

RESEARCH HIGHLIGHT

Blocking the receptor EP3 to PGE2 as a way to safely prevent atherothrombosis

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Aspirin inhibits the platelet production of thromboxane A2 and its beneficial effect on myocardial infarction was demonstrated more than two decades ago. This result validated the strategy aimed at targeting platelet function to prevent myocardial infarction. Since then, numerous drugs targeting various activators of platelets have been developed to further improve prevention. However, the beneficial effect of all these drugs on atherothrombosis is limited by an increased risk of bleeding, because they target thrombosis effectors which are also key players in hemostasis. Since aspirin blocks the generation of numerous prostanoids, including inhibitors of platelet activation, targeting one of them might allow the antithrombotic activity to be maintained without promoting bleeding. In examining the roles of various arachidonic acid metabolites on atherothrombosis, we studied the prostaglandin E2 (PGE2). *In vivo*, PGE2 facilitates the responses of platelets to all their various activators through its receptor EP3. PGE2 is produced in relatively high amounts in the context of chronic inflammation such as atherosclerosis, and aggravates murine atherothrombosis. Conversely, PGE2 is not involved in hemostasis. As expected, blocking EP3 strikingly reduced atherothrombosis in mice without impacting bleeding tests. In a recent paper published in *Prostaglandins & Other Lipid Mediators*, we reviewed literature data about the effect of PGE2 and its receptor EP3 on platelet thrombosis and hemostasis in mice and humans. We concluded that cumulated data now justifies validating the role of EP3 blockers with phase III trials to safely improve the prevention of myocardial infarction.

Keywords: prostanoids; PGE2; EP3; antiaggregant; hemostasis; DG-041; myocardial infarction

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Atherothrombosis, the occurrence of thrombosis on atherosclerotic plaques ^[1], is the main complication of atherosclerosis, responsible for myocardial infarction (MI) and stroke. As it remains the first cause of death worldwide, it is still a major public health goal to prevent it. Atherothrombosis is currently inhibited by anti-aggregant drugs, such as aspirin or clopidogrel for instance. This

approach has been proved to be successful ^[2, 3], as it decreased long term mortality from 30% in the 80's to less than 10% today. However, these drugs interfere also with hemostasis and induce bleeding. The more potent the antithrombotic drugs are, the more bleeding they induce, as shown for instance with the P2Y₁₂ blocker Prasugrel ^[4, 5]. This risk of bleeding induced by antithrombotic agents seems

