The Prostaglandin E2 Receptor EP4 Is Expressed by Human Platelets and Potently Inhibits Platelet Aggregation and Thrombus Formation*

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Objective—Low concentrations of prostaglandin (PG) E2 enhance platelet aggregation, whereas high concentrations inhibit it. The effects of PGE2 are mediated through 4 G protein-coupled receptors, termed E-type prostaglandin (EP) receptor EP1, EP2, EP3, and EP4. The platelet-stimulating effect of PGE2 has been suggested to involve EP3 receptors. Here we analyzed the receptor usage relating to the inhibitory effect of PGE2.

Methods and Results—Using flow cytometry, we found that human platelets expressed EP4 receptor protein. A selective EP4 agonist (ONO AE1-329) potently inhibited the platelet aggregation as induced by ADP or collagen. This effect could be completely reversed by an EP4 antagonist, but not by PGI2, PGD2, and thromboxane receptor antagonists or cyclooxygenase inhibition. Moreover, an EP4 antagonist enhanced the PGE2-induced stimulation of platelet aggregation, indicating a physiological antaggregatory activity of EP4 receptors. The inhibitory effect of the EP4 agonist was accompanied by attenuated Ca2+ flux, inhibition of glycoprotein IIb/IIIa, and downregulation of P-selectin. Most importantly, adhesion of platelets to fibrinogen under flow and in vitro thrombus formation were effectively prevented by the EP4 agonist. In this respect, the EP4 agonist synergized with acetylsalicylic acid.

Conclusion—These results are suggestive of EP4 receptor activation as a novel antithrombotic strategy. (Arterioscler Thromb Vasc Biol. 2010;30:2416-2423.)

Key Words: aspirin ▪ pharmacology ▪ platelet receptor blockers ▪ platelets ▪ prostacyclin ▪ prostainglandins ▪ thromboxanes

Subjects with increased platelet reactivity are at increased prospective risk for coronary events and death. A number of pathophysiological states, such as atherosclerosis, diabetes, and metabolic syndrome, are associated with increased platelet reactivity and thrombogenic potential. Under inflammatory conditions, the synthesis of prostanoids in endothelial cells and smooth muscle cells is highly increased. Predominantly, the biosynthesis of prostaglandin (PG) E2 is enhanced in vascular smooth muscle cells1 and macrophages2,3 by inflammatory mediators.

Prostanoids are involved in hemostasis by differentially influencing platelet aggregation. Although thromboxone (TX) A2, produced in platelets, and PGH2, released untransformed from activated/dysfunctional endothelium, are potent stimulators of platelet aggregation,4 PGI2 and PGD2 are known to inhibit platelet aggregation.5

PGE2 shows a biphasic, concentration-dependent effect on platelet aggregation. Although high concentrations inhibit platelet aggregation, lower concentrations enhance it.6-11 PGE2 binds and activates 4 G protein–coupled receptors, EP1, EP2, EP3, and EP4. Each of these receptors has a distinct pharmacological signature and intracellular signal transduction.12,13,14 Stimulation of the EP3 receptors results in elevation of free intracellular Ca2+ levels, whereas stimulation of the EP2 and EP4 receptors usually increases intracellular cAMP levels through activation of G3 protein,15 resulting in a decrease of intracellular Ca2+ levels.

Human platelets contain mRNA for EP1 receptors, all of the EP3 splice variants, and EP4; however, mRNA for EP2 receptors is lacking in platelets.16 The proaggregatory effect of PGE2 has been ascribed to the activation of the EP3 receptor, leading to inhibition of the increase in cAMP,16,17 an increased mobilization of Ca2+,18 and increased P-selectin expression on platelets.19,20 An EP3 antagonist has been proposed to be useful for antithrombotic therapy.19 Here we demonstrate that a selective EP4 agonist inhibits platelet aggregation, Ca2+ mobilization, and upregulation of P-selection. In November 2009, we first reported these novel findings in a meeting abstract.21 Thereafter, while the current report was in preparation, 3 articles were published that...
overlap with the data in our abstract and fully confirm our findings, as they report that an EP4 antagonist reverses the inhibition by PGE2 of U46619-induced platelet aggregation; that the EP4 agonist ONO AE1-329 prevents the platelet-activating factor (PAF)-induced platelet aggregation, U46619-induced P-selectin upregulation, and thrombin receptor-mediated Ca2+ flux in platelets; and that murine platelets are less sensitive to EP4 activation than human platelets. Regrettably, none of these authors acknowledged our work. In excess of these observations, we report here that (1) platelets express EP4 receptor protein, (2) activation of EP4 receptors counteracts the activation of glycoprotein [GP] IIb/IIa, and (3) hence prevents the binding of platelets to fibrinogen. Moreover, (4) these inhibitory effects of the EP4 receptor on platelet activation translate to potent antithrombotic activity of the EP4 agonist as shown by in vitro thrombus formation assays using whole blood, and (5) EP4 activation enhances the inhibitory effect of acetylsalicylic acid. Therefore, we propose that selective EP4 receptor agonists may provide a novel antithrombotic strategy in humans.

