Increased Bleeding Tendency and Decreased Susceptibility to Thromboembolism in Mice Lacking the Prostaglandin E Receptor Subtype EP₃

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Background—Among the prostanoids, thromboxane (TX) A₂ is a potent stimulator of platelets, whereas prostaglandin (PG) I₂ inhibits their activation. The roles of PGE₂ in the regulation of platelet function have not been established, however, and the contribution of PGE₂ in hemostasis and thromboembolism is poorly understood. The present study was intended to clarify these roles of PGE₂ by using mice lacking the PGE₂ receptor subtype 3 (EP₃⁻/⁻ mice).

Methods and Results—Expression of mRNAs for EP₃ in murine platelets was confirmed by quantitative reverse transcription–polymerase chain reaction. PGE₂ and AE-248, a selective EP₃ agonist, showed concentration-dependent potentiation of platelet aggregation induced by U46619, a TXA₂ receptor agonist, although PGE₂ alone could not induce aggregation. PGE₂ and AE-248 increased cytosolic calcium ion concentration ([Ca²⁺]ᵢ), and AE-248 inhibited the forskolin-induced increase in cytosolic cAMP concentration ([cAMP]ᵢ), suggesting Gᵢ coupling of EP₃. The potentiating effects of PGE₂ and AE-248 on platelet aggregation along with their effects on [Ca²⁺]ᵢ and [cAMP]ᵢ were absent in EP₃⁻/⁻ mice. In vivo, the bleeding time was significantly prolonged in EP₃⁻/⁻ mice. Moreover, when mice were challenged intravenously with arachidonic acid, mortality and thrombus formation in the lung were significantly reduced in EP₃⁻/⁻ mice.

Conclusions—PGE₂ potentiated platelet aggregation induced by U46619 via EP₃ by increasing [Ca²⁺]ᵢ, decreasing [cAMP]ᵢ, or both. This potentiating action of PGE₂ via EP₃ is essential in mediating both physiological and pathological effects of PGE₂ in vivo. (Circulation. 2001;104:1176-1180.)

Key Words: platelets • prostaglandins • thromboxane • hemorrhage • thrombosis

Among the prostanoids, thromboxane (TX) A₂ is a potent stimulator of platelets, whereas prostaglandin (PG) I₂ inhibits their activation.¹,² The role of PGE₂ in the regulation of platelet function, however, has not been established. In the circulatory system, PGE₂ is produced from several lines of cells. Activated platelets produce significant amounts of PGE₂,³,⁴ which increases 20-fold in the presence of a TXA₂ synthase inhibitor.⁵ Macrophages are another source of PGE₂, especially in an inflammatory situation. For example, although resident macrophages containing only cyclooxygenase 1 produce small amounts of PGE₂, induction of cyclooxygenase 2 with lipopolysaccharide results in the preferential production of PGE₂.⁶ Platelets play a critical role not only in hemostasis but also in many pathological conditions, such as cerebral thrombosis, ischemic heart disease, and atherosclerosis,⁷-⁹ in which they exert their actions by aggregating themselves and releasing bioactive substances.⁹-¹¹ Because of these roles of platelets, PGE₂ produced in the circulatory system may have a great influence on hemostasis and many pathological conditions through the control of platelet function.

The actions of PGE₂ are mediated through rhodopsin-type cell surface receptors.¹² There are 4 subtypes of the PGE₂ receptor: EP₁, EP₂, EP₃, and EP₄.¹² Signaling of EP₁ results in [Ca²⁺]ᵢ elevation, and those of EP₂ and EP₄ produce elevations of [cAMP]. EP₃, when transfected in Chinese hamster ovary cells, mediates inhibition of adenylate cyclase and an increase in [Ca²⁺]ᵢ.¹³ PGE₂ has been reported to have a biphasic effect on platelet response, potentiating their aggregation at low concentrations and inhibiting it at higher concentrations.¹⁴ It has been suggested that PGE₂ acts on a specific EP receptor to potentiate aggregation and that its inhibitory actions are mediated by the PG₁₂ receptor (ie, IP).¹⁵,¹⁶ In fact, a specific binding site for [³H]PGE₂ has been demonstrated on human...
platelets,17 and activation of this receptor has been suggested to lead to inhibition of adenylate cyclase through G5.18 Matthews and Jones19 compared the potentiating effects of various PGE analogues on aggregatory response and their effects on [cAMP]i in human platelets and suggested that the relevant receptor is “EP-like” and mediates the potentiating effect of PGE2 by inhibiting adenylate cyclase. The action of PGE2 on platelets has been controversial, however, and its role in the regulation of platelet function has been largely unknown. This is because there have been no known agonists or antagonists strictly specific to each of the 4 subtypes of EP, which has prevented characterization of the receptors participating in the regulation of platelet function.

