The Novel Prostaglandin I₂ Mimetic ONO-1301 Escapes Desensitization in an Antiplatelet Effect Due to Its Inhibitory Action on Thromboxane A₂ Synthesis in Mice

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ABSTRACT

ONO-1301 ([E]-5-[2-[1-phenyl-1-(3-pyridyl)methylideneaminooxy]ethyl]-7,8-dihydronaphthalene-1-yloxy)acetic acid) is a novel prostaglandin (PG) I₂ mimic with inhibitory activity on the thromboxane (TX) A₂ synthase. Interestingly, ONO-1301 retains its inhibitory effect on platelet aggregation after repeated administration, while beraprost, a representative agonist for the PG₁₂ receptor (IP), loses its inhibitory effect after repeated administration. In the present study, we intended to clarify the mechanism by which ONO-1301 escapes desensitization of an antiplatelet effect. In platelets prepared from wild-type mice, ONO-1301 inhibited collagen-induced aggregation and stimulated cAMP production in an IP-dependent manner. In addition, ONO-1301 inhibited arachidonic acid-induced TXA₂ production in platelets lacking IP. Despite the decrease in stimulatory action on cAMP production, the antiplatelet effect of ONO-1301 hardly changed after repeated administration for 10 days in wild-type mice. Noteworthy, beraprost could retain its antiplatelet effect after repeated administration in combination with a low dose of ozagrel, a TXA₂ synthase inhibitor. Therefore, we hypothesized that chronic IP stimulation by beraprost induces an increase in TXA₂ production, leading to reduction in the antiplatelet effect. As expected, repeated administration of beraprost increased the plasma and urinary levels of a TXA₂ metabolite, while ONO-1301 did not increase them significantly. In addition, beraprost could retain the ability to inhibit platelet aggregation after repeated administration in mice lacking the TXA₂ receptor (TP). These results indicate that TP-mediated signaling participates in platelet desensitization against IP agonists and that simultaneous inhibition of TXA₂ production confers resistance against desensitization on IP agonists.

Introduction

Prostanoids, including prostaglandins (PGs) and thromboxane (TX), are lipid mediators that exert a variety of actions in the body through their respective receptors (Narumiya et al., 1999). Among these prostaglandins, PG₁₂ and TXA₂ play an important role in the maintenance of cardiovascular homeostasis (Hamberg et al., 1975; Moncada et al., 1976). PG₁₂, which is produced mainly by vascular endothelial cells, is a vasodilator and an inhibitor of platelet aggregation (Moncada et al., 1976). In contrast, TXA₂, which is synthesized mainly by platelets, is a vasoconstrictor and a potent stimulator of platelet aggregation (Ellis et al., 1976). Accordingly, PG₁₂ and TXA₂ are critically involved in the pathogenesis of thrombotic disorders, such as atherosclerosis obliterans, acute myocardial infarction, and cerebral thrombosis (Coleman et al., 1994; Narumiya et al., 1999; Yuhki et al., 2011).

PG₁₂ is chemically unstable. Its half-life is only several minutes under physiologic conditions. In addition, circulatory PG₁₂ is biologically unstable because it is metabolized efficiently by 15-hydroxyprostaglandin dehydrogenase of the pulmonary vascular endothelium. To overcome the problem of this instability of PG₁₂, several PG₁₂ analogs, including illoprost and beraprost, have been developed. However, a clinically important issue is that signaling mediated by the PG₁₂ receptor IP is desensitized easily by repeated administration of these analogs (Smyth et al., 1996; Zucker et al., 1998). ONO-1301
Collagen was purchased from Nycodem Pharma (Munich, Germany). U-46619 [9,11-dideoxy-9ß,11a-methanoepoxy-prosta-5Z,13E-dien-1-oic acid] and arachidonic acid were purchased from Cayman Chemical (Ann Arbor, MI). Carboxymethyl cellulose sodium salt (CMC-Na) and acetylsalicylic acid (aspirin) were purchased from Nacalai Tesque (Kyoto, Japan). ONO-1301 and ozagrel were obtained from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Beraprost was obtained from Toray Industries, Inc. (Tokyo, Japan). Seratrodast was purchased from KRT Laboratories, Inc. (St. Paul, MN). Forskolin, 3-isobutyl-1-methylxanthine, murine fibrinogen, and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO).

