandins, influence blood flow and coagulation, in part by modulating platelet reactivity. Prostacyclin (PGI_2), the most studied of these prostanoids, inhibits platelet aggregation and adhesion.[6–8] The endothelium of the human microvasculature produces PGE_2,[9] and in healthy humans, both PGI_2 and PGE_2 modulate the coronary response to sympathetic stimulation[10], suggesting that PGE_2 contributes to vascular physiology and thrombosis.

Four G-protein-coupled receptors mediate the actions of PGE_2: EP1, EP2, EP3 (multiple splice variants), and EP4. In mice, low concentrations (*e.g.,* 100 nM) of PGE_2 potentiate platelet aggregation by activating EP3, whereas high concentrations (*e.g.,* 0.6 mM) inhibit aggregation by stimulating the prostacyclin receptor (IP).[11] $EP3^{-/-}$ mice have increased bleeding times and decreased susceptibility to thromboembolism when challenged with intravenous or periadventitial delivery of arachidonic acid.[12,13] Furthermore, local delivery of arachidonic acid to arterial walls stimulates production of PGE_2 in mice, and plaque-produced PGE_2 can activate platelet EP3, facilitating arterial thrombosis.[14] These animal data have driven the ongoing clinical development of an EP3 antagonist (DG-041) as an antiplatelet agent.[15,16].

In humans, the role of PGE_2 has been well studied in the context of plaque rupture, but its effect on platelet reactivity is not well understood. In atherosclerotic plaque, macrophages primarily produce PGE_2.[17,18] Studies in the early 1970s noted that the effect of PGE_2 on platelets was either inhibitory or stimulatory depending on its concentration and the species of animal examined.[19–22] Bruno *et al.* reported that low doses of PGE_2 did not potentiate aggregation induced by either ADP or collagen, but higher (supraphysiological) doses (>1 μM) inhibited aggregation.[19] Others have observed potentiation of ADP- or collagen-induced aggregation at low doses (10-100 nM PGE_2) and inhibition at high doses (10 μM PGE_2), similar to the data from mice.[23,24] Inferences from animal data suggest

Abbreviations: PGE_2, prostaglandin E_2; PCI, percutaneous coronary interventions; EP, PGE_2 receptor; IP, prostacyclin receptor; PGI_2, Prostacyclin; PRP, platelet-rich plasma; PPP, platelet-poor plasma; TP, thromboxane receptor; RT-PCR, reverse transcription-polymerization chain reaction.

☆ The work has been carried out at Vanderbilt University.

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doi:10.1016/j.thromres.2010.04.003

that these effects are mediated by EP3 and IP, respectively, but there are few studies in humans that characterize the mechanisms of PGE_2 action on platelet function, especially with regard to modulation of thromboxane-mediated events. Accordingly, we investigated the effects of PGE_2 on thromboxane-mediated human platelet activation using PGE_2 and selective agonists and antagonists of EP subtypes.

Materials and Methods

Materials

U46,619, PGE_2, butaprost free acid, sulprostone, and PGE_1-OH were obtained from Cayman Chemical (Ann Arbor, MI). DG-041 was synthesized by the Vanderbilt Institute of Chemical Biology Synthesis Core. MF-191 was kindly provided by Merck Frosst Canada Ltd. (Kirkland, Quebec). CHRONO-LUME® was purchased from Chrono-Log Corporation (Havertown, PA). PGE_2 stock (2 mg/ml in EtOH; 5.67 mM) was kept at -20 °C and serially diluted in PBS to 100X working concentrations.

Human Subjects and Blood Collection

This study was approved by the Vanderbilt University Institutional Review Board. Nineteen healthy adult volunteers (11 men, 8 women) participated after providing written informed consent. All denied taking any medications within the preceding 14 days. Blood was drawn using a 19-gauge butterfly cannula; the first 3 ml was discarded before drawing blood into a polypropylene syringe containing 3.2% sodium citrate (final dilution 1:9). Blood was centrifuged at $190\times g$ for 10 minutes at room temperature and the platelet-rich plasma (PRP) was transferred to a polypropylene container. The residual blood was centrifuged at $2000\times g$ for 10 minutes and the platelet-poor plasma (PPP) was transferred to a separate polypropylene container. The platelet concentration of PRP was determined using a Z1 Dual Threshold particle counter (Beckman-Coulter) and was adjusted to 2.5×10^8 platelets/ml with autologous PPP.