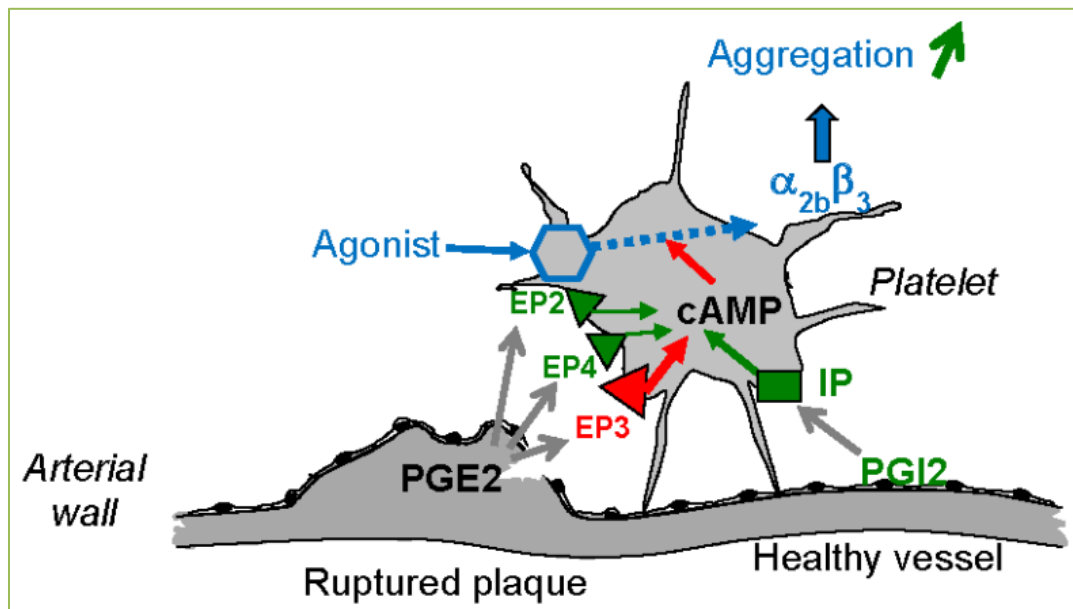


Figure 1. Diagram showing the effect of PGE2 on in vivo platelet function. PGE2 activates its three receptors EP2, EP3 and EP4 expressed on the membranes of platelets. The activation of EP3 receptors inhibits adenylate cyclase and thus decreases the amount of cAMP produced in platelets. cAMP has a braking effect on the platelet response mainly by inhibiting the cytoplasmic release of calcium, and thereby decreases the platelet aggregation. Thus, the activation of EP3 sensitizes the platelets, which means that platelets respond to a lower concentration of agonists. PGE2 also activates EP2 and EP4 receptors which conversely activate the adenylate cyclase, increase cAMP production and desensitize platelets to their activators. The net effect is however driven by EP3, as seen when platelets are stimulated with activator concentrations that are within the potentiation window. Red color: inhibition. Green color: stimulation. Blue color: the agonist effect. modified from ref (21).

unavoidable^[4] and is currently an obstacle in the efforts to improve MI prevention. However, this Charybdis-and-Scylla situation may not be ineluctable. Instead of targeting components that are common to thrombosis and hemostasis, seeking for a mediator produced mainly during inflammation and involved in thrombosis would be promising. Indeed, inflammation is known to alter thrombosis, but not to be part of efficient hemostasis. Plaques host chronic inflammation and may generate pro-thrombotic mediators which are not at play in hemostasis. In this case, inhibiting a mediator which links inflammation to thrombosis would allow atherothrombosis to be inhibited while leaving hemostasis fully functional. We showed that PGE2 produced during inflammation increases *in vivo* thrombosis but did not impact hemostasis. Targeting the PGE2 pathway is thus an ideal strategy for a new class of antithrombotic agents that does not interfere with hemostasis.

The platelet EP3 paradigm in mice

PGE2 has 4 receptors (EP1-EP4). It is involved in uterine contraction (EP2), in the closure of the ductus arteriosus (EP4), in bone formation (EP4), in the control of blood pressure during high-salt diet (EP2), and in gastric mucosal integrity although this latter role is still debated^[6]. Above all,

PGE2 has long-been known for its effect on platelets.

In the 70's and 80's, PGE2 was mainly identified as a weak platelet inhibitor in *in vitro* studies, as compared to prostacyclin (PGI2), and did not spark interest in *in vivo* investigations. Indeed, high amounts of PGE2 (> 10⁻⁵M) are needed to inhibit *in vitro* the response of platelets stimulated with working concentrations of full agonists (collagen, ADP, TXA2, thrombin)^[7-10]. Further studies showed that this effect is in fact due to unspecific binding to PGI2 receptors^[11]. Interestingly, at low concentrations PGE2 conversely potentiates, i.e. facilitates, the weak platelet response initiated by low concentrations of full agonists^[7-10]. Pharmacological and knocked-out studies showed that this effect is specific, resulting from the activation of EP3^[11, 12]. EP3 inhibits the activity of adenylate cyclase^[11, 12] and thereby decreases the amount of cAMP in platelets. Since cAMP inhibits the platelet ability to respond to proaggregant agents, decreasing its production in platelets facilitates the platelet response. Thus, the only specific action of PGE2 on platelets is to facilitate their aggregation and it could be seen as a weak agent that only modulates the platelet activity induced by full agonists. This seems to be the case in *in vitro* tests, which usually test agonists one by one. Conversely, *in vivo* injuries release several agonists which have redundant