Materials and Methods
Reagents are described in the supplemental materials, available online at http://atvb.ahajournals.org.

Platelet Aggregation
This study was approved by the ethics committee of the Medical University of Graz. Before blood sampling from healthy volunteers, all donors signed an informed consent form. Human platelet-rich and platelet-poor plasma were prepared from citrated whole blood by centrifugation. Platelet aggregation was recorded at 37°C with constant stirring (1000 rpm) in an Aggrecorder-II (KDK Corp, Kyoto, Japan) as described. Washed platelets were fixed, permeabilized, and blocked (Becton Dickinson). To record P-selectin expression, platelets were raining on an Olympus IX70 fluorescence microscope and an Olympus UPlanFI-20x/0.40 lens, using a Hamamatsu ORCA-ER digital camera and the Olympus CellP software. Cell images of 3 microscopic fields from each channel were captured, and images were analyzed using DucoCell software (Cellix).

In Vitro Thrombogenesis
VenaFluoro+ Biochips (Cellix) were coated with collagen (200 μg/mL) at 4°C overnight. Blocking was performed with bovine serum albumin (10 μg/mL) for 30 minutes at room temperature followed by washing steps. Whole blood collected in 3.8% sodium citrate was incubated with 3,3′-dihexyloxacarbocyanine iodide (1 μmol/L) in the dark for 10 minutes. To record inhibition of thrombus formation, whole blood was treated with PGE2 (10 μmol/L), the EP4 agonist ONO AE1-329 (300 nmol/L), or acetylsalicylic acid (1 mmol/L) for 10 minutes before the perfusion was started. The EP4 antagonist (ONO AE3-208; 1 and 10 μmol/L) or its vehicle was added 10 minutes before the respective agonist. CaCl2 at a final concentration of 1 mmol/L was added 2 minutes before ADP or collagen. In some experiments acetylsalicylic acid (1 mmol/L as lysine acetylsalicylic acid) was added to platelet rich plasma 30 minutes before the proaggregatory stimulus. To record inhibition of agonist-induced aggregation, PGE2, or other compounds were added 2 minutes before ADP or collagen. The antagonists or their vehicle were added 10 minutes before the respective agonist. Data were expressed as percentage of maximum light transmission, with nonstimulated platelet-rich plasma being 0% and platelet-poor plasma 100%.

Flow Cytometric Immunofluorescence Staining
Washed platelet preparations were fixed, permeabilized, and blocked using Ultra V blocking solution. Staining was done using an EP4 antibody or control IgG antibody (20 μg/mL) for 30 minutes on ice and subsequently incubated with an anti-rabbit Alexa Fluor-488 conjugated secondary antibody (4 μg/mL), with the required washing steps. The samples were read on a FACSCalibur flow cytometer (Becton Dickinson). To record P-selectin expression, platelets were activated by ADP (3 μmol/L) in the presence cytochalasin B (5 μg/mL) for 15 minutes at 37°C in the presence of the anti-CD62P–fluorescein isothiocyanate antibody. The samples were washed and fixed, and CD62P (P-selectin) upregulation was detected by flow cytometry. Activation of the fibrinogen receptor GPIIb/IIa was assayed using the PAC-1 antibody, which recognizes a conformation-dependent determinant on the GPIIb/IIa complex. Total receptor expression was determined with an anti-CD41 antibody directed against GPIIb. After incubation with PGE2 or other agonists for 10 minutes, the stimulation of the samples with ADP (3 μmol/L) was carried out at 37°C for 5 minutes in the presence of anti-CD41 or PAC-1 antibody, directly conjugated to fluorescein isothiocyanate. Pretreatment with antagonists or acetylsalicylic acid started 15 minutes before the agonist/vehicle treatment. The reaction was stopped; samples were washed and fixed and then analyzed by flow cytometry.