**Materials and Methods**

**Platelet Preparation.** Blood was drawn by cardiac puncture through a 21 G needle into a syringe containing 3.8% trisodium citrate from ether-anesthetized mice and was diluted immediately with an equal volume of an experimental buffer (20 mM HEPES, 140 mM NaCl, 5 mM MgCl₂, and 5 mM KCl, pH 7.4). Diluted blood was then centrifuged at 90g for 5 minutes, and platelet-rich plasma (PRP) was obtained by collecting the upper phase. Platelet-poor plasma (PPP) was prepared by further centrifuging the remaining lower phase at 1500g for 10 minutes. For repeated administration studies, 0.5% CMC-Na (10 ml/kg per day) was administered with or without ONO-1301 (20 mg/kg per day), beraprost (0.3 mg/kg per day), ozagrel, an inhibitor of TXA₂ synthase (3 mg/kg per day), aspirin (1 mg/kg per day), or seratrodast, a TP antagonist (30 mg/kg per day) by oral gavage once a day. At 2 hours after drug administration, blood was drawn. In platelet aggregation studies, the number of platelets in PRP was adjusted to 3 × 10⁵ platelets/µl with PPP, and the final concentration of trisodium citrate was adjusted to 0.38%. To prepare washed platelets, a one-tenth volume of 100 mM EDTA (pH 7.4) was added to PRP, and the mixture was centrifuged at 900g for 15 minutes. The platelet pellet was washed once with a washing buffer (135 mM NaCl, 5 mM KCl, 8 mM Na₂HPO₄, 2 mM NaH₂PO₄, and 10 mM EDTA, pH 7.2) and centrifuged at 90g for 15 minutes. Finally, the platelet pellet was suspended in modified Tyrode’s buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.42 mM Na₂HPO₄, and 1 mM MgCl₂, pH 7.4) and the number of platelets was adjusted to 1 × 10⁶ platelets/µl.

**Platelet Aggregation Study.** PRP (200 µl) was stirred at 37°C in a cuvette, and platelet aggregation was examined using an aggregometer (PAT-4A; Nihon Koden, Tokyo, Japan), which was set to show platelet aggregation rates in unstimulated PRP and PPP to be 0% and 100%, respectively (Ushikubi et al., 1987). PRP was preincubated for 5 minutes, and then ONO-1301 or beraprost was added to PRP 1 minute before the addition of collagen or U-46619, a TP agonist, which was added at a concentration to induce platelet aggregations of 50%–60% (collagen, 1.5–2.0 µg/ml or 45%–55% (U-46619, 2.5–3.0 µM), respectively. To examine platelet aggregations in repeated administration studies, collagen concentrations were fixed to 2.0 µg/ml in WT mice and 3.0 µg/ml in TP-/- mice and WT mice administered seratrodast, concentrations of which induced platelet aggregations of 50%–70%. U-46619 concentration was fixed to 3.0 µM to induce platelet aggregations of 40%–70%.

**Measurement of cAMP Production.** Washed platelets (100 µl) were preincubated in the presence of 1 mM 3-isobutyl-1-methylxanthine, an inhibitor of phosphodiesterase, for 10 minutes at 37°C, and then ONO-1301, beraprost, or forskolin, a direct activator of adenylate cyclase, was added. After further incubation for 10 minutes at 37°C, the reaction was terminated by the addition of 30% trichloroacetic acid (25 µl), and the platelets were disrupted by sonication. The solution was centrifuged at 20,400g for 10 minutes at 4°C, and the supernatant was washed three times with water-saturated diethyl ether. The content of cAMP was determined with a radioimmunoassay kit (Yamasa Shoyu, Chiba, Japan).

**Measurement of TXB₂ Content.** Washed platelets (100 µl) were preincubated in the presence of 1 mM CaCl₂ and 100 µM fibrinogen for 5 minutes at 37°C, and then ONO-1301 or ozagrel was added 1 minute before the addition of 0.2 mM arachidonic acid. After further incubation for 5 minutes at 37°C, the reaction was terminated by the addition of ice-cold 1 N HCl (25 µl), and the platelets were centrifuged at 20,400g for 10 minutes at 4°C. The supernatant was neutralized with one-fifth volume of 1 M Tris, and the content of TXB₂ was measured with a TXB₂ enzyme immunoassay (EIA) kit (Cayman Chemical).