actions on platelets and attenuate the effect of antiaggregant therapy targeting one or two pathways. PGE₂ tunes the sensitivity of platelets, meaning that it modulates the platelet aggregating response to all agonists. Thereby, the effect of PGE₂ is not altered by the redundancy of agonists, explaining its ability to significantly increase thrombosis *in vivo*. PGE₂ also binds the EP₂ and EP₄ receptors on platelets, which activate adenylate cyclase and increase cAMP. Thus, and as opposed to the action of EP₃, EP₂ and EP₄ activation inhibit platelet aggregation^[13]. However, the relative importance of this effect is unclear both in mice^[13-15] and humans^[13, 14, 16-20]. Murine observations revealed that the impact of PGE₂ on thrombosis, i.e. the potentiation effect, is predominantly driven by EP₃ activation^[13, 21, 22]. Indeed, *in vivo* thrombosis was significantly decreased in EP₃ deficient mice after periaidventitial delivery of arachidonic acid (AA) or ferric chloride^[21]. To sum up, the PGE₂/EP₃ pathway can contribute to increase the thrombotic response of platelets in mice and could thus play a role in atherothrombosis.

The chronic inflammatory context prevailing in plaques activates the AA pathway which produces prostanoids such as TXA₂, PGI₂, but also PGE₂^[21]. *In vitro*, peritoneal macrophages produce more PGE₂ than TXA₂ in response to prolonged inflammatory stimulation with LPS^[23]. This suggests that PGE₂ can be produced in large amounts in plaques. Indeed, the PGE₂ content found in aortic tissue was dramatically increased by the presence of atherosclerotic plaques^[21, 24]. We further showed that this plaque-produced PGE₂ does impact platelets and aggravates atherothrombosis via EP₃, since atherothrombosis triggered by scratching the surface of plaques in murine carotid arteries^[21] was almost abrogated when platelets lacked EP₃.

Exploring the involvement of PGE₂ in hemostasis, we failed to detect any increase in its production at the site of bleeding^[22]. This was expected, as *in vitro* significant production of PGE₂ requires prolonged stimulation^[23], which is difficult to reconcile with the context of emergency which prevails in hemostasis. The failure of EP₃-deprived platelets to impact bleeding time or bleeding loss supports this interpretation, and establishes that PGE₂ is not involved in murine hemostasis^[11, 22].

Consistently with this set of data, the EP₃ antagonist DG-041 inhibited the aggregation of platelets *in vitro*, decreased *in vivo* thrombosis induced by AA or ferric chloride and strikingly inhibited atherothrombosis in mice^[22]. Importantly, DG-041 did not prolong bleeding times, and did not impact the volumes of blood lost after cutaneous, liver or cerebral injuries in mice^[22]. These latter data establish that inhibiting the PGE₂/EP₃ pathway does not alter murine

hemostasis.

To recapitulate, the PGE₂/EP₃ pathway has the capacity to aggravate atherothrombosis along with the inability to interfere with hemostasis. We called this “the platelet EP₃ paradigm” to highlight the effects of PGE₂/EP₃ pathway on platelets which sharply contrast with other pro-thrombotic agents as it preserves hemostasis. This opens a new perspective for safely controlling atherothrombosis^[25].

Does the platelet EP₃ paradigm apply to human platelets?

Blocking EP₃ in patients in order to control atherothrombosis is appealing, but only if the platelet EP₃ paradigm translates to humans. The murine and human DNA sequences coding for EP receptors display a high degree of homology. The biphasic response to PGE₂ was observed in human platelets before being documented in murine platelets^[8-10, 26-28]. These similarities suggest that murine data on atherothrombosis could be transferable to humans. However, a number of publications^[9, 19, 29-32] questioned this translation. In short, they showed that the effect of PGE₂ on human platelets is restricted to inhibition, is dependent on the nature of the agonist, is variable amongst individuals or even is restricted to an *in vitro* feature. Nowadays, the reinterpretation and reconciliation of these conflicting reports are possible by using the notion of potentiation window and the initial level of cAMP^[25]. The potentiation window is the range of agonist concentrations in which the tested full agonist can trigger aggregation only with the help of a potentiating agent, such as PGE₂. In several publications, the concentrations of tested agonists were outside of the potentiation window, leading to data misinterpretation^[25]. Another source of misinterpretation is the initial level of cAMP in platelets, since any stimulation before tests decreases the cAMP level and masks the potentiating effect. Other hurdles may impede the evidence for potentiation, such as the technique used to record it. Indeed, for unclear reasons, the potentiating effect of PGE₂ is detected in PRP using light scattering earlier after stimulation than in whole blood using impedancemetry^[22]. Lastly, interindividual variations observed in the effect of PGE₂ on human platelets^[19, 32] can also be better understood by considering the variable initial amounts of cAMP in platelets rather than the variable innate ability of platelets to respond^[25]. Thus, the reinterpretation of inconsistent data with these analytical tools, potentiation window and initial cAMP levels, reveals that PGE₂ indeed potentiates the effect of agonists on human platelets in whole blood. The potentiating effect of PGE₂ therefore translates from mice to humans.