Platelet Adhesion
Vena8 biochips (Cellix Ltd, Dublin, Ireland) were coated with fibrinogen (100 μg/mL) at 4°C overnight. On the next day, the chips were blocked with BSA (10 μg/mL) for 30 minutes at room temperature and then washed once more with PBS containing 0.9 mmol/L Ca2+ and 0.5 mmol/L Mg2+. Eighty-microliter aliquots of platelet-rich plasma were pretreated with vehicle, iloprost (3 mmol/L), or ONO AE1-329 (300 nmol/L) for 5 minutes. Aliquots were then mixed with ADP (10 μmol/L) in the presence of 1 mmol/L CaCl2 (10 μL) and then immediately perfused over the fibrinogen-coated channels at constant shear stress of 0.5 dyne cm−2 for 3 minutes using the Mirus nanopump (Cellix). Platelet adhesion was recorded on an Olympus IX70 fluorescence microscope and an Olympus UPlanFL×200x/0.40 lens, using a Hamamatsu ORCA-ER digital camera and the Olympus CellP software. Cell images of 3 microscopic fields from each channel were captured, and images were analyzed using DucoCell software (Cellix).

Statistical Analyses
Data are shown as mean±SEM for n observations. Comparisons of groups were performed using 1-way or 2-way ANOVA with the Dunnett post test or the Wilcoxon signed rank test. Probability values of P<0.05 were considered statistically significant.

Results
EP4 Receptor Stimulation Inhibits Platelet Aggregation
First, we tested the hypothesis that activation of EP4 receptors modulates platelet aggregation. Platelet-rich plasma was treated with various concentrations of the EP4 selective agonist ONO AE1-329 (0.01 to 300 mmol/L) for 7 minutes before ADP or collagen was added to induce platelet aggregation. The concentrations of ADP (2.5 to 20 μmol/L) and collagen (1.25 to 10 μg/mL) were adjusted to give submaxi-
EP4 receptors expressed by human platelets inhibit platelet aggregation. A. Original tracing showing that the EP4 agonist ONO AE1-329 (3 to 300 nmol/L) inhibited platelet aggregation, as induced by ADP. B. ONO AE1-329 (3 nmol/L to 3 μmol/L) inhibited platelet aggregation as induced by ADP or collagen. The concentrations of ADP (2.5 to 20 μmol/L) and collagen (1.25 to 10 μg/mL) were adjusted to give submaximal aggregation. ONO AE1-329 itself did not induce platelet aggregation at concentrations up to 1 μmol/L (n=6, data not shown) but concentration-dependently attenuated both the ADP- and collagen-induced aggregation (Figure 1A and 1B). To investigate the role of EP4 receptors in the PGE2-induced effects on platelet function, samples were pretreated with the EP4 antagonist ONO AE3-208 (300 nmol/L) for 10 minutes and then treated with PGE2 (3 to 300 nmol/L) for 7 minutes. Finally, platelet aggregation was initiated with ADP at concentrations that yielded half-maximal platelet aggregation. PGE2 alone did not trigger platelet aggregation under these conditions (n=6, data not shown) but slightly enhanced the ADP-induced aggregation. This effect was dramatically enhanced in the presence of the EP4 antagonist (Figure 1C). A similar observation was made when collagen was used as a proaggregatory stimulus (n=6, data not shown). Consistently, EP4 receptor protein was detectable on platelets in flow cytometry as revealed by a polyclonal anti-EP4 antibody that gave considerably higher staining than the respective isotype-matched control antibody (Figure 1D).