To explore the physiological and pathophysiological roles of PGE2, we generated mice lacking EP3 (EP3−/− mice).20 Moreover, AE-248,21 a recently developed compound, shows higher selectivity to EP3 compared with those of known EP3 agonists such as sulprostone and M&B-28767.22 In this report, we characterize the EP3 participating in the potentiating action of PGE2 on platelet aggregation and clarified the roles of PGE2 in vivo by using EP3−/− mice and AE-248.

Methods

Animals and Reagents

The generation and maintenance of EP3−/− mice have been reported previously.20,21 All studies were performed on 8- to 12-week-old male and female mice except for the experiments involving arachidonic acid– and collagen/epinephrine–induced thromboembolism, in which only male mice were used. All experiments in this study were approved by the Asahikawa Medical College Committee on Animal Research. ADP, epinephrine, and PGE2 were purchased from Sigma Chemical Co, and U46619 was from Cayman Chemical. Collagen reagent was purchased from Hormon Chemie. AE-248 was a gift from ONO Pharmaceuticals, Osaka, Japan.

Platelet Aggregation

Blood was taken by cardiac puncture from ethanesthetized mice with a syringe containing 50 μL of 3.8% trisodium citrate and was diluted with an equal volume of a buffer: 20 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L MgCl2, and 5 mmol/L KCl (pH 7.4). The final concentration of trisodium citrate was adjusted to 0.38%. Platelet-rich plasma (PRP) was prepared by centrifugation at 800 rpm for 5 minutes. Platelet-poor plasma was obtained by further centrifugation at 3000 rpm for 5 minutes. The number of platelets in the PRP was adjusted to 3×1010 platelets/μL. Platelet aggregation was measured with an aggregometer (PAT-4A, Nihon Koden). 23 U46619 was a representative agonist for the TXA2 receptor (TP), which was used to activate the receptor. U46619 is not as potent a stimulator of murine platelets as it is of human platelets, however, inducing full aggregation of murine platelets at concentrations >20 μmol/L.

Reverse Transcription–Polymerase Chain Reaction

PRP was carefully prepared to avoid contamination with leukocytes. After the addition of EDTA (7.7 mmol/L), PRP was centrifuged at 2000 rpm for 10 minutes. The platelet pellet was washed once and suspended in PBS. The purity of platelets estimated by hemocytometry was >99.9%. Total RNA was isolated from the platelets with TriReagent (Molecular probe), and reverse transcription was performed according to the protocol of the manufacturer. The mRNA levels were determined by real-time PCR using Taqman dye and specific primer sets for EPs, as we previously reported.24 The amplified product was sequenced to confirm the specificity of the primers and probes used.

Calcium Measurements

Platelets were prepared as described previously25,26 and loaded with 10 μmol/L fura 2-AM (Dojindo) for 45 minutes and resuspended in a buffer containing 10 mmol/L HEPES, 145 mmol/L NaCl, 1 mmol/L MgCl2, 5 mmol/L KCl, and 1 mmol/L CaCl2 (pH 7.4). The fluorescence was measured with a fluorometer (CAF-110, Japanese Spectroscopic Co). The Ca2+ content of the platelet was calculated according to a previously reported method.27 The [Ca2+]i reached a peak value within 2 minutes of administration of the reagents and then declined quickly. The data for [Ca2+]i represent peak values.

CAMP Measurements

PRP was preincubated for 5 minutes at 37°C with 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) and then exposed to various concentrations of PGE2 for 5 minutes at 37°C. In experiments examining whether AE-248 inhibits the increase in [cAMP], 1 μmol/L forskolin was simultaneously incubated with IBMX for 5 minutes at 37°C and then exposed to the agent for 5 minutes at 37°C. The content of cAMP was determined by a radioimmunoassay kit (Yamasa Shoyu).

Bleeding Time

Bleeding times were assessed according to a previously reported method.28 In brief, mice were placed in a holder, and their tails were transected 1 cm proximal from the tip. The remaining tail was immersed immediately into PBS maintained at 37°C, and the time during which visible bleeding was observed was measured.

Thromboembolism Induced by Arachidonic Acid and Collagen/Epinephrine

Acute thromboembolism was assessed with an established model.29 Into conscious male mice, 62.5 mg/kg body wt of arachidonic acid was injected into the tail vein. Survival was evaluated 1 hour after injection, because the mice alive at 1 hour usually recovered from this challenge. For histological examination, mice were humanely killed 3 minutes after injection, and the lungs were excised. Tissue preparations were stained with hematoxylin and eosin. We also assessed acute thromboembolism in another model.27 Into conscious male mice, 2 mg/kg of collagen and 120 mg/kg of epinephrine dissolved in a buffer included in the collagen reagent were injected into the tail vein. The amount of collagen and epinephrine used was determined as that which induced mortality of 80% to 90% in wild-type mice.