Thus, the signaling involved in the potentiating effect of PGE₂ should also translate between the two species. The

importance of the anti-aggregatory role of EP2 and EP4 in murine or human atherothrombosis is currently unclear, making interspecies comparisons unreliable. Conversely, in both murine and human platelets, EP3 facilitates platelet aggregation [11-14, 16, 17, 19, 20, 31, 33] and sensitizes platelets by decreasing cAMP production, even when platelets are blocked by agents such as aspirin or clopidogrel [16, 17, 22]. Therefore, the signaling pathway activated by EP3 translates well from murine to human platelets and, as in mice, PGE2 does facilitate the human platelet response by activating its receptor EP3. In addition, as it was clearly shown that human plaques produce PGE2 [22, 24, 34], atherothrombosis could be aggravated by PGE2 also in humans.

The inhibition or inactivation of EP3 in mice failed to alter hemostasis. Similarly, a phase I trial did not show any impact on bleeding time in volunteers [22, 35] who received high doses of the EP3 blocker DG-041. The maximal tested dose reached 8 times the dose needed to fully inhibit the *in vitro* potentiating effect of PGE2 on human platelets. Thus, the non-involvement of the PGE2/EP3 pathway in hemostasis also translates from mice to humans.

To summarize, EP3 facilitates aggregation of platelets similarly in mice and humans, EP2 and EP4 inhibit aggregation of platelets similarly in mice and humans, and PGE2 fails to impact hemostasis in both mice and volunteers. This good translation from mice to humans suggests that human atherothrombosis could also be PGE2-dependent and thus the EP3 paradigm be applicable in patients. However this must be demonstrated in a phase III trial.

What would be the advantages of an EP3 blocker as compared to established drugs? Aspirin attenuates thrombosis through the inhibition of TXA2 which is a key factor in hemostasis. In addition, aspirin blocks the production of PGI2 and PGD2 which exerts beneficial inhibitory effects. Targeting EP3 efficiently inhibits thrombosis at the site of chronic inflammation where PGE2 is produced, such as atherosclerotic plaques, via the desensitization of platelets to all their agonists, including TXA2. In addition, targeting only EP3 has the advantage to keep effective the platelet inhibition mediated by PGI2, PGD2 and PGE2 binding EP4 and EP2. Importantly, blocking EP3 does not interfere with hemostasis. Thus, inhibiting the PGE2/EP3 pathway is expected to prevent thrombosis as or more efficiently than aspirin, while not inducing bleeding.

The advantage of an EP3 blocker as compared to recently developed drugs is also mainly the lack of hemostasis impact. Indeed, all recently developed drugs target important players in hemostasis and induce bleeding, as opposed to the

PGE2/EP3 strategy. The inhibitory effect of blocking EP3 is expected to be powerful on human thrombosis, as shown in mice, since it is insensitive to the redundancy effect resulting from the numerous agonists released at the injury site.

Conclusions

PGE2 mediates the effect of chronic inflammation on thrombosis, as shown by its aggravating role in murine atherothrombosis, and clearly potentiates aggregation of human platelets. Since it is involved neither in murine nor in human hemostasis, the PGE2/EP3 pathway is an attractive target to improve prevention of MI or stroke and to be tested in clinical phase III trials.

Author contributions

M.A.M. and J.E.F. worked together on the manuscript.

Conflicting interests

The authors have declared that no conflict of interests exists.

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Abbreviations

TXA2: Thromboxane A2; PGE2: Prostaglandin E2; MI: Myocardial Infarction; PGI2: Prostacyclin; ADP: Adenosine DiPhosphate cyclic; Camp: Adenosine MonoPhosphate.

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