In further experiments, we addressed the possibility that the inhibitory effect of the EP4 agonist ONO AE1-329 was mediated by alternative inhibitory PG receptors such as the EP2 receptor, I-prostanoid receptor (IP), or D-prostanoid receptor (DP). Butaprost, a selective EP2 agonist at concentrations up to 300 nmol/L, did not mimic the inhibitory effect of the EP4 agonist, as it left the ADP-induced aggregation unaltered (Figure 2A). Pretreatment of the samples with the IP antagonist CAY10441 (300 nmol/L) did not reverse the inhibitory effect of the EP4 agonist ONO AE1-329 (3 to 300 nmol/L) on ADP-induced aggregation. The concentration of ADP (2.5 to 20 μmol/L) was adjusted to give submaximal aggregation. C, Pretreatment of platelets with the TP antagonist SQ29548 (1 μmol/L) or acetylsalicylic acid (ASA, 1 mmol/L) did not affect the inhibition of ADP-induced aggregation by the EP4 agonist ONO AE1-329 (3 to 300 nmol/L). D, Pretreatment of platelets with the EP4 antagonists ONO AE3-208 (300 nmol/L or 1 μmol/L) or GW627368x (10 μmol/L) prevented the inhibitory effect of ONO AE1-329 (3 to 300 nmol/L) on ADP-induced aggregation. E, Pretreatment of platelets with the TP antagonist CAY10441 (300 nmol/L) prevented the inhibitory effect of the IP agonist iloprost (0.03 to 0.3 nmol/L) on ADP-induced aggregation, whereas the EP4 antagonists ONO AE3-208 and GW627368x had no effect. Data were expressed as percentage of the control response to ADP or collagen and are shown as the mean±SEM of 4 to 12 experiments. *P<0.05 versus pretreatment with vehicle.
moderately reduced the ADP-induced aggregation by 20% to 30% (P<0.05, n=6, data not shown), they did not influence the inhibitory effect of the EP4 agonist ONE-329 (Figure 2C). Moreover, the EP4 antagonists ONO AE3-208 (300 nmol/L and 1 μmol/L) and GW627368x (10 μmol/L) both reversed the inhibitory effect of the EP4 agonist (Figure 2D). Conversely, ONO AE3-208 and GW627368x had no effect on the iloprost-induced inhibition of platelet aggregation (Figure 2E). The proaggregatory effect of EP3 stimulation was confirmed by the EP3 agonist sulprostone, which potently enhanced the ADP-induced aggregation (Figure 2A), reminiscent of the proaggregatory effect of PGE2 in the presence of the EP4 agonist shown in Figure 1C.

Next, we investigated whether platelets from rats, guinea pigs, and mice responded to activation of EP4 receptors. In fact, flow cytometric immunostaining showed that platelets from all 3 species expressed EP4 receptors (Supplemental Figure I). Unexpectedly, the EP4 agonist ONE AE1-329 at a concentration of 300 nmol/L was unable to inhibit ADP-induced aggregation in any of the 3 species, although iloprost was effective (Supplemental Figure II). Collectively, these data suggested that, in contrast to rodents, EP4 receptors on human platelets play a prominent antiaggregatory role.

**Cellular Mechanisms Linked to EP4-Mediated Inhibition of Platelet Aggregation**

Because platelet aggregation essentially depends on increased free intracellular Ca2+ levels, we investigated how EP4 receptors modulate Ca2+ responses to ADP using a flow cytometric Ca2+ flux assay. ADP was highly efficient to stimulate Ca2+ flux in platelets in a concentration-dependent manner (10 to 1000 nmol/L). Whereas iloprost caused almost complete inhibition, ONO AE1-329 significantly attenuated this response (Figure 3A). Similarly, PGE2 in the concentration range of 0.1 and 10 μmol/L reduced the Ca2+ responses to 100 nmol/L ADP. This effect of PGE2 was largely prevented in the presence of the EP4 antagonist GW627368x (Figure 3B).

Because elevation of intracellular free Ca2+ levels is crucial for upregulation of P-selectin and activation of GPIIb/IIIa, we further investigated the effects of EP4 activation on these responses. Stimulation of platelets with ADP (3 μmol/L) increased the surface expression of CD62P 8-fold as detected by an anti-CD62P antibody directly conjugated to fluorescein isothiocyanate (Figure 3C). The activation of GPIIIa was detected with the use of a conformation-dependent antibody, PAC-1 (Figure 3D). ONO AE1-329 (300 nmol/L) effectively prevented the CD62P upregulation and the activation of GPIIIa. In contrast, relatively high concentrations of PGE2 (10 μmol/L) were required to achieve similar inhibition. The TP antagonist SQ29548 (100 nmol/L) by itself had no effect on ADP-induced CD62P upregulation and activation of GPIIIa (data not shown) and also did not prevent the inhibition obtained by ONO AE1-329 (300 nmol/L; Figure 3C and 3D). The inhibitory effects of PGE2 (10 μmol/L) and the EP4 agonist ONE AE1-329 (300 nmol/L) were also seen in platelets treated with acetylsalicylic acid (1 mmol/L; Figure 3E and 3F). To further substantiate the role of EP4 receptors, platelets were pretreated with the