Results

Expression of mRNAs of Prostanoid Receptors in Murine Platelets

We first examined whether mRNAs of the EPs were expressed in murine platelets by the RT-PCR method (Figure 1). We found expression of mRNAs of EP2, EP4, and EP3 along with those of TP and IP. Expression of DP, EP1, and EP5 was not detected. We next quantified the expression levels of mRNAs of the EPs with competitive RT-PCR. The expres-
Figure 1. Expression of mRNAs for prostanoid receptors. Expression of mRNAs of prostanoid receptors was assessed by RT-PCR. Amplification products in lane 1 are nonspecific. Although primers used for amplification of EP1, DP, and FP worked well with cDNAs prepared from kidney, ileum, and aorta, respectively, these primers failed to amplify respective band with cDNAs from platelets.

Figure 2. U46619-induced platelet aggregations and effects of PGE2 on them in wild-type and EP3−/− mice. A, U46619-induced platelet aggregations in wild-type and EP3−/− mice. There were no significant differences in platelet responses to U46619 between wild-type and EP3−/− mice (Figure 2A), indicating that there was no difference in the sensitivity of platelets to U46619 between these mice. In wild-type mice, PGE2 potentiated U46619-induced platelet aggregation concentration-dependently, with an EC50 value of 10 μmol/L (Figure 2B). Similar potentiating effects of PGE2 were observed on ADP-induced aggregation (data not shown). PGE2 at concentrations of 30 μmol/L or higher showed inhibitory effects on aggregation (data not shown), probably because of the cross-action of PGE2 on IP. In wild-type mice, PGE2 itself, however, could not induce platelet aggregation at up to 30 μmol/L concentration. In EP3−/− mice, the potentiating action of PGE2 disappeared completely, and an inhibitory action, probably via IP, was disclosed (Figure 2B). In wild-type mice, AE-248 also potentiated the U46619-induced aggregation (Figure 3A). In EP3−/− mice, however, AE-248 lost this action completely (Figure 3B). Although AE-248 itself could not induce platelet aggregation, it did induce a shape change at concentrations of ≥30 μmol/L (data not shown). These results clearly show that EP3 mediates the potentiating effect of PGE2 on platelet aggregation. It is notable that U46619 at concentrations of ≤2 μmol/L could induce full aggregation in the presence of PGE2 or AE-248, because U46619 alone at these concentrations could induce only small, reversible aggregations (Figures 2B and 3A).

Figure 3. Effects of AE-248 on U46619-induced platelet aggregation in wild-type and EP3−/− mice. A, Effect of AE-248 on U46619 (2 μmol/L)-induced platelet aggregation in wild-type mice. B, Effect of AE-248 on U46619 (2 μmol/L)-induced platelet aggregation in EP3−/− mice. Arrows indicate time of U46619 addition, and total duration of aggregations is 5 minutes. Concentrations of AE-248 are shown near each aggregation curve; these agents were added 1 minute before addition of U46619. Data are representative of 6 experiments with similar results. 

Signaling of the Potentiating Effect of PGE2 Mediated by EP3

In wild-type mice, both PGE2 and AE-248 induced a significant increase in [Ca2+]. In EP3−/− mice, however, these agonists failed to increase [Ca2+], (Figure 4A). PGE2 itself at 1 μmol/L increased [cAMP], in wild-type mice, probably because of the cross-action on IP. This increase in [cAMP], however, was significantly augmented in EP3−/− mice (Figure 4B), suggesting that the inhibitory action of PGE2 on [cAMP], occurred via EP3. In accordance with this finding, AE-248 suppressed the forskolin-induced increase in [cAMP], in wild-type mice. In contrast, in EP3−/− mice, AE-248 failed to suppress this increase in [cAMP], (Figure 4C), indicating that EP3 indeed mediated the inhibitory action of PGE2 on [cAMP], along with its stimulatory effect on [Ca2+]. These results indicate that the potentiating action of PGE2 on platelet aggregation is mediated by EP3 via an elevation in [Ca2+]i.

Increased Bleeding Tendency and Decreased Susceptibility to Thromboembolism in EP3−/− Mice

The bleeding times were 141 ± 17 and 353 ± 46 seconds in wild-type and in EP3−/− mice, respectively (Figure 5A). This...
result clearly showed that endogenous PGE₂ plays an important role in hemostasis via EP₃. Because it is generally accepted that TXA₂ plays a major role in hemostasis, this result is surprising and may suggest that the concentration of TXA₂ generated in this condition could not fully activate the platelets by itself but required the potentiating action of PGE₂.

