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Title Page

The novel prostaglandin I_2 mimetic ONO-1301 escapes desensitization in an anti-platelet effect due to its inhibitory action on thromboxane A_2 synthesis in mice

Hitoshi Kashiwagi, Koh-ichi Yuhki, Fumiaki Kojima, Shima Kumei, Osamu Takahata, Yoshiki Sakai, Shuh Narumiya and Fumitaka Ushikubi

Department of Pharmacology, Asahikawa Medical University, Asahikawa, Japan (H. K., K. Y., F. K., S. K., O. T., F. U., Primary Laboratory of Origin)

JST, CREST, Tokyo, Japan (H. K., K. Y., F. K., S. K., S. N., F. U.)

Ono Pharmaceutical Co., Ltd., Research Headquarters, Osaka, Japan (Y. S.)

Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto, Japan (S. N.)

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Running Title Page

Running title: A novel action of ONO-1301 in platelet aggregation

Correspondence to: Fumitaka Ushikubi, Department of Pharmacology, Asahikawa Medical University, Midorigaoka-Higashi 2-1-1-1, Asahikawa 078-8510, Japan.

Phone: 81-166-68-2360; Fax: 81-166-68-2369; E-mail: ushikubi@asahikawa-med.ac.jp

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Abbreviations:

CMC-Na, carboxymethyl cellulose sodium salt; GPCR, G protein-coupled receptor; IBMX, 3-isobutyl-1-methylxanthine; ONO-1301, (E)-[5-[2-[1-phenyl-1-(3-pyridyl)methylidene-aminooxy]ethyl]-7,8-dihydronaphthalene-1-yloxy]acetic acid; PG, prostaglandin; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TX, thromboxane; WT, wild-type.

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Abstract

ONO-1301 is a novel prostaglandin (PG) I_2 mimetic with inhibitory activity on thromboxane (TX) A_2 synthase. Interestingly, ONO-1301 retains its inhibitory effect on platelet aggregation after repeated administration, while beraprost, a representative agonist for the PGI_2 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 anti-platelet 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 TXA_2 production in platelets lacking IP. Despite the decrease in stimulatory action on cAMP production, the anti-platelet effect of ONO-1301 hardly changed after repeated administration for 10 days in wild-type mice. Noteworthy, beraprost could retain its anti-platelet effect after repeated administration in combination with a low dose of ozagrel, a TXA_2 synthase inhibitor. Therefore, we hypothesized that chronic IP stimulation by beraprost induces an increase in TXA_2 production, leading to reduction in the anti-platelet effect. As expected, repeated administration of beraprost increased the plasma and urinary levels of a TXA_2 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 TXA_2 receptor TP. These results indicate that TP-mediated signaling participates in platelet desensitization against IP agonists and that simultaneous inhibition of TXA_2 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, PGI_2 and TXA_2 play an important role in the maintenance of cardiovascular homeostasis (Hamberg et al., 1975; Moncada et al., 1976). PGI_2, which is produced mainly by vascular endothelial cells, is a vasodilator and an inhibitor of platelet aggregation (Moncada et al., 1976). In contrast, TXA_2, which is synthesized mainly by platelets, is a vasoconstrictor and a potent stimulator of platelet aggregation (Ellis et al., 1976). Accordingly, PGI_2 and TXA_2 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).

PGI_2 is chemically unstable; its half-life is only several minutes under physiological conditions. In addition, circulatory PGI_2 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 PGI_2, several PGI_2 analogues, including illoprost and beraprost, have been developed. However, a clinically important issue is that signaling mediated by the PGI_2 receptor IP is desensitized easily by repeated administration of these analogues (Smyth et al., 1996; Zucker et al., 1998). ONO-1301 was originally developed as a PGI_2 mimetic that has a non-prostanoid structure with TXA_2 synthase inhibitory activity (Kondo et al., 1995). Interestingly, we found that the inhibitory effect of ONO-1301 on platelet aggregation, in contrast to that of beraprost, was

not attenuated by repeated administration. However, the mechanism remains to be determined.

In addition to a potent anti-platelet effect, ONO-1301 has been reported to have several cardiovascular actions. ONO-1301 protected the myocardium from ischemia/reperfusion injury (Hirata et al., 2012), enhanced angiogenesis through up-regulation of endogenous growth factors in the ischemic heart (Nakamura et al., 2007) and suppressed the development of monocrotaline-induced pulmonary hypertension (Kataoka et al., 2005; Obata et al., 2008). Like other PGI_2 analogues, ONO-1301 also has an anti-fibrotic action, suppressing the development of bleomycin-induced pulmonary fibrosis (Murakami et al., 2006) and ureter obstruction-induced tubulointerstitial fibrosis (Nasu et al., 2012). Therefore, ONO-1301 is promising as a therapeutic agent for a variety of cardiovascular diseases.

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 defined pharmacologically the IP-dependent action of ONO-1301 on aggregation response and on cAMP production in murine platelets and the IP-independent action of ONO-1301 on TXA_2 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 TXA_2 synthase inhibitory activity of ONO-1301 and TXA_2 receptor TP-mediated signaling play a key role in retention of the anti-platelet effect of ONO-1301 after repeated administration.

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Materials and Methods

Materials

Collagen was purchased from Nycomed Pharma (Munich, Germany). U-46619 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. (St. Paul, MN). Forskolin, 3-isobutyl-1-methylxanthine (IBMX), murine fibrinogen and indomethacin were purchased from Sigma-Aldrich (St. Louis, MO).