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** EP4 stimulation attenuates Ca2+ mobilization, the upregulation of P-selectin (CD62P), and activation of GPIIb/IIIa in washed human platelets. A, Ca2+ flux induced by ADP (10 to 1000 nmol/L) was significantly attenuated by ONE AE1-329 (300 nmol/L) and iloprost (0.3 nmol/L). Ca2+ responses were detected by flow cytometry as changes in fluorescence of the Ca2+-sensitive dye Fluo-3 by flow cytometry and are presented as percentage of baseline fluorescence. B, Pretreatment of platelets with the EP4 antagonist GW627368x (10 μmol/L) prevented the PGE2-induced (0.01 to 10 μmol/L) attenuation of platelet Ca2+-flux, as elicited by 100 nmol/L ADP. C and D, ADP (3 μmol/L) increased the surface expression of CD62P and the activation of GPIIb/IIIa in platelets. For CD62P expression, platelets were primed with cytochalasin B (5 μg/mL). Activation of GPIIb/IIIa was detected using the conformation-sensitive antibody PAC-1. Iloprost (3 nmol/L), PGE2 (10 μmol/L), and the EP4 agonist ONE AE1-329 (300 nmol/L) caused significant inhibition of CD62P upregulation and GPIIb/IIIa activation. Pretreatment with the TP antagonist SQ29548 (1 μmol/L) did not abrogate the effect of the EP4 agonist ONE AE1-329 (300 nmol/L). E and F, In acetylsalicylic acid–treated platelets, iloprost (3 nmol/L), PGE2 (10 μmol/L), and the EP4 agonist ONE AE1-329 (300 nmol/L) still reduced the upregulation of CD62P and GPIIb/IIIa activation. The EP4 antagonist ONE AE3-208 (1 and 10 μmol/L) abrogated the effect of the EP4 agonist ONE AE1-329 (300 nmol/L) and PGE2 (10 μmol/L). Data were expressed as percentage of the control response to ADP and are shown as the mean±SEM of 6 to 12 experiments. *P<0.05 versus pretreatment with vehicle; #P<0.05 versus the respective agonist.
EP4 antagonist ONO AE3-208 (1 or 10 µmol/L), which abolished the inhibitory effects of the EP4 agonist ONO AE1-329 and PGE₂ (Figure 3E and 3F). The total amount GPIIb/IIIa was not changed by any of these treatments as determined using an anti-CD41 antibody (Supplemental Figure III). These observations suggest that activation of EP4 receptors attenuates platelet aggregation by the control of Ca²⁺ mobilization and the ensuing upregulation of P-selectin and activation of GPIIb/IIIa.

**Effect of EP4 Activation on Platelet Adhesion and In Vitro Thrombus Formation**

Because we observed that stimulation of EP4 receptors downregulates the expression of adhesion molecules and prevents activation of the fibrinogen receptor GPIIb/IIIa, we investigated the adhesion of platelets to fibrinogen under flow conditions. Platelet-rich plasma was perfused under shear stress over fibrinogen-coated surfaces, and platelet adhesion was recorded by microscopy. Addition of 10 µmol/L ADP to the samples rapidly triggered the adhesion of platelets to fibrinogen within 3 minutes (Figure 4A). In samples treated with ONO AE1-329 (300 nmol/L), this was largely prevented, and only individual small aggregates were observed. Iloprost (3 nmol/L) likewise prevented platelet adhesion. Computerized image analysis of the photographs taken after 3 minutes of platelet perfusion confirmed that the EP4 agonist reduced the platelet-covered area by 90% (Figure 4B).

