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)methylideneaminoxy]ethyl]-7,8-dihydronaphthalene-1-yloxy]acetic acid] is a novel prostaglandin (PG) I₂ mimetic 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 PG I₂ 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 prostanoids, PG I₂ and TX A₂ play an important role in the maintenance of cardiovascular homeostasis (Hamberg et al., 1975; Moncada et al., 1976). PG I₂, 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, PG I₂ and TXA₂ are critically involved in the pathogenesis of thrombotic disorders, such as arteriosclerosis obliterans, acute myocardial infarction, and cerebral thrombosis (Coleman et al., 1994; Narumiya et al., 1999; Yuhki et al., 2011).

PG I₂ is chemically unstable. Its half-life is only several minutes under physiologic conditions. In addition, circulatory PG I₂ 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 PG I₂, several PG I₂ analogs, including illoprost and beraprost, have been developed. However, a clinically important issue is that signaling mediated by the PG I₂ receptor IP is desensitized easily by repeated administration of these analogs (Smyth et al., 1996; Zucker et al., 1998). ONO-1301

ABBREVIATIONS: CMC-Na, carboxymethyl cellulose sodium salt; EIA, enzyme immunoassay; GPCR, G protein-coupled receptor; IP, prostaglandin I₂ receptor; ONO-1301, [(E)-5-[2-[1-phenyl-1-(3-pyridyl)methylideneaminoxy]ethyl]-7,8-dihydronaphthalene-1-yloxy]acetic acid; PG, prostaglandin; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TP, thromboxane A₂ receptor; TX, thromboxane; U-46619, 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid; WT, wild-type.
In this study, we attempted to clarify the mechanism by which ONO-1301 retains its inhibitory effect on platelet aggregation when administered repeatedly. We first pharmacologically defined the IP-dependent action of ONO-1301 on aggregation response and cAMP production in murine platelets and the IP-independent action of ONO-1301 on TXA2 production. Then, we confirmed that ONO-1301 could retain its inhibitory effect on platelet aggregation after repeated oral administration to mice. Finally, we examined whether TXA2 synthase inhibitory activity of ONO-1301 and TXA2 receptor (TP)-mediated signaling play a key role in the retention of the antiplatelet effect of ONO-1301 after repeated administration.

Materials and Methods

Collagen was purchased from Nycomed Pharma (Munich, Germany). U-46619 [9,11-dideoxy-9α,11α-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 LKT Laboratories, Inc. (Tokyo, Japan).

Mice. Generation and maintenance of mice lacking the PGE2 receptor subtype EP2 (EP2−/− mice), EP4 (EP4−/− mice), IP (IP−/− mice), or TP (TP−/− mice) have been reported (Murata et al., 1997; Segi et al., 1998; Hizaki et al., 1999; Kabashima et al., 2003). These mice and wild-type (WT) control mice, but with the exception of EP4−/− mice, have a genetic background similar to that of C57BL/6 mice. Ep2−/− mice have a mixed genetic background of C57BL/6 and 129/Ola mice (Segi et al., 1998). For the experiments using EP4−/− mice, F2-WT mice having a similar genetic background were used as controls. All mice experiments, which were approved by the Asahikawa Medical University Committee on Animal Research, were carried out using 12- to 20-week-old male mice.

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 (0.3 mg/kg per day), aspirin (1 mg/kg per day), or seratrodast (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 × 10^5 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 × 10^5 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 μM 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 (Yamaso 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 (ELA) 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) 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).

**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.
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 mice (WT washed platelets) and EP4–/– mice (IP–/– washed platelets). In WT washed platelets, both ONO-1301 and beraprost potently increased intracellular cAMP levels (Fig. 3). In IP–/– washed platelets, however, these increases were completely abolished (Fig. 3). These results clearly 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 $T_{\text{max}}$ (1.4 ± 0.2 hours) and $t_{1/2}$ (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
Effects of Repeated Administration of ONO-1301 and Beraprost on cAMP Production. To gain further insight into the difference between the long-term actions of ONO-1301 and beraprost, we next examined the effects of repeated administration of ONO-1301 and beraprost on succeeding cAMP production in WT washed platelets. After repeated administration of beraprost for 10 days, increases in platelet cAMP levels induced by both ONO-1301 and beraprost were significantly blunted compared with that in the vehicle control (Fig. 6, A and B), suggesting that desensitization of IP signaling observed by repeated administration of beraprost is reversible, we stopped administration of beraprost from days 11 to 14 and examined the inhibitory effect of beraprost at day 15. The antiplatelet effect of beraprost had recovered completely at day 15, indicating that beraprost-induced desensitization is a reversible phenomenon (Fig. 5).