Mice

Generation and maintenance of mice lacking the PGE_2 receptor subtype EP_2 ($EP_2^{-/-}$ mice), EP_4 ($EP_4^{-/-}$ mice), IP ($IP^{-/-}$ mice), or TP ($TP^{-/-}$ mice) have been reported (Hizaki et al., 1999; Segi et al., 1998; Murata et al., 1997; Kabashima et al., 2003). These mice and wild-type (WT) control mice, but with the exception of $EP_4^{-/-}$ mice, have a genetic background similar to that of C57BL/6 mice. $EP_4^{-/-}$ mice have a mixed genetic background of C57BL/6 and 129/ola mice (Segi et al., 1998). For the experiments using $EP_4^{-/-}$ mice, F2-WT mice having a similar genetic background were used as controls. All mice experiments, which were approved by 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 $MgCl_2$ and 5 mM KCl, pH 7.4). Diluted blood was then centrifuged at 90 g for 5 min, 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 1,500 g for 10 min. For repeated administration studies, 0.5% CMC-Na (10 mL/kg/day) was administered with or without ONO-1301 (20 mg/kg/day), beraprost (0.3 mg/kg/day), ozagrel, an inhibitor of TXA_2 synthase (3 mg/kg/day), aspirin (1 mg/kg/day) or seratrodast, a TP antagonist (30 mg/kg/day), by oral gavage once a day. At 2 hr 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, one-tenth volume of 100 mM EDTA (pH 7.4) was added to PRP and the mixture was centrifuged at 900 g for 15 min. The platelet pellet was washed once with a washing buffer (135 mM NaCl, 5 mM KCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4 and 10 mM EDTA, pH 7.2) and centrifuged at 900 g for 15 min. Finally, the platelet pellet was suspended in modified Tyrode's buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.42 mM NaH_2PO_4 and 1 mM $MgCl_2$, pH 7.4) and the number of platelets was adjusted to 1×10^6 platelets/μL.

Platelet aggregation study

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PRP (200 μL) was stirred at 37ºC 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 min and then ONO-1301 or beraprost was added to PRP 1 min 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 to 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 IBMX, an inhibitor of phosphodiesterase, for 10 min at 37ºC, and then ONO-1301, beraprost or forskolin, a direct activator of adenylate cyclase, was added. After further incubation for 10 min at 37ºC, 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 20,400 g for 10 min at 4ºC, 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).

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Measurement of TXB_2 content

Washed platelets (100 μL) were preincubated in the presence of 1 mM $CaCl_2$ and 100 μg/mL fibrinogen for 5 min at 37ºC, and then ONO-1301 or ozagrel was added 1 min before the addition of 0.2 mM arachidonic acid. After further incubation for 5 min at 37ºC, the reaction was terminated by the addition of ice-cold 1 N HCl (25 μL), and the platelets were centrifuged at 20,400 g for 10 min at 4ºC. The supernatant was neutralized with one-fifth volume of 1 M Tris, and the content of TXB_2 was measured with a TXB_2 EIA Kit (Cayman Chemical).

When determining plasma TXB_2 level after repeated administration, 0.5% CMC-Na (10 mL/kg/day) was administered with or without ONO-1301 (20 mg/kg/day), beraprost (0.3 mg/kg/day), ozagrel (3 mg/kg/day) or aspirin (1 mg/kg/day) to WT mice for 10 days by oral gavage once a day. At 2 hr 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 1,500 g for 20 min at 4ºC, 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 volume of ethanol was added to plasma and the mixture was centrifuged at 3,000 g for 10 min at 4ºC 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 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

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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 TXB_2 EIA kit. After the plasma had been purified, plasma content of TXB_2 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, blood pressure of conscious mice was measured by the tail-cuff method with a BP-98A instrument (Softron, Tokyo, Japan).

Measurement of 11-dehydro TXB_2 and creatinine contents in urine

WT mice were administered 0.5% CMC-Na (10 mL/kg/day) with or without ONO-1301 (20 mg/kg/day) or beraprost (0.3 mg/kg/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-hr urine samples were collected. Urinary content of 11-dehydro TXB_2 was measured with an 11-dehydro TXB_2 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 $PGF_1\alpha$ content in plasma

WT mice were administered 0.5% CMC-Na (10 mL/kg/day) with or without

ONO-1301 (20 mg/kg/day) or beraprost (0.3 mg/kg/day) for 10 days by oral gavage once a day. At 2 hr 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 1,500 g for 20 min at 4ºC, and plasma was obtained by collecting the upper phase. Plasma was purified by the preferred method of a 6-keto $PGF_1\alpha$ EIA Kit (Cayman Chemical). Briefly, four-times volume of acetone was added to plasma and the mixture was centrifuged at 3,000 g for 10 min at 4ºC to remove precipitated proteins. Acetone of the upper phase was evaporated under a gentle stream of nitrogen, and 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 $PGF_1\alpha$ was measured with the EIA kit.

Measurement of PGE_2 content in plasma

WT mice were administered 0.5% CMC-Na (10 mL/kg/day) with or without ONO-1301 (20 mg/kg/day) for 10 days by oral gavage once a day. At 2 hr 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 1,500 g for 20 min at 4ºC, and plasma was obtained by collecting the upper

phase. After purification by a C-18 solid phase extraction column, plasma PGE_2 content was measured with a PGE_2 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 ANOVA or one-way ANOVA 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 IC_{50} value of 460 ± 66 nM (Figure 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 IC_{50} value of beraprost was 6.8 ± 1.7 nM (Figure 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 IC_{50} values of 397 ± 123 and 6.0 ± 1.1 nM (Supplemental Figure 1).