Finally, we assessed whether EP4 activation also prevented thrombogenesis. To this end, whole blood was perfused through collagen-coated channels, and thrombus formation was recorded by fluorescence microscopy. Adhesion of platelets to collagen was observed after 1 minute, subsequently leading to pronounced thrombus formation as shown in Figure 5 at 3 minutes. In samples pretreated with the EP4 agonist ONO AE1-329 (300 nmol/L) or PGE₂ (10 µmol/L), thrombus formation was largely prevented, and only small aggregates were observed (Figure 5A). The effects of the EP4 agonist and PGE₂ were reversed by the EP4 antagonist ONO AE3-208 (1 µmol/L and 10 µmol/L). As expected, thrombus formation was likewise attenuated by acetylsalicylic acid (1 mmol/L), and its combination with the EP4 agonist ONO AE1-329 almost completely abolished the thrombus formation (Figure 5A). Computerized image analysis revealed that the EP4 agonist reduced the area of the thrombi by 79%, whereas the combination of ONO AE1-329 plus acetylsalicylic acid reduced it by 93% (Figure 5B).

**Discussion**

In the present study, we suggest a novel path for antithrombotic intervention, as we show that EP4 receptors expressed on human platelets confer inhibitory signals. On the one hand, we observed that EP4 receptors play an important role in the complex action of PGE₂ in the regulation of platelet function, because an EP4 antagonist accentuated the PGE₂-induced enhancement of agonist-induced platelet aggregation. On the other hand, selective stimulation of EP4 receptors using an EP4 agonist potently inhibited platelet aggregation in response to ADP or collagen. Furthermore, we could decipher the cellular mechanisms linked to EP4-
mediated platelet inhibition: ligation of EP4 receptors apparently attenuates Ca\(^{2+}\) mobilization in platelets and curbs the agonist-induced upregulation of P-selectin and the dimerization/activation of the fibrinogen receptor GPIIb/IIIa. On a functional level, these changes lead to a reduced capacity of platelets to adhere to surfaces and to form thrombi under flow conditions.

PGE\(_2\) has long been known to attenuate platelet aggregation at high concentrations, ie, in the micromolar range; at lower concentrations, however, PGE\(_2\) is promoting aggregatory responses induced by platelet activators, such as ADP or collagen.\(^6,7,9,10,32\) Therefore, the primary action of PGE\(_2\) was suggested to be proaggregatory. The EP3 receptor was subsequently characterized as the receptor that mediates the PGE\(_2\)-induced augmentation of platelet aggregation, and EP3 receptor antagonists are currently under consideration as potential novel antithrombotic treatment.\(^19\) This notion was also confirmed in our study, because the EP3 agonist sulprostone potently attenuated the ADP-induced platelet aggregation with an estimated EC\(_{50}\) value of 3 nmol/L. How the inhibitory role of EP4 receptors has until now gone unnoticed might be explained by studies in PGE\(_2\) receptor and IP knockout mice, suggesting that it is the IP rather than EP2 or EP4 receptors that underlie the inhibitory effect of PGE\(_2\) on murine platelet aggregation.\(^24,33\)

In the current study, we show for the first time that human platelets express EP4 receptors, which is in line with a previous report of EP4 mRNA detected in platelets.\(^{15}\) Led by this observation, we tested an EP4 selective agonist, ONO AE1-329, in assays of platelet aggregation. In fact, ONO AE1-329 potently attenuated agonist-induced platelet aggregation with apparent IC\(_{50}\) values of 3 and 0.1 nmol/L against the proaggregatory stimuli ADP and collagen, respectively. Conversely, 2 chemically distinct EP4 antagonists, GW627368x and ONO AE3-208, abrogated the inhibitory effect of ONO AE1-329 on platelet aggregation. Moreover, the specific IP and DP antagonists, CAY10441 and BWA868c, showed no tendency to reverse the inhibitory effect of the EP4 agonist, although they prevented the inhibitory effects of the IP agonist iloprost (this study) and the DP agonist PGD\(_2\).\(^{27}\) Similarly, the TP antagonist SQ29548 or acetylsalicylic acid had no effect on the EP4-mediated inhibition of platelet aggregation. Therefore, these data unequivocally demonstrate that selective activation of EP4 receptors on platelets restrains platelet aggregation in humans without involving other inhibitory prostanoid receptors or modulating thromboxane A\(_2\) release or TP activity.