When determining the plasma TXB₂ level after repeated administration, 0.5% CMC-Na (10 ml/kg per day) was administered with or without ONO-1301 (20 mg/kg per day), beraprost (0.3 mg/kg per day), ozagrel (3 mg/kg per day), or aspirin (1 mg/kg per day) to WT mice for 10 days by oral gavage once a day. At 2 hours after drug administration on the 10th day, blood was drawn with a syringe containing 3.8% trisodium citrate and 100 µM indomethacin, and then the final concentrations of trisodium citrate and indomethacin were adjusted to 0.38% and 10 µM, respectively. Blood was immediately
centrifuged at 1500g for 20 minutes at 4°C, and plasma was obtained by collecting the upper phase. Plasma was purified by a C-18 solid phase extraction column (Cayman Chemical). Briefly, four times the volume of ethanol was added to plasma and the mixture was centrifuged at 3000g for 10 minutes at 4°C to remove precipitated proteins. Ethanol of the upper phase was evaporated under a gentle stream of nitrogen, and 1 M acetic acid was added to the sample to adjust the pH to 3.5–4.0. The sample was passed through a C-18 solid phase extraction column, and the column was washed with ultrapure water followed by hexane. To elute the sample, ethyl acetate containing 1% methanol was added to the column. Finally, an organic solvent of the eluate was evaporated under a nitrogen stream and the sample was suspended in a buffer of a TXB2 EIA kit. After the plasma had been purified, plasma content of TXB2 was measured with the EIA kit.

**Measurement of Blood Pressure.** WT mice were administered 0.5% CMC-Na (10 ml/kg per day) with or without ONO-1301 (20 or 100 mg/kg) or beraprost (0.3 or 3 mg/kg) by oral gavage. After drug administration, the blood pressure of conscious mice was measured by the tail-cuff method with a BP-98A instrument (Softron, Tokyo, Japan).

**Measurement of 11-Dehydro TXB2 and Creatinine Contents in Urine.** WT mice were administered 0.5% CMC-Na (10 ml/kg per day) with or without ONO-1301 (20 mg/kg per day) or beraprost (0.3 mg/kg per day) by oral gavage once a day. After repeated administration for 10 days, mice were moved individually to plastic metabolic cages with a wire mesh floor and 24-hour urine samples were collected. Urinary content of 11-dehydro TXB2 was measured with an 11-dehydro TXB2 EIA kit (Cayman Chemical), and the content of creatinine in urine was measured with a QuantiChrom creatinine assay kit (BioAssay Systems, Hayward, CA).

**Measurement of 6-Keto PGF1α Content in Plasma.** WT mice were administered 0.5% CMC-Na (10 ml/kg per day) with or without ONO-1301 (20 mg/kg per day) or beraprost (0.3 mg/kg per day) for 10 days by oral gavage once a day. At 2 hours after drug administration on the 10th day, blood was drawn with a syringe containing 3.8% trisodium citrate and 100 μM indomethacin, and then the final concentrations of trisodium citrate and indomethacin were adjusted to 0.38% and 10 μM, respectively. Blood was immediately centrifuged at 1500g for 20 minutes at 4°C, and plasma was obtained by collecting the upper phase. Plasma was purified by the preferred method of a 6-keto PGF1α EIA kit (Cayman Chemical). Briefly, four times the volume of acetone was added to plasma, and the mixture was centrifuged at 3000g for 10 minutes at 4°C to remove precipitated proteins. Acetone of the upper phase was evaporated under a gentle stream of nitrogen, and a saturated NaCl solution was added to the sample. To wash the sample, ethyl acetate–acetone (3:1) was added and the upper phase was collected twice. Finally, an organic solvent was evaporated under a nitrogen stream and the sample was suspended in a buffer of the EIA kit. After the plasma had been purified, plasma content of 6-keto PGF1α was measured with the EIA kit.

**Measurement of PGE2 Content in Plasma.** WT mice were administered 0.5% CMC-Na (10 ml/kg per day) with or without ONO-1301 (20 mg/kg per day) for 10 days by oral gavage once a day. At 2 hours after drug administration on the 10th day, blood was drawn with a syringe containing 3.8% trisodium citrate and 100 μM indomethacin, and then the final concentrations of trisodium citrate and indomethacin were adjusted to 0.38% and 10 μM, respectively. Blood was immediately centrifuged at 1500g for 20 minutes at 4°C, and plasma was obtained by collecting the upper phase. After purification by a C-18 solid phase extraction column, plasma PGE2 content was measured with a PGE2 EIA kit (Cayman Chemical) with ultrapure water followed by hexane.

**Data Analysis.** All data are expressed as means ± S.E.M. Statistical comparisons of data were made by Student’s t test or the Mann-Whitney test after the F test for equal variance. For multiple comparisons, repeated two-way analysis of variance or one-way analysis of variance followed by the Tukey-Kramer test was used. P < 0.05 was considered statistically significant.