Contribution of prostanoid receptors to the inhibitory effect of ONO-1301 on platelet aggregation

It has been reported that inhibitory PGE_2 receptor subtypes, EP_2 and EP_4, in addition to IP are expressed in platelets (Paul et al., 1998). Accordingly, selective activation of EP_2 or EP_4 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 EP_2 ($EP_2^{-/-}$ platelets), EP_4 ($EP_4^{-/-}$ platelets) or IP ($IP^{-/-}$ platelets). Collagen induced

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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 (Figure 2A), indicating that IP plays a pivotal role in the inhibitory action of ONO-1301 on collagen-induced platelet aggregation. In $EP_2^{-/-}$ platelets, the inhibitory potency of ONO-1301 did not change significantly compared with that in WT platelets: the IC_{50} values were 622 ± 166 and 460 ± 66 nM, respectively (Figure 2A). In addition, the inhibitory potency of ONO-1301 in $EP_4^{-/-}$ platelets was not significantly different from that in F2-WT platelets: the IC_{50} values were 639 ± 152 and 501 ± 95 nM, respectively (Figure 2B). These results indicate that EP_2 and EP_4 expressed in platelets are not involved in the inhibitory effect 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 $IP^{-/-}$ mice ($IP^{-/-}$ washed platelets). In WT washed platelets, both ONO-1301 and beraprost potently increased intracellular cAMP levels (Figure 3). In $IP^{-/-}$ washed platelets, however, these increases were completely abolished (Figure 3). These results clearly indicate that ONO-1301, as well as the IP agonist beraprost, increase cAMP production in an IP-dependent manner.

Inhibitory effect of ONO-1301 on TXA_2 production in $IP^{-/-}$ washed platelets

To verify the action of ONO-1301 as a TXA_2 synthase inhibitor, we examined the effect of ONO-1301 on arachidonic acid-induced TXA_2 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 TXA_2 production, we measured contents of TXB_2, a stable TXA_2 metabolite, in the medium. Both ONO-1301 and ozagrel, a representative TXA_2 synthase inhibitor, inhibited TXA_2 production in a concentration-dependent manner: the IC_{50} values were 12 ± 2.2 and 3.4 ± 0.6 μM, respectively (Figure 4). These results indicate that ONO-1301 works as a TXA_2 synthase inhibitor as potently as ozagrel. Therefore, we confirmed that ONO-1301 has two pharmacological actions as an IP agonist and a TXA_2 synthase inhibitor, while the IC_{50} value of ONO-1301 as a TXA_2 synthase inhibitor was 30-fold higher than that as an IP agonist (Figure 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 min after administration (Supplemental Figure 2). This time course is compatible with T_{max} (1.4 ± 0.2 hr) and $t_{1/2}$ (1.1 ± 0.1 hr) of beraprost (Kato et al., 1989). However, ONO-1301 (100 mg/kg) or beraprost (3 mg/kg) induced marked reduction in blood pressure (Supplemental

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Figure 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 mg/kg/day and 0.3 mg/kg/day, respectively) were determined to cause inhibition of collagen-induced platelet aggregation to similar degrees at day 1, which reflect the difference in their IC_{50} values of platelet aggregation *in vitro* (Figure 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 (Figure 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 (Figure 5). Similar results were observed when U-46619 was used to induce platelet aggregation (Supplemental Figure 3). Furthermore, to determine whether 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 anti-platelet effect of beraprost had recovered completely at day

15, indicating that beraprost-induced desensitization is a reversible phenomenon (Figure 5).

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 (Figure 6A and B), suggesting that desensitization of 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 (Figure 6A and B), indicating that desensitization of IP signaling was induced also by ONO-1301 despite the retention of its anti-platelet effect. Furthermore, repeated administration of ONO-1301 and beraprost led to significant decreases in forskolin-induced cAMP production (Figure 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 anti-platelet 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 TXA_2 synthase inhibitor

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contributes to the retention of its anti-platelet effect, we examined the effect of low-dose ozagrel on decreased response of platelets to beraprost after repeated administration. At a low dose of 3 mg/kg/day, ozagrel alone did not affect collagen-induced platelet aggregation (Figure 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 anti-platelet effect at day 10 (Figure 7A). This result suggests that the inhibitory action of ONO-1301 on TXA_2 synthase contributes to maintenance of its anti-platelet effect during repeated administration.

To exclude direct and acute action of ozagrel on beraprost-induced signaling, we examined the anti-aggregatory action of beraprost in platelets prepared at 2 hr after single administration of low-dose ozagrel. There was no significant difference in dose-dependent anti-platelet effects of beraprost between low-dose ozagrel-treated and vehicle-treated groups (Figure 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 anti-platelet 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/day) could prevent desensitization of the anti-platelet effect by beraprost after repeated administration (Figure 7C).

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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-treated and beraprost-treated WT mice. Repeated administration of beraprost for 10 days significantly increased plasma TXB_2 level compared with that of the vehicle-treated control; the levels were 250 ± 34 and 147 ± 18 pg/mL, respectively (Figure 8A). As expected, however, repeated administration of ONO-1301 did not significantly increase plasma TXB_2 level (182 ± 13 pg/mL, Figure 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 urinary 11-dehydro TXB_2 level, but repeated administration of ONO-1301 did not (Figure 8B). The urinary 11-dehydro TXB_2 levels of vehicle-, ONO-1301- and beraprost-treated mice were 2.1 ± 0.1, 2.2 ± 0.1 and 2.9 ± 0.3 ng/mg creatinine, respectively. In addition, we examined the effects of low-dose ozagrel and aspirin on the increase in plasma TXB_2 level induced by repeated administration of beraprost. Repeated administration of ozagrel or aspirin at low doses with beraprost for 10 days significantly suppressed the increase in plasma TXB_2 level induced by beraprost to the control level (Figure 8C), while ozagrel or aspirin alone did not affect plasma TXB_2 level (data not shown). The plasma TXB_2 levels of mice treated with a vehicle, beraprost, beraprost plus ozagrel and beraprost plus aspirin were 170 ± 30, 262 ± 32, 150 ± 43 and 172 ± 39 pg/mL, respectively. These results suggest that repeated IP stimulation by beraprost increases TXA_2 level, leading to the development of decreased platelet response to beraprost.

