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.⁷,⁸,⁹ 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 adenylyl 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 PGI₂ receptor (ie, IP).¹⁵,¹⁶ In fact, a specific binding site for [³H]PGE₂ has been demonstrated on human...
platelets, and activation of this receptor has been suggested to lead to inhibition of adenylate cyclase through G\(\text{i}\). Matthews and Jones\(^{19}\) compared the potentiating effects of various PGE analogues on aggregatory response and their effects on [cAMP], in human platelets and suggested that the relevant receptor is “EP\(_1\)-like” and mediates the potentiating effect of PGE\(_2\) by inhibiting adenylate cyclase. The action of PGE\(_2\) 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 PGE\(_2\), we generated mice lacking EP\(_3\) (EP\(_{3}\)\(^{-/-}\) mice).\(^{20}\) Moreover, AE-248,\(^{21}\) a recently developed compound, shows higher selectivity to EP\(_3\) compared with those of known EP\(_3\) agonists such as sulprostone and M&B-28767.\(^{22}\) In this report, we characterized the EP participating in the potentiating action of PGE\(_2\) on platelet aggregation and clarified the roles of PGE\(_2\) in vivo by using EP\(_{3}\)\(^{-/-}\) mice and AE-248.

**Methods**

**Animals and Reagents**

The generation and maintenance of EP\(_{3}\)\(^{-/-}\) mice have been reported previously.\(^{20}\) 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 PGE\(_2\) 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 ether-anesthetized mice with a syringe containing 50 \(\mu\)L of 3.8% trisodium citrate and was diluted with an equal volume of a buffer: 20 mmol/L HEPES, 140 mmol/L NaCl, 5 mmol/L MgCl\(_2\), 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 further obtained by centrifugation at 3000 rpm for 10 minutes. The number of platelets in the PRP was adjusted to 3 \(\times\) 10\(^{10}\) platelets/\(\mu\)L. Platelet aggregation was measured with an aggregometer (PAT-4A, Nihon Koden).\(^{23}\) U46619, a representative agonist for the TXA\(_2\) receptor (TP), was used to activate the receptor. U46619 is not as potent a stimulator of murine platelets at concentrations \(>20\) mmol/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 Isogen (Nippon Gene). Total RNA (2 \(\mu\)g) was reverse-transcribed (RT) with Moloney murine leukemia virus reverse transcriptase (Toyobo) and oligo-d\(T\) primers (Gibco-BRL). The resulting cDNA was amplified by 35 polymerase chain reaction (PCR) cycles with an annealing temperature of 60\(^\circ\)C with primer sets specific for each prostanoid receptor. To quantify expression levels of the mRNAs for the EPs, we adopted competitive RT-PCR methods that included a competitive DNA construction kit (Takara). Primer sequences used were as follows: (1) for EP\(_1\), 5’ primer 5’-CGCGAGGC-TTACGAGGCA-CAGA-3’, 3’ primer 5’-GGTTGCTGGGCGAA-CAGTCG-3’; (2) for EP\(_3\), 5’ primer 5’-AG-CATCTGCTAGGCGT-AGAGAC-3’, 3’ primer 5’-AGACCCCTT-TACACCTTCTCAATGG-3’; (3) for EP\(_5\), 5’ primer 5’-GGTAT-GGCGGAAATCTAAGAC-3’, 3’ primer 5’-GAAATAGCAG-CAACCAG-GGAAGTGGTTTT-3’; (4) for EP\(_5\), 5’ primer 5’-TTCGCGTGTTGG-TGCGAGTTC-3’, 3’ primer 5’-GAGTGTGGTGTGG-CTGGTGAC-3’; (5) for FP, 5’ primer 5’-GCTCTTGTTTG-TCTCCTGTGT-3’, 3’ primer 5’-GTCACTCGA- GAAATAGCAGCACCAC-3’; (6) for TP, 5’ primer 5’-CTCCGACACTGGTGAC-3’, 3’ primer 5’-GATATAGGACC- CAGGGTTCAACAG-3’; (7) for IP, 5’ primer 5’-GGCGAGGGA- GTGAGATTTT-3’, 3’ primer 5’-GTCACTCGAAGCACCCAG- TCAATGG-3’; and (8) for DP, 5’ primer 5’-GCTTITTGTGCG- CTCCCCCTTGTG-3’, 3’ primer 5’-CATCCGGAATACTGAAGT- TCCCTG-3’.

**Calcium Measurements**

Washed platelets prepared as described previously\(^{24}\) were loaded with 10 \(\mu\)mol/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 MgCl\(_2\), 5 mmol/L KCl, and 1 mmol/L CaCl\(_2\) (pH 7.4). The fluorescence was measured with a fluorometer (CAF-110, Japanese Spectroscopic Co). The [Ca\(^{2+}\)]\(_{\text{i}}\) was calculated according to a previously reported method.\(^{24}\) The [Ca\(^{2+}\)]\(_{\text{i}}\) reached a peak value within 2 minutes of administration of the reagents and then declined quickly. The data for [Ca\(^{2+}\)]\(_{\text{i}}\) represent peak values.