### Results

**Inhibitory Effect of ONO-1301 on Platelet Aggregation.** We first examined the inhibitory effect of ONO-1301 on collagen-induced aggregation of platelets prepared from WT mice (WT platelets). ONO-1301 inhibited collagen-induced aggregation in a concentration-dependent manner, with an IC50 value of 460 ± 66 nM (Fig. 1). We also examined the inhibitory effect of beraprost, a representative IP agonist, on collagen-induced platelet aggregation and found that its inhibitory potency was almost two rank orders higher than that of ONO-1301. The IC50 value of beraprost was 6.8 ± 1.7 nM (Fig. 1). Moreover, we examined the inhibitory effects of ONO-1301 and beraprost on platelet aggregation induced by U-46619. ONO-1301 and beraprost inhibited U-46619-induced platelet aggregation in concentration-dependent manners, with respective IC50 values of 397 ± 123 and 6.0 ± 1.1 nM (Supplemental Fig. 1).

**Contribution of Prostanoid Receptors to the Inhibitory Effect of ONO-1301 on Platelet Aggregation.** It has been reported that inhibitory PGE2 receptor subtypes, EP2 and EP4, in addition to IP are expressed in platelets (Paul et al., 1998). Accordingly, selective activation of EP2 or EP4 leads to the inhibition of platelet aggregation (Kuriyama et al., 2010). Therefore, to determine which prostanoid receptors contribute to the inhibitory effect of ONO-1301, we examined the effects of ONO-1301 on collagen-induced aggregation of platelets lacking EP2 (EP2–/– platelets), EP4 (EP4–/– platelets), or IP (IP–/– platelets). Collagen induced aggregation in a similar manner among these platelets as well as in WT platelets and platelets prepared from F2-WT mice (F2-WT platelets). In IP–/– platelets, the inhibitory effect of ONO-1301 was entirely abolished (Fig. 2A), indicating that IP plays a pivotal role in the inhibitory action of ONO-1301 on collagen-induced platelet aggregation. In EP2–/– platelets, the inhibitory potency of ONO-1301 did not change significantly compared with that in WT platelets. The IC50 values were 622 ± 166 and 460 ± 66 nM, respectively (Fig. 2A). In addition, the inhibitory potency of ONO-1301 in EP4–/– platelets was not significantly different from that in F2-WT platelets. The IC50 values were 639 ± 152 and 501 ± 95 nM, respectively (Fig. 2B). These results indicate that EP2 and EP4 expressed in platelets are not involved in the inhibitory effect of ONO-1301.

![Fig. 1.](image-url) Effects of ONO-1301 and beraprost on collagen-induced aggregation of platelets prepared from WT mice. Collagen was added at a concentration to induce platelet aggregation of 50%–60% (1.5–2.0 μg/ml). ONO-1301 or beraprost was added at indicated concentrations to PRP 1 minute before the addition of collagen. C of the horizontal axis represents control. Each value is the mean ± S.E.M. (n = 4).
of ONO-1301 on collagen-induced platelet aggregation and that IP is the receptor mediating the inhibitory effect of ONO-1301.

Effect of ONO-1301 on cAMP Production. To further confirm the IP-mediated action of ONO-1301, we examined whether ONO-1301 could increase intracellular concentrations of cAMP, the second messenger of IP signaling, in an IP-dependent manner. The effects of ONO-1301 on cAMP production were examined in washed platelets prepared from WT, EP2+/−, and IP−/− mice (A) and F2-WT and EP4−/− mice (B). Collagen was added at a concentration to induce platelet aggregation of 50%–60% (1.5–2.0 μg/ml). ONO-1301 was added at indicated concentrations to PRP 1 minute before the addition of collagen. C on the horizontal axis represents control. Each value is the mean ± S.E.M. (n = 4).

Inhibitory Effect of ONO-1301 on TXA2 Production in IP−/− Washed Platelets. To verify the action of ONO-1301 as a TXA2 synthase inhibitor, we examined the effect of ONO-1301 on arachidonic acid–induced TXA2 production in platelets. To exclude an IP-mediated action of ONO-1301, IP−/− washed platelets were used for the experiment. To estimate the degree of TXA2 production, we measured the contents of TXB2, a stable TXA2 metabolite, in the medium. Both ONO-1301 and ozagrel, a representative TXA2 synthase inhibitor, inhibited TXA2 production in a concentration-dependent manner. The IC50 values were 12 ± 2.2 and 3.4 ± 0.6 μM, respectively (Fig. 4). These results indicate that ONO-1301 works as a TXA2 synthase inhibitor as potently as ozagrel. Therefore, we confirmed that ONO-1301 has two pharmacological actions as an IP agonist and a TXA2 synthase inhibitor, while the IC50 value of ONO-1301 as a TXA2 synthase inhibitor was 30-fold higher than that as an IP agonist (Fig. 1).