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Furthermore, we measured the plasma level of 6-keto $PGF_1\alpha$, a major metabolite of PGI_2, to determine whether the inhibitory activity of ONO-1301 on TXA_2 synthase causes a shift of arachidonic acid metabolism toward PGI_2. Repeated administration of ONO-1301 and beraprost did not change the plasma 6-keto $PGF_1\alpha$ level significantly (Supplemental Figure 4). This result suggests that retention of the anti-platelet effect of ONO-1301 is due not to a direct increase in PGI_2 synthesis but to maintenance of the PGI_2/TXA_2 balance by suppressed TXA_2 production. We also measured the plasma PGE_2 level to determine whether redirection of arachidonic acid metabolism towards PGE_2 occurred after repeated administration of ONO-1301. Repeated administration of ONO-1301 significantly increased the plasma PGE_2 level compared with that in the vehicle-treated controls; the values were 51 ± 7.8 and 30 ± 4.1 pg/mL, respectively (Supplemental Figure 5). This result indicates that ONO-1301 can escape desensitization of an anti-platelet effect under the condition of redirected arachidonic acid metabolism towards PGE_2.

Involvement of TXA_2-mediated signaling in reduction of the anti-platelet effect of beraprost after repeated administration

To confirm the involvement of TXA_2-mediated signaling, we examined whether the anti-platelet effect of beraprost diminishes after repeated administration to $TP^{-/-}$ mice. Since collagen-induced platelet aggregation depends partially on TP signaling, 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 (Figures 5 and 7A), beraprost could retain an inhibitory effect on collagen-induced platelet

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aggregation for 10 days in $TP^{-/-}$ mice (Figure 9A). We further examined whether beraprost can retain the anti-platelet 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 anti-platelet effect for 10 days (Figure 9B), reproducing pharmacologically the result obtained in the experiment using $TP^{-/-}$ mice (Figure 9A). These results clearly indicate that TXA_2 plays a crucial role in reduction of the anti-platelet effect of beraprost after repeated administration.

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Discussion

Previous studies indicated that PGI_2 and its analogues 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 anti-platelet effect after repeated administration. We first confirmed that ONO-1301 has an IP-independent inhibitory action on TXA_2 production in platelets as well as an IP-dependent anti-aggregatory action. Accordingly, an increase in plasma and urinary levels of a TXA_2 metabolite induced by repeated IP stimulation by the IP agonist beraprost was not observed after repeated administration of ONO-1301, suggesting participation of TP signaling in the development of desensitization. As expected, beraprost could retain an anti-platelet effect after repeated administration when used in combination with the TXA_2 synthase inhibitor ozagrel. In addition, beraprost escaped desensitization of the anti-platelet effect after repeated administration in $TP^{-/-}$ mice, indicating further the importance of TP signaling in the development of desensitization. These results provide a novel insight into the mechanism underlying desensitization of IP signaling in general and will be helpful for overcoming clinically important issues accompanying therapies using IP agonists.

ONO-1301 has been developed as a molecule having a dual function: stimulation of IP and inhibition of TXA_2 synthase. We showed that ONO-1301 inhibits collagen-induced platelet aggregation in a concentration-dependent manner (Figure 1). Recent studies have shown that platelet function is regulated via several prostanoid receptors expressed in

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platelets, including EP_2 (Kuriyama et al., 2010; Smith et al., 2010), EP_3 (Fabre et al., 2001; Ma et al., 2001), EP_4 (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, EP_2 and EP_4 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 $EP_2^{-/-}$ or $EP_4^{-/-}$ 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 (Figure 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 anti-aggregatory action, ONO-1301 increased platelet cAMP concentration in an IP-dependent manner (Figure 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 Figure 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 TXA_2 production in a concentration-dependent and IP-independent manner (Figure 4). The inhibitory potency of ONO-1301 in TXA_2 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 TXA_2 synthesis would also contribute to determining the pharmacological nature of ONO-1301.

Noteworthy, the anti-platelet effect of ONO-1301 did not decrease significantly after repeated administration, although that of beraprost decreased markedly (Figure 5). This result indicates the possibility that the activity of ONO-1301 that inhibits TXA_2 synthesis prevented the anti-platelet effect of ONO-1301 from being desensitized. It should also be noted that the anti-platelet effect of beraprost was restored after 4 days of drug withdrawal (Figure 5), suggesting that desensitization of the anti-platelet 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 anti-platelet 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 (Figure 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 (Figure 6A 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 (Figure 6C), indicating involvement of adenylate cyclase and/or its downstream signaling in desensitization of IP/cAMP signaling. Although we did not examine the mechanism of this desensitization, mechanisms common to GPCRs, such as diminished response of adenylate cyclase (Jaschonek et al., 1988), down-regulation of G_s (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 anti-platelet effects after repeated administration of these agents. Therefore, a mechanism other than IP/cAMP signaling would explain the difference in the anti-platelet effects after repeated administration between ONO-1301 and beraprost.

We examined whether TP signaling contributes to retention of the anti-platelet effect after repeated administration of ONO-1301. Noteworthy, low-dose ozagrel could rescue beraprost from losing the anti-platelet effect (Figure 7A), suggesting that suppression of TXA_2 production is critically involved in retention of the anti-platelet effect. In line with this result, low-dose aspirin also rescued beraprost from the desensitization (Figure 7C). From these results, we expected that TXA_2 synthesis would be promoted by repeated administration of beraprost, leading to a decrease in the anti-platelet effect. As expected, repeated administration of beraprost increased the plasma and urinary levels of TXA_2 metabolite, while repeated administration of ONO-1301 did not induce such increases (Figure 8A, C). This result is consistent with a previous finding that continuous administration of beraprost increases TXA_2 production in rats (Kataoka et al., 2005). Interestingly, low-dose ozagrel and aspirin suppressed only the increase in plasma level of TXB_2 induced by beraprost (Figure 8C) without an effect on plasma TXB_2 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 PGE_2 level; the level was 51 pg/mL (145 pM) (Supplemental Figure 5), which is

consistent with a previous finding that inhibition of TXA_2 formation by dazoxiben, an inhibitor of TXA_2 synthase, redirected arachidonic acid metabolism from TXA_2 towards PGE_2 in human clotting blood in vitro (Watts et al., 1991). We previously reported that PGE_2 potentiated platelet aggregation at concentrations above 1 nM via EP_3 (Ma et al., 2001) and inhibited it at concentrations above 300 nM via EP_2 and EP_4 (Kuriyama et al., 2010). These concentrations of PGE_2 were higher than the plasma PGE_2 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 anti-platelet effect after repeated administration in $TP^{-/-}$ mice, which lack TP-mediated signaling completely. Notably, beraprost could retain its anti-platelet effect, inhibiting platelet aggregation at even the 10th day of repeated administration (Figure 9A). In addition, this finding was well reproduced when TP signaling was blocked pharmacologically by the TP antagonist seratrodast (Figure 9B). These results clearly indicate that the TXA_2/TP system plays a critical role in the development of desensitization in the anti-platelet effects of beraprost and that the action that inhibits TXA_2 production confers resistance against desensitization of the anti-platelet effect on ONO-1301.