ONO AE1-329 had no inhibitory effect on platelets from rats, guinea pigs, and mice, although iloprost was effective in all 3 species (cf. supplemental data). One possible explanation might be the expression levels of EP4 receptors in platelets from different species.
thrombus formation, which allows quantification of platelet function under flow conditions. In fact, adhesion of platelets and subsequent thrombogenesis were effectively prevented by the EP4 agonist.

Agonist-induced Ca²⁺ flux was also attenuated by the EP4 agonist and also by PGE₂, and this effect was largely prevented by the EP4 antagonist GW627368x. However, the effect of EP4 receptor activation on Ca²⁺ flux was considerably smaller compared with the pronounced inhibition of platelet aggregation, suggesting that additional mechanisms are likely to be in place. P-selectin (CD62P) plays an important role in the interaction of platelets with leukocytes and endothelial cells and is upregulated in activated platelets. The ultimate step in platelet aggregation is activation of GPIIb/IIIa, which then binds fibrinogen with high avidity and thus enables thrombus formation. Most substantially, the EP4 agonist ONO AE1-329 and, to a lesser extent, PGE₂ were able to prevent the agonist-induced upregulation of P-selectin and activation of GPIIb/IIIa. Again, the EP4 antagonist completely abolished the inhibitory effect of the EP4 agonist on upregulation of P-selectin and GPIIb/IIIa activation, whereas the TP antagonist and acetylsalicylic acid had no effect. These observations were in good agreement with reduced platelet adhesion to fibrinogen, the ligand of GPIIb/IIIa. Finally, flow chamber experiments using perfusions of human whole blood over collagen confirmed that the inhibitory effects of ONO AE1-329 on platelet function translated to potent antithrombogenic effects. In this respect, the EP4 agonist matched the effect of acetylsalicylic acid, and in combination even enhanced the inhibitory effect of that clinically relevant antiplatelet drug.

EP4 receptors have been shown to couple to G₄ proteins and adenylyl cyclase–mediated cAMP production. The antiaggregatory effects of PGL₃ and PGD₂ have been demonstrated to rely on cAMP and subsequent attenuation of Ca²⁺ mobilization, suggesting that cAMP is an important negative regulator of platelet function. Unexpectedly, we observed that pretreatment with the adenylyl cyclase inhibitor SQ22536 (100 µmol/L) was unable to prevent the inhibitory effect of ONO AE1-329 on platelet aggregation (data not shown, n=6). The disparity of IP and EP4 signaling with respect to the adenylyl cyclase/cAMP pathways might thus account for the higher potency of iloprost as platelet inhibitor. In contrast, the nonselective protein kinase C (PKC) inhibitor chelerythrine (100 µmol/L) abolished the EP4-mediated effects on platelets (data not shown, n=6), which was in line with PKC being linked to EP4 signaling in HL-60 cells and eosinophils. PKC is a major regulator of platelet granule secretion, integrin activation, and aggregation, whereby individual members of the PKC family play distinct, sometimes opposing roles. For instance, PKC-δ, a classic isofrom, is an essential positive regulator of granule secretion and thrombus formation, whereas the novel isoform PKC-δ negatively regulates filopodia formation and thromboxane A₂ release and thus reduces platelet aggregation and thrombus formation. Therefore, the involvement of PKC isoforms in EP4 signaling of platelets remains a substantial field for further investigation.

In conclusion, our data suggest that EP4 receptors might play an important role in the control of hemostasis by mediating the inhibitory effect of PGE₂, thereby balancing out the proaggregatory effect of EP3 receptors. When activated separately using selective agonists, EP4 receptors afford potent inhibition of platelet function with respect to platelet adhesion, aggregation, and thrombus formation. Hence, EP4 agonists might constitute a novel class of antithrombotic agents and might be clinically useful in cases where acetylsalicylic acid or ADP antagonists are not warranted or are insufficient.

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Disclosures

None.