Effect of ONO-1301 on Blood Pressure. To examine whether ONO-1301 affects vascular tone via cAMP-mediated signaling, we measured blood pressure in WT mice after administration of ONO-1301. At a dose of 20 mg/kg, ONO-1301 had little effect on blood pressure. On the other hand, beraprost (0.3 mg/kg) caused a slight decrease in blood pressure from 30 to 90 minutes after administration (Supplemental Fig. 2). This time course is compatible with the T1/2 (1.4 ± 0.2 hours) and T1/2 (1.1 ± 0.1 hours) of beraprost (Kato et al., 1989). However, ONO-1301 (100 mg/kg) or beraprost (3 mg/kg) induced a marked reduction in blood pressure (Supplemental Fig. 2).

Inhibitory Effect of ONO-1301 on Platelet Aggregation Was Retained after Repeated Administration. It is common to observe desensitization of IP signaling, as was reported also for other G protein–coupled receptors (GPCRs) (Sibley and Lefkowitz, 1985; Freedman et al., 1995; Sulakhe et al., 1997). Therefore, we examined whether the inhibitory effect of ONO-1301 on collagen-induced platelet aggregation is diminished after repeated oral administration in WT mice. The doses of ONO-1301 and beraprost (20 and 0.3 mg/kg per day, respectively) were determined to cause inhibition of collagen-induced platelet aggregation to similar degrees at day 1, which reflect the difference in their IC50 values of platelet aggregation in vitro (Fig. 1). The inhibitory effect of beraprost on collagen-induced platelet aggregation was reduced significantly at day 7 and had almost completely disappeared at day 10, indicating that desensitization of IP signaling had developed (Fig. 5). In sharp contrast to the case of beraprost, the inhibitory effect of ONO-1301 was maintained to a similar degree during the experimental period, indicating that desensitization of IP signaling had not apparently occurred in the case of ONO-1301 (Fig. 5). Similar results were observed when U-46619 was used to induce platelet aggregation (Supplemental Fig. 3). Furthermore, to
Effect of Low-Dose Ozagrel on Platelet Response to Beraprost after Repeated Administration. To determine whether the action of ONO-1301 as a TXA\(_2\) synthase inhibitor contributes to the retention of its antiplatelet effect, we examined the effect of low-dose ozagrel on the decreased response of platelets to beraprost after repeated administration. At a low dose of 3 mg/kg per day, ozagrel alone did not affect collagen-induced platelet aggregation (Fig. 7A). However, this dose of ozagrel administered concomitantly with beraprost could maintain the inhibitory effect of beraprost on platelet aggregation for 10 days, while beraprost alone almost completely lost its antiplatelet effect at day 10 (Fig. 7A). This result suggests that the inhibitory action of ONO-1301 on TXA\(_2\) synthase contributes to the maintenance of its antiplatelet effect during repeated administration.

To exclude the direct and acute action of ozagrel on beraprost-induced signaling, we examined the antiaggregatory action of beraprost in platelets prepared at 2 hours after single administration of low-dose ozagrel. There was no significant difference in dose-dependent antiplatelet effects of beraprost between low-dose ozagrel-treated and vehicle-treated groups (Fig. 7B), indicating there was no direct action of low-dose ozagrel on beraprost-induced signaling.

On the other hand, aspirin also decreases TXA\(_2\) production by inhibiting cyclooxygenase, a rate-limiting enzyme of prostanoid synthesis. Therefore, we further examined whether beraprost could retain the antiplatelet effect after repeated administration in combination with aspirin. We determined the dose of aspirin as a dose that would not affect collagen-induced platelet aggregation when used alone. As expected, low-dose aspirin (1 mg/kg per day) could prevent desensitization of the antiplatelet effect by beraprost after repeated administration (Fig. 7C).