Previous studies demonstrated reciprocal regulation between IP and TP. TP activation resulted in sensitization of 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 co-expressed 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 TXA_2 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 TXA_2 synthase inhibitor rescues an IP agonist from losing the anti-platelet effect after repeated administration. Given an important application of PGI_2 analogues as anti-platelet agents, it is a clinically very important issue to develop a method for preventing reduction in the effect of PGI_2 analogues. The results of the present work provide a novel insight in this issue and will contribute to broadening of the clinical application of PGI_2 analogues.

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Authorship Contributions

Participated in research design: Kashiwagi, Sakai, Narumiya and Ushikubi.

Conducted experiments: Kashiwagi, Yuhki, Kojima, Kumei and Takahata.

Contributed to supply of new reagents or analytic tools: Sakai.

Performed data analysis: Kashiwagi and Kumei.

Contributed to preparation of the manuscript: Kashiwagi, Yuhki, Kojima and Ushikubi.

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Footnotes

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Reprint requests to: Fumitaka Ushikubi, Department of Pharmacology, Asahikawa Medical University, Midorigaoka-Higashi 2-1-1-1, Asahikawa 078-8510, Japan.
E-mail: ushikubi@asahikawa-med.ac.jp

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Figure Legends

Figure 1. Effects of ONO-1301 and beraprost on collagen-induced platelet aggregation

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 min before the addition of collagen. C of the horizontal axis represents control. Each value is the mean ± S.E.M. (n = 4).

Figure 2. IP mediates the inhibitory effects of ONO-1301 on collagen-induced platelet aggregation

Effects of ONO-1301 on collagen-induced aggregation in platelets prepared from WT, $EP_2^{-/-}$ and $IP^{-/-}$ mice (A) and from F2-WT and $EP_4^{-/-}$ 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 min before the addition of collagen. C of the horizontal axis represents control. Each value is the mean ± S.E.M. (n = 4).

Figure 3. Effects of ONO-1301 and beraprost on cAMP production

Effects of ONO-1301 and beraprost on cAMP production in washed platelets prepared from WT and $IP^{-/-}$ mice. ONO-1301 (10 μM) or beraprost (1 μM) was added to washed platelets to induce cAMP production. Each value is the mean ± S.E.M. WT: control, n = 8; ONO-1301, n = 8; beraprost, n = 6. $IP^{-/-}$: control, n = 5; ONO-1301, n = 5; beraprost, n

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= 5. *P < 0.05 vs. control of WT.

Figure 4. Effects of ONO-1301 and ozagrel on arachidonic acid-induced TXA_2 production

Effects of ONO-1301 and ozagrel on arachidonic acid-induced TXA_2 production in washed platelets prepared from $IP^{-/-}$ mice. To estimate the degree of TXA_2 production, we measured the content of TXB_2, a stable TXA_2 metabolite, in the medium. Arachidonic acid (0.2 mM) was added to washed platelets to induce TXA_2 production. ONO-1301 or ozagrel was added at indicated concentrations to washed platelets 1 min before the addition of arachidonic acid. C of the horizontal axis represents control. Each value is the mean ± S.E.M. (n = 4).

Figure 5. Effects of repeated administration of ONO-1301 and beraprost on collagen-induced platelet aggregation

Inhibitory effects of ONO-1301 and beraprost on collagen-induced platelet aggregation at indicated time points are presented. ONO-1301 (20 mg/kg/day) or beraprost (0.3 mg/kg/day) was administered orally to WT mice once a day, and PRP was prepared 2 hr after the administration. Administration of each drug was withdrawn from the 11th to 14th days. Collagen (2.0 μg/mL) was added to PRP to induce platelet aggregation. Each value is the mean ± S.E.M. Vehicle, n = 6; ONO-1301, n = 4; beraprost, n = 6. *P < 0.05 vs. beraprost at 1st day.

Figure 6. Effects of repeated administration of ONO-1301 and beraprost on cAMP production

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/day) or beraprost (0.3 mg/kg/day) was administered orally to WT mice once a day, and washed platelets were prepared 2 hr 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). *$P < 0.05$ vs. vehicle.

Figure 7. Effects of low-dose ozagrel and aspirin on anti-platelet 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/day), ozagrel (3 mg/kg/day) or beraprost plus ozagrel was administered orally to WT mice once a day and PRP was prepared 2 hr 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 anti-platelet action of beraprost. Ozagrel (3 mg/kg/day) was administered orally to WT mice and PRP was prepared 2 hr 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 min 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/day), aspirin (1 mg/kg/day) or beraprost plus aspirin was administered orally to WT mice once a day and PRP was prepared 2 hr 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.

