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

Hong Ma, MD; Akiyoshi Hara, PhD; Chun-Yang Xiao, PhD; Yuji Okada, MD; Osamu Takahata, MD; Kazuhiro Nakaya, PhD; Yukihiro Sugimoto, MD; Atsushi Ichikawa, MD; Shuh Narumiya, MD; Fumitaka Ushikubi, MD

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
platelets, and activation of this receptor has been suggested to lead to inhibition of adenylate cyclase through G\textsubscript{i}. Matthews and Jones 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\textsubscript{P}”, and mediates the potentiating effect of PGE\textsubscript{2} by inhibiting adenylate cyclase. The action of PGE\textsubscript{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 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\textsubscript{2}, we generated mice lacking EP\textsubscript{3} (EP\textsubscript{3}\textsuperscript{−/−}). Moreover, AE-248, a recently developed compound, shows higher selectivity to EP\textsubscript{3}, compared with those of known EP\textsubscript{3} agonists such as sulprostone and M&B-28767. In this report, we characterized the EP participating in the potentiating action of PGE\textsubscript{2} on platelet aggregation and clarified the roles of PGE\textsubscript{2} in vivo by using EP\textsubscript{3}\textsuperscript{−/−} mice and AE-248.

**Methods**

**Animals and Reagents**

The generation and maintenance of EP\textsubscript{3}\textsuperscript{−/−} mice have been reported previously. 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\textsubscript{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\textsubscript{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 obtained by further centrifugation at 3000 rpm for 10 minutes. The number of platelets was counted. Platelet aggregation was measured with an aggregometer (PAT-4A, Nihon Koden). 23 U46619 was a representative agonist for the TXA\textsubscript{2} receptor (TP), 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 platelets. In order to lead to inhibition of adenylate cyclase through G\textsubscript{i}.18

**cAMP Measurements**

PRP was preincubated for 5 minutes at 37°C 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\textsubscript{2}, 5 mmol/L KCl, and 1 mmol/L CaCl\textsubscript{2} (pH 7.4). The fluorescence was measured with a fluorescent (CAF-110, Japanese Spectroscopic Co). The [Ca\textsuperscript{2+}]) was calculated according to a previously reported method. The [Ca\textsuperscript{2+}] reached a peak value within 2 minutes of administration of the reagents and then declined quickly. The data for [Ca\textsuperscript{2+}] represent peak values.

**Bleeding Time**

Bleeding times were assessed according to a previously reported method. 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. 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. 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\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4} along with those of TP and IP. Expression of DP, EP\textsubscript{1}, and FP was not detected. We next quantified the expression levels of mRNAs of the EPs with competitive RT-PCR.
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 IP1,15,16 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).

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+]i. In EP3−/− mice, however, these agonists failed to increase [Ca2+]i (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+]i. These results indicate that the potentiating action of PGE2 on platelet aggregation is mediated by EP1 via an elevation in [Ca2+]i, 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).
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 EP3 and EP4 has recently been reported in human platelets, but precise expression levels of these receptors are unclear. We found significant expression of EP3 mRNA, whereas those for EP1 and EP2 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 EP3, 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.

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 
$[cAMP]_{i}$. We also found that EP1 mediates the decrease in 
$[cAMP]_{i}$. Moreover, we demonstrated that PGE2 induces the 
increase in $[Ca^{2+}]_{i}$ via EP3. To the best of our knowledge, this 
is the first report demonstrating the participation of Ca$^{2+}$ in 
signaling of EP3 in platelets. Whether the decrease in $[cAMP]_{i}$, 
or the increase in $[Ca^{2+}]_{i}$, 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 
for platelet function in various pathological conditions.

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. EP3, 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.