Effects of Repeated Administration of ONO-1301 and Beraprost on TXA\(_2\) Synthesis. To further confirm participation of TXA\(_2\) in the regulation of platelet responses, we examined whether the degree of TXA\(_2\) synthesis differs in ONO-1301- and beraprost-treated WT mice. Repeated administration of beraprost for 10 days significantly increased the plasma TXB\(_2\) level compared with that of the vehicle-treated control. The levels were 250 ± 34 and 147 ± 18 pg/ml, respectively (Fig. 8A). As expected, however, repeated administration of ONO-1301 did not significantly increase the plasma TXB\(_2\) level (182 ± 13 pg/ml) (Fig. 8A). We also examined the effects of repeated administration of ONO-1301 and beraprost on the level of 11-dehydro TXB\(_2\), one of the main metabolites of TXA\(_2\), in urine. Repeated administration of beraprost for 10 days increased the urinary 11-dehydro TXB\(_2\) level, but repeated administration of ONO-1301 did not
ONO-1301 significantly suppressed the increase in the plasma TXB2 level induced by repeated administration of beraprost. Repeated administration of ONO-1301 and beraprost did not change the plasma 6-keto PGF1α level significantly (Supplemental Fig. 4). This result suggests that retention of the antiplatelet effect of ONO-1301 is not due to a direct increase in PGI2 synthesis but to maintenance of the PGI2/TXA2 balance by suppressed TXA2 production. We also measured the plasma PGE2 level to determine whether redirection of arachidonic acid metabolism toward PGE2 occurred after repeated administration of ONO-1301. Repeated administration of ONO-1301 significantly increased the plasma PGE2 level compared with that in the vehicle-treated controls. The values were 51 ± 7.8 and 30 ± 4.1 pg/ml, respectively (Supplemental Fig. 5). This result indicates that ONO-1301 can escape desensitization of an antiplatelet effect under the condition of redirected arachidonic acid metabolism toward PGE2.

**Involvement of TXA2-Mediated Signaling in Reduction of the Antiplatelet Effect of Beraprost after Repeated Administration.** To confirm the involvement of TXA2-mediated signaling, we examined whether the antiplatelet effect of beraprost diminishes after repeated administration to TP−/− mice. Since collagen-induced platelet aggregation depends partially on TP signaling, the collagen concentration was increased to 3.0 μg/ml to induce a degree of aggregation in TP−/− control platelets similar to that in WT control platelets. In sharp contrast to WT mice (Figs. 5 and 7A), beraprost could retain an inhibitory effect on collagen-induced platelet aggregation for 10 days in TP−/− mice (Fig. 9A). We further examined whether beraprost can retain the antiplatelet effect after repeated administration in WT mice when TP-mediated signaling was blocked pharmacologically by seratrodast, a TP antagonist. Beraprost administered in combination with seratrodast could retain its antiplatelet effect for 10 days (Fig. 9B), pharmacologically reproducing the result obtained in the experiment using TP−/− mice (Fig. 9A). These results clearly indicate that TXA2 plays a crucial role in the reduction of the antiplatelet effect of beraprost after repeated administration.

**Discussion**

Previous studies indicated that PGI2 and its analogs require repeated or continuous administration because of their instability and that repeated IP stimulation easily induces desensitization of IP signaling (Smyth et al., 1996; Zucker et al., 1998). In the present study, we intended to clarify the mechanism by which ONO-1301 could escape desensitization of the antiplatelet effect after repeated administration. We first confirmed that ONO-1301 has an IP-independent inhibitory action on TXA2 production in platelets as well as an IP-dependent antiaggregatory action. Accordingly, an increase in plasma and urinary levels of a TXA2 metabolite induced by repeated IP stimulation by the IP agonist beraprost was not observed after repeated administration of ONO-1301, suggesting participation of TP

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**Fig. 6.** Effects of repeated administration of ONO-1301 and beraprost on cAMP production induced by ONO-1301 (A), beraprost (B), and forskolin (C) are presented. ONO-1301 (20 mg/kg per day) or beraprost (0.3 mg/kg per day) was administered orally to WT mice once a day, and washed platelets were prepared 2 hours after administration on the 10th day. ONO-1301 (10 μM), beraprost (1 μM), or forskolin (10 μM) was added to washed platelets to induce cAMP production. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 versus vehicle.
Fig. 7. Effects of low-dose ozagrel and aspirin on antiplatelet action of beraprost. (A) Time courses of the effects of beraprost, low-dose ozagrel, and beraprost plus low-dose ozagrel on collagen-induced platelet aggregation. Beraprost (0.3 mg/kg per day), ozagrel (3 mg/kg per day), or beraprost plus ozagrel was administered orally to WT mice once a day and PRP was prepared 2 hours after the administration. Collagen (2.0 μg/ml) was added to PRP to induce platelet aggregation. Each value is the mean ± S.E.M. Vehicle, n = 6; beraprost, n = 6; low-dose ozagrel, n = 3; beraprost plus low-dose ozagrel, n = 4. *P < 0.05. (B) Effects of low-dose ozagrel on antiplatelet action of beraprost. Ozagrel (3 mg/kg) was administered orally to WT mice and PRP was prepared 2 hours after the administration. Collagen (2.0 μg/ml) was added to PRP to induce platelet aggregation. Beraprost was added at indicated concentrations to PRP 1 minute before the addition of collagen. C of the horizontal axis represents control. Each value is the mean ± S.E.M. (n = 4). (C) Time courses of the effects of beraprost, low-dose aspirin, and beraprost plus low-dose aspirin on collagen-induced platelet aggregation. Beraprost (0.3 mg/kg per day), aspirin (1 mg/kg per day), or beraprost plus aspirin was administered orally to WT mice once a day, and PRP was prepared 2 hours after the administration. Collagen (2.0 μg/ml) was added to PRP to induce platelet aggregation. Each value is the mean ± S.E.M. Vehicle, n = 4; beraprost, n = 4; low-dose aspirin, n = 4; beraprost plus low-dose aspirin, n = 6. *P < 0.05.