To validate this assumption, we next examined the acute thromboembolism induced by arachidonic acid, in which TXA₂ is known to play a main role. As shown in Figure 4B, 8 of 10 wild-type mice died within 10 minutes of injection of arachidonic acid. In contrast, 8 of 9 EP₃⁻/⁻ mice survived. Histological examination showed marked thrombus formation in the arterioles of the lung from wild-type mice. Alveolar hemorrhage was also observed in broad areas, which frequently accompanied massive pulmonary thrombosis (Figure 5C and 5D). In contrast, little evidence of such thrombus formation or alveolar hemorrhage was found in the lungs from EP₃⁻/⁻ mice (Figure 5E and 5F). We further examined the acute thromboembolism induced by collagen and epinephrine, in which the mediator of thromboembolism is independent of prostanoid production. There was no difference in mortality between the wild-type and the EP₃⁻/⁻ mice: 10 of 12 and 9 of 12 mice, respectively, died within 15 minutes of injection. These findings suggest an important pathological role for PGE₂ in acute thromboembolism, again via EP₃, and suggest that this role is dependent on the production of PGE₂ in relevant pathological conditions.

Discussion

The expression of mRNAs of EP₁ and EP₃ has recently been reported in human platelets, but precise expression levels of these receptors are unclear. We found significant expression of EP₁ mRNA, whereas those for EP₂ and EP₄ were much lower. There is a limitation, however, to the use of RT-PCR for determination of expression level of mRNA in platelets, because of possible contamination of mRNAs from leukocytes. This limitation, however, could be overcome by examining the platelet function in mice lacking EP₁ and the role of the EP₃ expressed in platelets was verified functionally in this study.

To assess the in vitro and in vivo roles of PGE₂ in the regulation of platelet function, we used mice lacking EP₁ and a specific EP₃ agonist, AE-248. We first examined the effects of PGE₂ on platelet aggregation and demonstrated for the first time that EP₃ mediates the potentiating effect of PGE₂ on platelet aggregation. Although extremely high concentrations of PGE₂ inhibited the aggregations induced by U46619 and ADP, this inhibitory effect may be derived from cross-action of PGE₂ on IP as suggested.

We next examined the signaling of PGE₂ in platelets. The potentiating effect of PGE₂ on platelet aggregation has been
reported to be mediated by inhibition of the increase in [cAMP].\textsuperscript{10,28} We also found that EP\textsubscript{1} mediates the decrease in [cAMP]. Moreover, we demonstrated that PGE\textsubscript{2} induces the increase in [Ca\textsuperscript{2+}]\textsubscript{i} via EP\textsubscript{3}. To the best of our knowledge, this is the first report demonstrating the participation of Ca\textsuperscript{2+} in signaling of EP\textsubscript{3} in platelets. Whether the decrease in [cAMP], or the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, is important in the stimulatory effect of PGE\textsubscript{2} via EP\textsubscript{3}, however, remains to be determined.

Although the in vitro effects of PGE\textsubscript{2} on platelet aggregation have been reported, the roles of PGE\textsubscript{2} in the regulation of platelet function in vivo have not been known. To clarify these roles of PGE\textsubscript{2}, we tried 2 models in which platelet activation is thought to contribute critically: bleeding time and acute thromboembolism. Surprisingly, the bleeding time was significantly increased in EP\textsubscript{3}\textsuperscript{−/−} mice compared with that in wild-type mice. Moreover, EP\textsubscript{3}\textsuperscript{−/−} mice displayed a strong resistance to arachidonic acid–induced thromboembolism. These results show that PGE\textsubscript{2} via EP\textsubscript{3} plays a key role in hemostasis and acute thromboembolism. TXA\textsubscript{2}, however, has been thought to be a major player in these experimental models\textsuperscript{20} and in vivo. In fact, mice lacking TP have recently been reported to show increased bleeding time and decreased resistance to thromboembolism,\textsuperscript{29} and we reported that naturally occurring mutations of the TP gene cause an abnormal bleeding tendency.\textsuperscript{31}

Taken together, these results may suggest a novel mechanism involved in hemostasis and acute thromboembolism; that is, although both TXA\textsubscript{2} and PGE\textsubscript{2} are produced at the site of hemostasis or thrombus formation, the concentration of TXA\textsubscript{2} achieved is too low to fully activate the platelets by itself, requiring the potentiating action of PGE\textsubscript{2}.

The roles of PGE\textsubscript{2} demonstrated in this study probably could not be directly applied to humans, because there may be some differences in the expression of the prostanoid receptors between human and mouse platelets. EP\textsubscript{1}, however, might play a role in mediating the action of PGE\textsubscript{2} in physiological and pathological conditions. Thus, the roles established here for PGE\textsubscript{2} may lead to the development of novel drugs, which would act specifically on EP\textsubscript{3} and could modulate platelet function in various pathological conditions.

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References
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