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Supplement Material

The prostaglandin E\textsubscript{2} receptor EP4 is expressed by human platelets and potently inhibits platelet aggregation and thrombus formation

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MATERIALS AND METHODS

Reagents

All laboratory reagents were from Sigma (Vienna, Austria), unless specified. Assay buffer as used in Ca\(^{2+}\) flux and flow cytometrical immunostaining experiments was made from Dulbecco’s modified phosphate-buffered saline (PBS; with 0.9 mM Ca\(^{2+}\) and 0.5 mM Mg\(^{2+}\); Invitrogen, Vienna, Austria). Polyclonal rabbit anti-EP4 antibody, isotype-matched control antibody, CAY10441, BWA868c, SQ29548 and GW627368x were purchased from Cayman (Ann Arbor, MI, USA). Fixative solution was prepared by adding 9 ml distilled water and 30 mL FACS-Flow® to 1 mL CellFix®. ADP and equine fibrillar collagen were obtained from Probe&Go (Endingen, Germany). Drugs were dissolved in water, ethanol or dimethyl sulfoxide (DMSO) and further diluted in assay buffer to give a final concentration of the solvents < 0.1%. The EP4 agonist ONO AE1-329 (2-[3-[(1R,2S,3R)-3-hydroxy-2-[(E,3S)-3-hydroxy-5-[2-(methoxymethyl)phenyl]pent-1-enyl]-5-oxo-cyclopentyl]sulfanyl)propylsulfanyl]acetic acid) and the EP4 antagonist ONO AE3-208 (2-[[2-[2-(2-methylnaphthalen-1-yl)propanoylamino]phenyl]methyl]benzoic acid) were a kind gift from ONO Pharmaceutical (Osaka, Japan).

Platelet aggregation

Male Sprague-Dawley rats, Balb/c mice or guinea pigs were sacrificed by an overdose of pentobarbital. Blood was drawn by cardiac puncture into 3.8% sodium citrate. Platelet aggregation of washed platelets was recorded at 37 °C with constant stirring (1000 rpm), in a four-channel Aggrecorder II aggregometer (KDK Corp., Kyoto, Japan) as described\(^1\)\(^-\)\(^3\). Platelet aggregation was measured as the increase in light transmission for 5 min, starting with the addition of ADP (2.5-20 μM) as pro-aggregatory stimulus. CaCl\(_2\) at a final concentration of 1
mM was added 2 min before ADP. To record inhibition of agonist-induced aggregation, PGE$_2$ or other compounds were added 2 min before ADP. Data were expressed as percent of maximum light transmission, with non-stimulated platelet-rich plasma being 0% and platelet-poor plasma 100%.

**Flow cytometric immunofluorescence staining**

EP4 receptors on platelets were determined using indirect immunofluorescence staining. Washed platelet preparations were fixed, permeabilized and blocked by Ultra V blocking solution. Staining was done using an EP4 antibody or control IgG antibody (20 µg/ml) for 30 min on ice and subsequently incubated with an anti-rabbit Alexa Fluor-488 conjugated secondary antibody (4 µg/ml), with the required washing steps. The samples were read on a FACSCalibur flow cytometer (Becton-Dickinson).

**Statistical analyses**

Data are shown as mean±SEM for $n$ observations. Comparisons of groups were performed using one-way ANOVA with Dunnett’s post test. Probability values of P<0.05 were considered as statistically significant.
Supplemental Figure I. Expression of EP4 receptors on platelets from rat (A), mouse (B) or guinea pig (C) was determined by indirect flow cytometry. The graph is representative of 3 stainings from different animals. For mouse EP4 staining the samples from 5 mice were pooled.
Supplemental Figure II. Lack of inhibitory effect of EP4 stimulation on platelet aggregation in the rat, mouse and guinea pig. Washed platelets were stimulated with 30 µM of ADP and platelet aggregation was recorded. The concentration of ADP (2.5-20 µM) was adjusted to give submaximal aggregation. Data were expressed as percent of the control response to ADP and are shown as the mean±SEM of 3 experiments (rat), 4 experiments (guinea pig) or 2 experiments (pooled of 4-5 mice, each). * P<0.05 versus pretreatment with vehicle.
**Supplemental Figure III. EP4 stimulation does not change total GPIIb expression.**

Platelets were treated with ONO AE1-329 (300 nM), iloprost (3 nM) or PGE₂ (10 µM) and then activated with 3 µM of ADP. In some experiments, platelets were additionally pretreated with the TP receptor antagonist SQ29548 (1 µM), acetylsalicylic acid (1 mM) and/or the EP4 antagonist ONO AE3-208 (1 and 10 µM). GPIIb expression was determined using an anti-CD41 antibody in flow cytometry. Data were expressed as percent of the control response to ADP and are shown as the mean±SEM of 6 experiments.

**Supplemental References**