Figure 8. Effects of repeated administration of ONO-1301 and beraprost on TXA_2 production

(A) Effects of repeated administration of ONO-1301 and beraprost on plasma TXB_2 levels were examined. ONO-1301 (20 mg/kg/day) or beraprost (0.3 mg/kg/day) was administered orally to WT mice once a day, and plasma was prepared 2 hr after administration on the 10th day. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 vs. vehicle. (B) Effects of repeated administration of beraprost, beraprost plus low-dose ozagrel and beraprost plus low-dose aspirin on plasma TXB_2 levels were examined. Beraprost (0.3 mg/kg/day), beraprost plus ozagrel (3 mg/kg/day) or beraprost plus aspirin (1 mg/kg/day) was administered orally to WT mice once a day, and plasma was prepared 2 hr after administration on the 10th day. Each value is the mean ± S.E.M. (n = 4). *P < 0.05. (C) Effects of repeated administration of ONO-1301 and beraprost on urinary 11-dehydro TXB_2 levels were examined. ONO-1301 (20 mg/kg/day) or beraprost (0.3 mg/kg/day) was administered orally to WT mice once a day, and a 24-hr urine sample was collected on the 10th day. Each value is the mean ± S.E.M. (n = 6). *P < 0.05 vs.

vehicle.

Figure 9. Effects of TXA_2 on an anti-platelet action of beraprost

(A) Time courses of the effects of beraprost on collagen-induced aggregation of platelets prepared from $TP^{-/-}$ mice. Beraprost (0.3 mg/kg/day) was administered orally to $TP^{-/-}$ mice once a day and PRP was prepared 2 hr after the administration. Collagen (3.0 μg/mL) was added to PRP to induce platelet aggregation. Each value is the mean ± S.E.M. Vehicle, n = 4; beraprost, n = 7. (B) Time courses of the effects of beraprost, seratrodast, and beraprost plus seratrodast on collagen-induced aggregation of platelets prepared from WT mice. Beraprost (0.3 mg/kg/day), seratrodast (30 mg/kg/day) or beraprost plus seratrodast was administered orally to WT mice once a day and PRP was prepared 2 hr after the administration. Collagen (3.0 μg/mL) was added to PRP to induce platelet aggregation. Each value is the mean ± S.E.M. (n = 4).

Figure 1

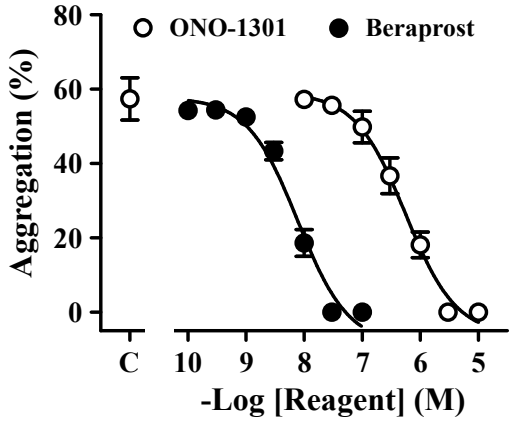
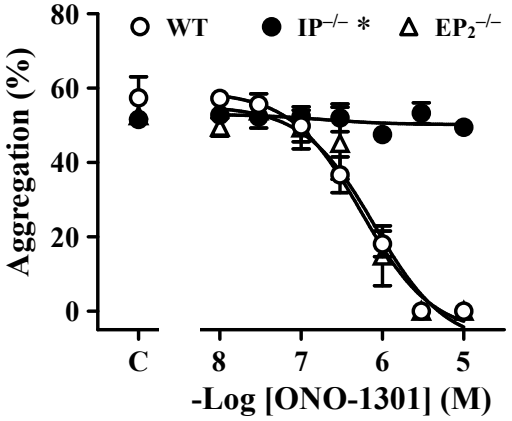

ONO-1301
Beraprost
Aggregation (%)
80
60
40
20
0
C
10
9
8
7
6
5
-Log [Reagent] (M)

Figure 2

A


WT
IP−/− *
EP2−/−
Aggregation (%)
80
60
40
20
0
C
8
7
6
5
-Log [ONO-1301] (M)

B

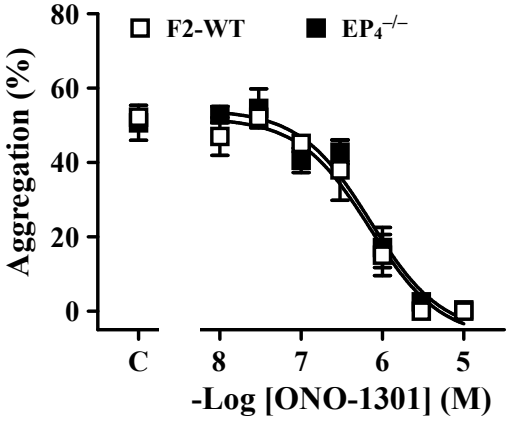

F2-WT
EP4−/−
Aggregation (%)
80
60
40
20
0
C
8
7
6
5
-Log [ONO-1301] (M)

Figure 3

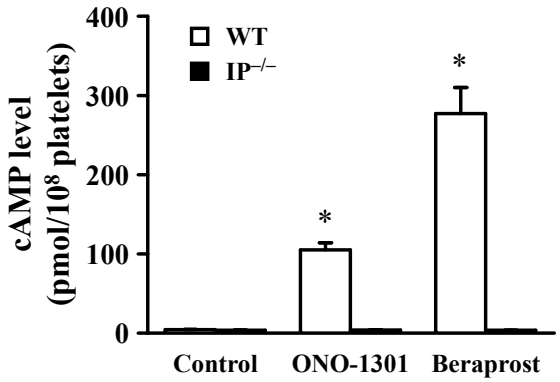

400
300
200
100
0
cAMP level
(pmol/10^8 platelets)
WT
IP−/−
*
*
Control
ONO-1301
Beraprost

Figure 4

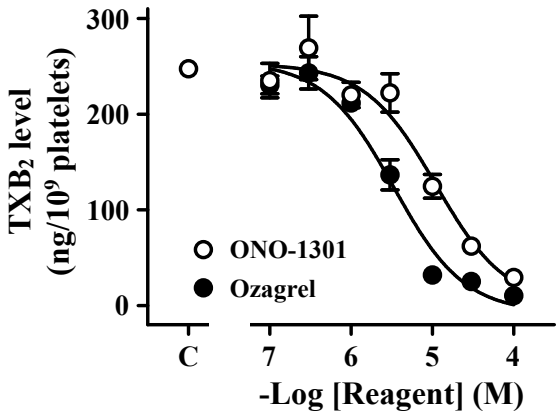

300
200
100
0
TXB2 level
(ng/10^9 platelets)
ONO-1301
Ozagrel
C
7
6
5
4
-Log [Reagent] (M)