**cAMP Measurements**

PRP was preincubated for 5 minutes at 37°C with 1 mmol/L forskolin was simultaneously incubated with IBMX for 5 minutes at 37°C. In experiments examining whether AE-248 inhibits the increase in [cAMP], 1 \(\mu\)mol/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 as that which induced mortality of 80% to 90% in male mice. 20

**Bleeding Time**

Bleeding times were assessed according to a previously reported method.\(^{25}\) 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.\(^{26}\) 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 EP\(_2\), EP\(_{5}\), and EP\(_{5}\) along with those of TP and IP. Expression of DP, EP\(_{3}\), and FP was not detected. We next quantified the expression levels of mRNAs of the EPs with competitive RT-PCR. The expres-
Potentiating Effect of PGE₂ on Platelet Aggregation Is Mediated by EP₃

U46619-induced platelet aggregations were similar 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.¹⁵,¹⁶ PGE₂ itself, however, could not induce platelet aggregation at up to 30 μmol/L concentration. In EP₁⁻/⁻ 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).

Signaling of the Potentiating Effect of PGE₂ Mediated by EP₁

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 EP₁. 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 EP₁ 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 EP₁ via an elevation in [Ca²⁺], a decrease in [cAMP], or both.

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 EP₂. 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 EP₂⁻/⁻ 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 EP₂⁻/⁻ 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 EP₂⁻/⁻ 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 EP₂, and suggest that this role is dependent on the production of PGE₂ in relevant pathological conditions.

**Discussion**

The expression of mRNAs of EP₁ and EP₂ has recently been reported in human platelets, but precise expression levels of these receptors are unclear. We found significant expression of EP₂ mRNA, whereas those for EP₁ 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 EP₂, 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 EP₃ 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.¹⁵,¹⁶

We next examined the signaling of PGE₂ in platelets. The potentiating effect of PGE₂ on platelet aggregation has been

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*Figure 4. Signal transduction of prostanoid receptors in platelets. A, Effects of AE-248 (10 μmol/L) or PGE₂ (10 μmol/L) on [Ca²⁺]i in wild-type (●) and EP₂⁻/⁻ (●) mice. Data are expressed as mean ± SEM (n=6 to 8). Basal [Ca²⁺]i was similar between wild-type and EP₂⁻/⁻ mice and was 87 ± 4 nmol/L in wild-type mice (n=8). **P<0.01 vs wild-type mice (unpaired Student’s t test). B and C, Stimulatory effects of PGE₂ (1 μmol/L) on cAMP formation (B) and inhibitory effects of AE-248 (10 μmol/L) on forskolin-stimulated cAMP formation (C) in wild-type (●) and EP₂⁻/⁻ (●) mice. Basal cAMP levels in wild-type and EP₂⁻/⁻ mice were 339 ± 21 and 342 ± 19 pmol/10⁹ platelets, respectively. cAMP levels stimulated by forskolin in wild-type and EP₂⁻/⁻ mice were 913 ± 112 and 824 ± 36 pmol/10⁹ platelets, respectively. Data are mean ± SEM (n=5). *P<0.05; **P<0.01 vs wild-type mice (unpaired Student’s t test).

*Figure 5. Hemostasis and arachidonic acid–induced thromboembolism in wild-type and EP₂⁻/⁻ mice. A, Bleeding times for wild-type (WT; n=24) and EP₂⁻/⁻ (n=18) mice. Data are mean ± SEM. **P<0.01 vs wild-type mice (unpaired Student’s t test). B, Thrombotic challenge in wild-type (WT; n=10) and EP₂⁻/⁻ (n=9) mice. Data represent percentage of deaths after injection of 62.5 mg/kg arachidonic acid. C through F, Histological examination of lungs from wild-type (C, D) and EP₂⁻/⁻ (E, F) mice. Marked thrombus formation in arterioles and alveolar hemorrhage are seen in wild-type lung. Arrows indicate arterioles. Boxed areas in C and E (×33) are magnified and presented as D and F (×132), respectively. Representative data from several independent experiments are presented.*
reported to be mediated by inhibition of the increase in [cAMP]. We also found that EP1 mediates the decrease in [cAMP]. Moreover, we demonstrated that PGE2 induces the increase in [Ca2+]i through EP3. To the best of our knowledge, this is the first report demonstrating the participation of Ca2+ in signaling of EP3 in platelets. Whether the decrease in [cAMP], or the increase in [Ca2+]i, is important in the stimulatory effect of PGE2 via EP3, however, remains to be determined.

Although the in vitro effects of PGE2 on platelet aggregation have been reported, the roles of PGE2 in the regulation of platelet function in vivo have not been known. To clarify these roles of PGE2, 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 EP3−/− mice compared with that in wild-type mice. Moreover, EP3−/− mice displayed a strong resistance to arachidonic acid–induced thromboembolism. These results show that PGE2 via EP3 plays a key role in hemostasis and acute thromboembolism. TXA2, however, has been thought to be a major player in these experimental models and in vivo. In fact, mice lacking TP have recently been reported to show increased bleeding time and decreased resistance to thromboembolism, and we reported that naturally occurring mutations of the TP gene cause an abnormal bleeding tendency. Taken together, these results may suggest a novel mechanism involved in hemostasis and acute thromboembolism; that is, although both TXA2 and PGE2 are produced at the site of hemostasis or thrombus formation, the concentration of TXA2 achieved is too low to fully activate the platelets by itself, requiring the potentiating action of PGE2.

The roles of PGE2 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. EP1, however, might play a role in mediating the action of PGE2 in physiological and pathological conditions. Thus, the roles established here for PGE2 may lead to the development of novel drugs, which would act specifically on EP3 and could modulate platelet function in various pathological conditions.

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