A Novel Action of ONO-1301 in Platelet Aggregation

ONO-1301 has been developed as a molecule having a dual function: stimulation of IP and inhibition of TXA2 synthase. We showed that ONO-1301 inhibits collagen-induced platelet aggregation in a concentration-dependent manner (Fig. 1). Recent studies have shown that platelet function is regulated via several prostanoid receptors expressed in platelets, including EP2 (Kuriyama et al., 2010; Smith et al., 2010), EP3 (Fabre et al., 2001; Ma et al., 2001), EP4 (Iyú et al., 2010; Kuriyama et al., 2010; Philipose et al., 2010; Smith et al., 2010), IP (Murata et al., 1997), and TP (Thomas et al., 1998). Among these receptors, EP2 and EP4, in addition to IP, mediate inhibitory signaling in platelets. Therefore, we investigated which inhibitory receptor(s) mediates the inhibitory action of ONO-1301 on platelet aggregation using mice lacking each inhibitory receptor. In EP2–/– or EP4–/– platelets, the inhibitory potency of ONO-1301 was not significantly different from that in respective control platelets. In contrast, the inhibitory action of ONO-1301 in IP–/– platelets disappeared completely (Fig. 2). This result indicates that the inhibitory action of ONO-1301 on collagen-induced platelet aggregation was mediated specifically by IP. In accordance with the antiaggregatory action, ONO-1301 increased platelet cAMP concentration in an IP-dependent manner (Fig. 3). Apart from the effect on platelets, we found that ONO-1301 could decrease blood pressure when used at a higher dose of 100 mg/kg (Supplemental Fig. 2), suggesting IP-mediated vasodilation via cAMP signaling. Although ONO-1301 did not affect blood pressure at the dose used in the present study, we should take notice of its potential effect on blood pressure. We also showed that ONO-1301 inhibited arachidonic acid–induced TXA2 production in a concentration-dependent and IP-independent manner (Fig. 4). The inhibitory potency of ONO-1301 in TXA2 production is 30-fold lower than that in platelet aggregation, suggesting that ONO-1301 works mainly as an IP agonist. However, the action of ONO-1301 as an inhibitor of TXA2 synthesis would also contribute to determining the pharmacological nature of ONO-1301.

Notably, the antiplatelet effect of ONO-1301 did not decrease significantly after repeated administration, although that of beraprost decreased markedly (Fig. 5). This result indicates the possibility that the activity of ONO-1301 that inhibits TXA2 synthase prevented the antiplatelet effect of ONO-1301 from being desensitized. It should also be noted that the antiplatelet effect of beraprost was restored after 4 days of drug withdrawal (Fig. 5), suggesting that...
desensitization of the antiplatelet effect of beraprost is a reversible phenomenon. This result is consistent with previous findings in human platelets (Modesti et al., 1987; Fisch et al., 1997). To try to determine the mechanism underlying desensitization of the antiplatelet effect, we examined whether repeated administration of ONO-1301 and beraprost affects succeeding cAMP production. Unexpectedly, cAMP production decreased significantly in platelets prepared from mice repeatedly treated with ONO-1301, as was the case with beraprost (Fig. 6). The extents of decreases in cAMP production were similar in platelets prepared from mice treated with ONO-1301 and beraprost when stimulated by respective agents. Notably, repeated administration of ONO-1301 and beraprost induced decreased cAMP responses against each of the agents (Fig. 6, A and B), indicating the development of cross-desensitization in IP/cAMP signaling. Moreover, decreases in cAMP response against forskolin were induced by repeated administration of ONO-1301 and beraprost (Fig. 6C), indicating involvement of adenylate cyclase and/or its downstream signaling in the desensitization of IP/cAMP signaling. Although we did not examine the mechanism of this desensitization, mechanisms common to GPCRs, such
as a diminished response of adenylate cyclase (Jaschonek et al., 1988), downregulation of Gα (Finney et al., 2000), and activation of phosphodiesterase (Seybold et al., 1998), would be involved. These results indicate that ONO-1301 and beraprost induce desensitization of IP-mediated signaling to similar extents, although there is a distinctive difference in the antiplatelet effects after repeated administration of these agents. Therefore, a mechanism other than IP/cAMP signaling would explain the difference in the antiplatelet effects between ONO-1301 and beraprost after repeated administration.