Figure 5

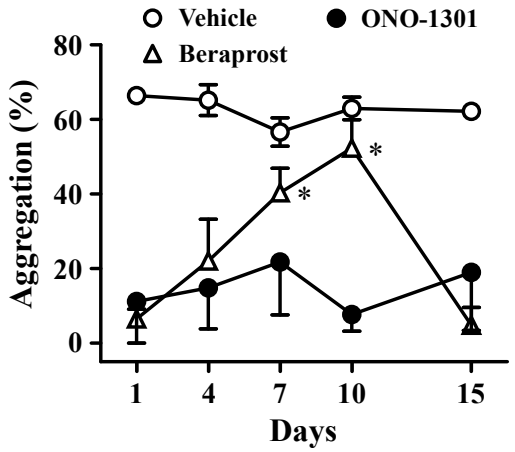
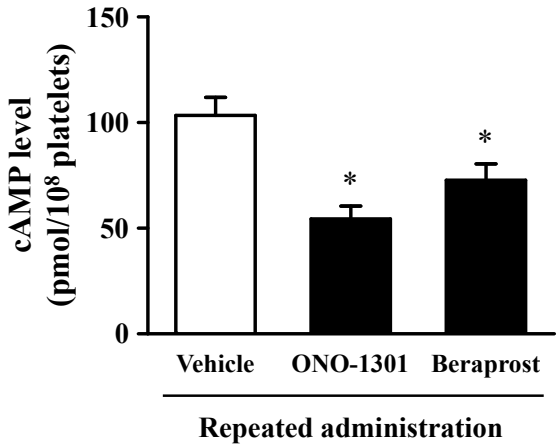
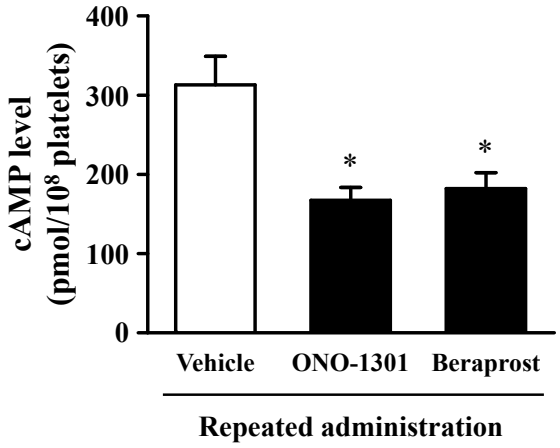

Vehicle
ONO-1301
Beraprost
Aggregation (%)
80
60
40
20
0
1
4
7
10
15
Days
*
*

Figure 6

A


150
100
50
0
cAMP level
(pmol/10^8 platelets)
*
*
Vehicle
ONO-1301
Beraprost
Repeated administration

B


400
300
200
100
0
cAMP level
(pmol/10^8 platelets)
*
*
Vehicle
ONO-1301
Beraprost
Repeated administration

C

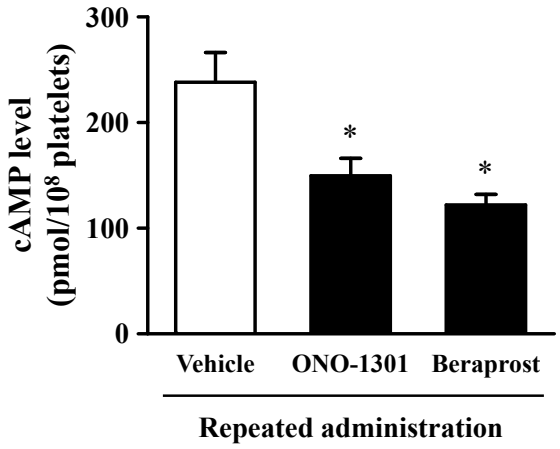
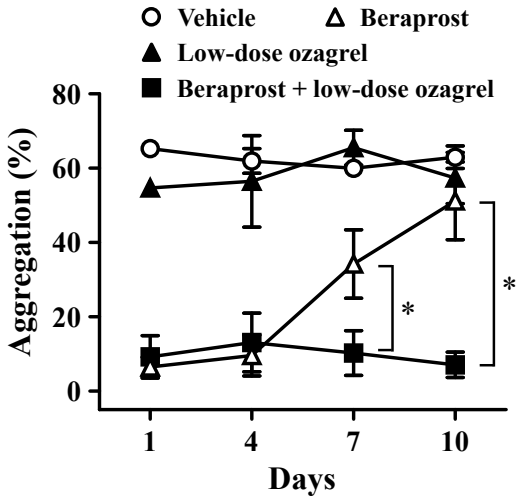
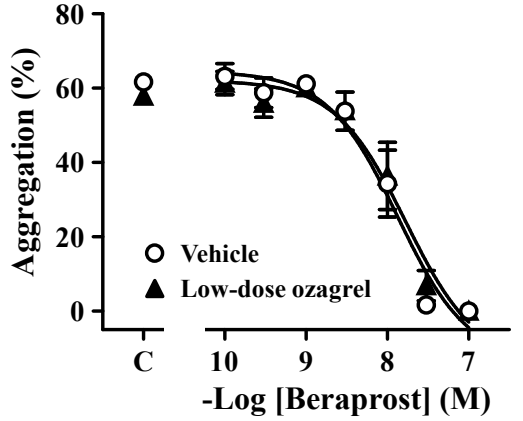

300
200
100
0
cAMP level
(pmol/10^8 platelets)
*
*
Vehicle
ONO-1301
Beraprost
Repeated administration