Effects of Repeated Administration of ONO-1301 and Beraprost on TXA2 Synthesis. To further confirm partic-

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 TXA2 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).

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IP signaling was induced by beraprost. Interestingly, the increases in cAMP levels induced by both ONO-1301 and beraprost after repeated administration of ONO-1301 were also significantly blunted (Fig. 6, A and B), indicating that desensitization of IP signaling was also induced by ONO-1301, despite the retention of its antiplatelet effect. Furthermore, repeated administration of ONO-1301 and beraprost led to significant decreases in forskolin-induced cAMP production (Fig. 6C), implying that adenylate cyclase and/or its downstream signaling were involved in desensitization induced by these agents. These results indicate that a mechanism other than IP/cAMP-mediated signaling contributes to the retention of the antiplatelet effect of ONO-1301.

Effect of Low-Dose Ozagrel on Platelet Response to Beraprost after Repeated Administration. To determine whether the action of ONO-1301 as a TXA2 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 TXA2 synthase contributes to the maintenance of its antiplatelet effect during repeated administration.

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The plasma TXB2 levels of mice treated with a vehicle, beraprost, beraprost plus ozagrel, and beraprost significantly suppressed the increase in the plasma TXB2 level induced by repeated IP stimulation by beraprost to the control level (Fig. 8C), while ozagrel or aspirin alone did not affect the plasma TXB2 level (data not shown). These results suggest that repeated IP stimulation by beraprost increases the TXA2 level, leading to the development of a decreased platelet response to beraprost.

Furthermore, we measured the plasma level of 6-keto PGFα, a major metabolite of PGI2, to determine whether the inhibitory activity of ONO-1301 on TXA2 synthase causes a shift of arachidonic acid metabolism toward PGI2. Repeated administration of ONO-1301 and beraprost did not change the plasma 6-keto PGFα 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 TXA2−/− 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 TXA2−/− 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 TXA2−/− 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.
the addition of collagen. Beraprost was added at indicated concentrations to PRP 1 minute before aspirin (1 mg/kg per day), or beraprost plus aspirin was administered collagen-induced platelet aggregation. Beraprost (0.3 mg/kg per day), ozagrel (3 mg/kg per day), or beraprost plus low-dose aspirin on collagen-induced platelet aggregation. Each value is the mean ± S.E.M. Vehicle, n = 4; beraprost, n = 6; beraprost plus low-dose ozagrel, n = 3; beraprost plus low-dose aspirin, n = 4. *P < 0.05. (B) Effects of low-dose ozagrel 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 of aspirin (Fig. 5). This result indicates the possibility that the activity of ONO-1301 that inhibits TXA2 synthase synthesis 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 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.

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 mg/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 mg/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.
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 on plasma TXB₂ levels were examined. 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 plasma was prepared 2 hours after administration on day 10. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 versus vehicle. (B) Effects of repeated administration of ONO-1301 and beraprost on urinary 11-dehydro TXB₂ levels were examined. 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 a 24-hour urine sample was collected on day 10. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 versus vehicle. (C) Effects of repeated administration of beraprost, beraprost plus low-dose ozagrel, and beraprost plus low-dose aspirin on plasma TXB₂ levels were examined. Beraprost (0.3 mg/kg per day), beraprost plus ozagrel (3 mg/kg per day), or beraprost plus aspirin (1 mg/kg per day) was administered orally to WT mice once a day, and plasma was prepared 2 hours after administration on day 10. Each value is the mean ± S.E.M. (n = 4). *P < 0.05.

Fig. 8. (A) Effects of repeated administration of ONO-1301 and beraprost on plasma TXB₂ levels were examined. 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 plasma was prepared 2 hours after administration on day 10. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 versus vehicle. (B) Effects of repeated administration of ONO-1301 and beraprost on urinary 11-dehydro TXB₂ levels were examined. 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 a 24-hour urine sample was collected on day 10. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 versus vehicle. (C) Effects of repeated administration of beraprost, beraprost plus low-dose ozagrel, and beraprost plus low-dose aspirin on plasma TXB₂ levels were examined. Beraprost (0.3 mg/kg per day), beraprost plus ozagrel (3 mg/kg per day), or beraprost plus aspirin (1 mg/kg per day) was administered orally to WT mice once a day, and plasma was prepared 2 hours after administration on day 10. Each value is the mean ± S.E.M. (n = 4). *P < 0.05.
as a diminished response of adenylate cyclase (Jaschonek et al., 1988), downregulation of Gs (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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