We examined whether TP signaling contributes to retention of the antiplatelet effect after repeated administration of ONO-1301. Noteworthy, low-dose ozagrel could rescue beraprost from losing the antiplatelet effect (Fig. 7A), suggesting that suppression of TXA2 production is critically involved in the retention of the antiplatelet effect. In line with this result, low-dose aspirin also rescued beraprost from the desensitization (Fig. 7C). From these results, we expected that TXA2 synthesis would be promoted by repeated administration of beraprost, leading to a decrease in the antiplatelet effect. As expected, repeated administration of beraprost increased the plasma and urinary levels of the TXA2 metabolite, while repeated administration of ONO-1301 did not induce such increases (Fig. 8, A and C). This result is consistent with a previous finding that continuous administration of beraprost increases TXA2 production in rats (Kataoka et al., 2005). Interestingly, low-dose ozagrel and aspirin suppressed only the increase in the plasma level of TXB2 induced by beraprost (Fig. 8C) without an effect on the plasma TXB2 level when used alone. The mechanism of these effects remains to be determined.

On the other hand, repeated administration of ONO-1301 significantly increased the plasma PGE2 level. The level was 51 pg/ml (145 pM) (Supplemental Fig. 5), which is consistent with a previous finding that inhibition of TXA2 formation by dazoxiben, an inhibitor of TXA2 synthase, redirected arachidonic acid metabolism from TXA2 toward PGE2 in human clotting blood in vitro (Watts et al., 1991). We previously reported that PGE2 potentiated platelet aggregation at concentrations above 1 nM via EP3 (Ma et al., 2001) and inhibited it at concentrations above 300 nM via EP2 and EP4 (Kuriyama et al., 2010). These concentrations of PGE2 were higher than the plasma PGE2 concentration detected after repeated administration of ONO-1301, suggesting that redirection of arachidonic acid metabolism does not affect the mechanism by which ONO-1301 escapes desensitization, at least under the present experimental conditions.

To further confirm the involvement of TP signaling, we examined whether beraprost could retain its antiplatelet effect after repeated administration in TP−/− mice, which lack TP-mediated signaling completely. Notably, beraprost could retain its antiplatelet effect, inhibiting platelet aggregation at even the 10th day of repeated administration (Fig. 9A). In addition, this finding was well reproduced when TP signaling was blocked pharmacologically by the TP antagonist seratrodast (Fig. 9B). These results clearly indicate that the TXA2/TP system plays a critical role in the development of desensitization in the antiplatelet effects of beraprost and that the action that inhibits TXA2 production confers resistance against desensitization of the antiplatelet effect on ONO-1301.

Previous studies demonstrated reciprocal regulation between IP and TP. TP activation resulted in sensitization of the cAMP response to an IP agonist in human platelets (Murray et al., 1990), and IP stimulation induced desensitization of the human TPα isoform in a protein kinase A–dependent manner (Walsh et al., 2000). It is known that several GPCRs form heterodimers and interact with one another, modifying their function (Devi, 2001; Breitwieser, 2004). Heterodimerization of IP with TP has also been reported. Although major signaling of TP is Gq-mediated inositol phosphate generation, IP-TP heterodimerization facilitated TP-mediated cAMP generation when these receptors were coexpressed in human embryonic kidney cells (Wilson et al., 2004). Noteworthy, TP activation induced heterologous internalization of IP followed by decreased response of the cell to an IP agonist in an IP–TP dimerization-dependent fashion (Wilson et al., 2007). This finding raises the possibility that reduction in the effect of beraprost found in this study depends on facilitated IP internalization through TP activation induced by increased TXA2 production. However, the precise mechanism by which TP signaling facilitates the development of desensitization against IP agonists remains to be determined.

In conclusion, this is the first report demonstrating that a TXA2 synthase inhibitor rescues an IP agonist from losing the antiplatelet effect after repeated administration. Given an important application of PGI2 analogs as antiplatelet agents, it is a clinically very important issue to develop a method for preventing reduction in the effect of PGI2 analogs. The results of the present work provide a novel insight in this issue and will contribute to broadening of the clinical application of PGI2 analogs.

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