Acknowledgments

This work was supported by a grant-in-aid for scientific research 
from the Ministry of Education, Science, Sports, and Culture 
of Japan and by a research grant for cardiovascular diseases (11C-1) 
from the Ministry of Health and Welfare. This work was also supported 
by grants from the Ono Medical Research Foundation, 
the Takeda Science Foundation, the Suhara Memorial Foundation, 
and the Smoking Research Foundation.

References

1. Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of 
biologically active compounds derived from prostaglandin endoperoxides. 
arteries transforms prostaglandin endoperoxides to an unstable substance 
3. Smith JB, Willis AL. Formation and release of prostaglandins by platelets 
platelets: redirection by the thromboxane synthetase inhibitor UK 37,248. 
Br J Pharmacol. 1983;79:356P.
6. Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferen-
tially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin 
contraction by a substance released from platelets: evidence that it is 
and actions of prostaglandins, endoperoxides, and thromboxanes. 
340:115–126.
10. Grainger DJ, Wakefield L, Bethell HW, et al. Release and activation of 
platelet latent TGF-beta in blood clots during dissolution with plasmin. Nat 
lysophosphatidic acid is released from activated platelets. Biochem J. 
12. Narumiya S, Sugimoto Y, Ushikubi F. Prostanoid receptors: structures, 
 subtype mediates calcium signals via G, in cDNA-transfected Chinese 
14. Gray S, Heptinstall S. The effects of PGE2 and CL 115,347, an antithy-
 pertensive PGE1 analogue, on human blood platelet behavior and vascular 
15. Andersen NH, Eggertsen TL, Harker LA, et al. On the multiplicity of 
platelet prostaglandin receptors. I: evaluation of competitive antagonism 
prostaglandin receptors, II: the use of N-0164 for distinguishing the loci 
of action for PGI2, PGD2, PGE2, and hydantoin analogs. Prostaglandins. 
17. Eggertsen TL, Andersen NH, Robertson RP. Separate receptors for 
prostacyclin and prostaglandin E2 on human gel-filtered platelets. J 
18. Ashby B. Cyclic AMP turnover in response to prostaglandins in intact 
platelets: evidence for separate stimulatory and inhibitory prostaglandin 
19. Matthews JS, Jones RL. Potentiation of aggregation and inhibition of 
adrenylate cyclase in human platelets by prostaglandin E analogues. Br 
receptor subtypes (EP1, EP2, EP3 and EP4) in bone resorption: 
the eight types and subtypes of the mouse prostaglandin receptors expressed in 
with platelet thromboxane A2 receptor abnormality: defective signal transduction 
animals, II: a comparison of different assay conditions in rats. Thromb 
26. Kohler C, Wooding W, Eilenbogen L. Intravenous arachidonate in the 
mouse: a model for the evaluation of antithrombotic drugs. Thromb Res. 
27. DiMinno G, Silver MJ. Mouse antithrombotic assay: a simple method for 
the evaluation of antithrombotic agents in vivo. J Pharmacol Exp Ther. 
1983;225:57–60.
adrenylate cyclase on platelet membranes, and inhibits forskolin-stimulated 
adenylate cyclase in human platelets by prostaglandin E analogues. Br 
30. Thomas DW, Bannon RB, Bannon PJ, et al. Coagulation defects and 
altered hemodynamic responses in mice lacking receptors for 
31. Hirata T, Kakizuka A, Ushikubi F, et al. Arg1$^{\alpha}$ to Leu mutation of 
the human thromboxane A2 receptor in a dominantly inherited bleeding 
Increased Bleeding Tendency and Decreased Susceptibility to Thromboembolism in Mice Lacking the Prostaglandin E Receptor Subtype EP₃
Hong Ma, Akiyoshi Hara, Chun-Yang Xiao, Yuji Okada, Osamu Takahata, Kazuhiro Nakaya, Yukihiko Sugimoto, Atsushi Ichikawa, Shuh Narumiya and Fumitaka Ushikubi

Circulation. 2001;104:1176-1180
doi: 10.1161/hc3601.094003

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/104/10/1176

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/