Platelet Aggregation

Measurements of platelet aggregation in PRP were made using light transmission aggregometry using a Chrono-Log lumi-aggregometer (Model 460VS). The aggregometer was calibrated for each sample to read 0-10% light transmission for PRP and 90-100% light transmission for PPP. Aliquots of PRP were placed in siliconized glass cuvettes containing teflon-coated stir bars and incubated for 2 minutes at 37 °C. When measurement of concomitant ATP release was desired, CHRONO-LUME® reagent was added per the manufacturer's protocol and incubated for an additional 2 minutes. Compounds of interest and aggregation-inducing agonists were added to the sample after initiation of recording. Samples were stirred at 1200 rpm throughout. Maximum percentage aggregation (0-100%) and ATP release were calculated using Aggrolink software (Chrono-Log). Experiments were completed within 2.5 hours of phlebotomy.

Single-Platelet Counting

In contrast to optical aggregometry, which measures macroaggregation, single-platelet counting measures microaggregation. After incubating PRP with compounds of interest for 6 minutes at 37 °C, stirred throughout at 1200 rpm as above, a 50 µl aliquot was removed for single-platelet counting. This aliquot was added to 5 µl of 10 X fixation buffer (0.1% formaldehyde in 3 mM sodium EDTA) and incubated at room temperature for at least 30 min. The fixed platelets were counted within one hour using a Z1 Dual Threshold particle counter (Beckman-Coulter) with a 50 µm aperture and monitoring the volumes between 3 and 30 fl.

Preparation of EP Agonists/Antagonists

All EP agonists/antagonists were freshly diluted in PBS to avoid vehicle-mediated effects on platelet aggregation (tested in parallel aggregation reactions).

Statistics

Normal distribution was assessed using Shapiro-Wilk test. Data are presented as mean ± SEM. Differences between two groups were analyzed using the Student's *t*-test for normally distributed data and using the Mann-Whitney test when a normal distribution was not assumed. Differences between more than two groups were analyzed by repeated measures ANOVA (or Friedman test) when samples from individual volunteers were subjected to several treatments in a single experiment; *post hoc* comparisons were made using Newman-Keuls (or Dunn's) multiple comparison test. $P<0.05$ was considered statistically significant.

Results

Effect of PGE_2 on Platelet Activation

We activated platelets in PRP with the thromboxane receptor agonist U46,619. For each of the 19 subjects studied, we determined the dose-response curve for U46,619 with respect to platelet aggregation (maximum chart deflection within 6 minutes). We consistently observed maximal aggregation responses with 1 µM U46619 ("maximal" dose) and "submaximal" aggregation responses with 300-500 nM U46,619. Most individuals had minimal, if any, platelet aggregation induced by <300 nM U46,619.

We investigated the effects of PGE_2 on platelets with three measures of platelet activation in citrated PRP: (1) macroaggregation using optical aggregometry, (2) release of dense granules, and (3) single-platelet counting. In 5 of 19 (26%) individuals, 100 nM PGE_2 inhibited ~85% of U46,619-induced platelet aggregation at doses of U46,619 that produced submaximal responses in the absence of PGE_2 (Fig. 1A). We refer to these individuals as "PGE_2 responders"; a typical aggregation curve is shown in Fig. 1B (left panel). All subjects were tested on more than one day, and each individual's response to PGE_2 was consistent. Pre-incubation with the IP antagonist CAY10441 (1 µM) or 100 µM aspirin did not affect this inhibition of U46,619-induced aggregation by PGE_2 (data not shown). Furthermore, the inhibitory effect of PGE_2 depended on the dose of the platelet agonist: in these same individuals, 100 nM PGE_2 inhibited only 12% of U46,619-induced platelet aggregation when a maximal dose of U46,619 was used (Fig. 1A); increasing the dose of PGE_2 to 5 µM produced similar results (data not shown).

In 14 of 19 (74%) individuals, 100 nM PGE_2 did not significantly attenuate the magnitude of U46,619-induced platelet aggregation regardless of the dose of U46,619, even when we increased the dose of PGE_2 to 5 µM. In 6 of these 14 individuals, PGE_2 slowed the rate of aggregation, but the maximal aggregation achieved was similar to that when PGE_2 was not present (Fig. 1B, middle panel). In the remaining 8 of these 14 individuals, PGE_2 seemed to have no effect on the rate or magnitude of platelet aggregation (Fig. 1B, right panel). Throughout the current report, we refer collectively to these 14 individuals as "PGE_2 nonresponders," recognizing that the "partial responders" appear to have an intermediate phenotype that requires further exploration.

Because the inhibitory effect of PGE_2 among responders is dependent on the concentration of U46,619, we investigated whether differential sensitivity to U46,619 may explain the interindividual variation in response to PGE_2. The U46,619 dose-response curves were similar between PGE_2 responders and nonresponders (Fig. 1C and D), making this an unlikely explanation for our observations.