Figure 7

A


Vehicle
Beraprost
Low-dose ozagrel
Beraprost + low-dose ozagrel
Aggregation (%)
80
60
40
20
0
1
4
7
10
Days
*
*

B


Aggregation (%)
80
60
40
20
0
Vehicle
Low-dose ozagrel
C
10
9
8
7
-Log [Beraprost] (M)

C

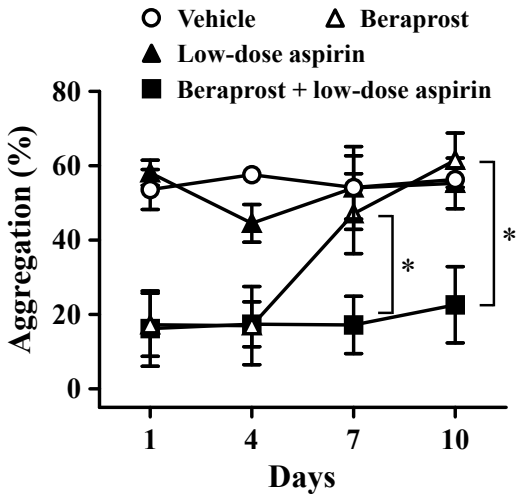

Vehicle
Beraprost
Low-dose aspirin
Beraprost + low-dose aspirin
Aggregation (%)
80
60
40
20
0
1
4
7
10
Days
*
*

Figure 8

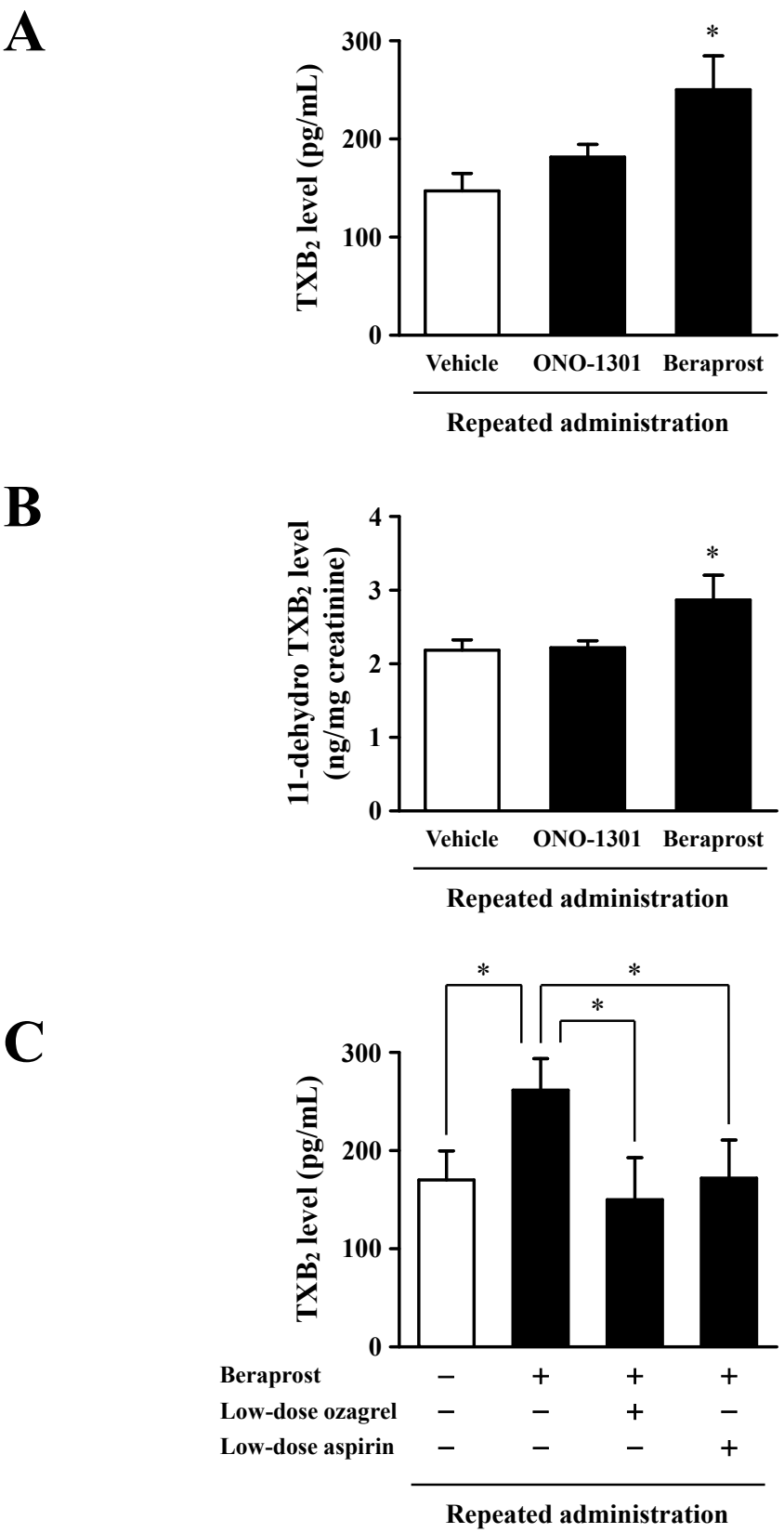
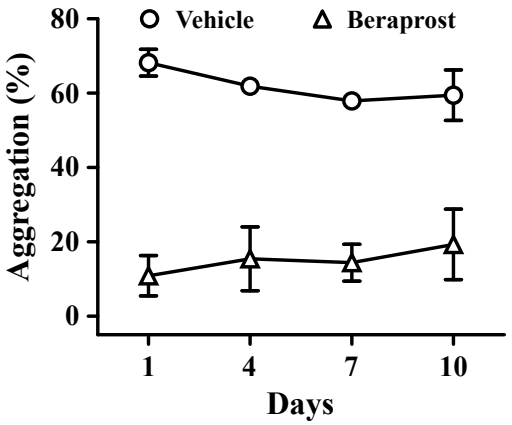
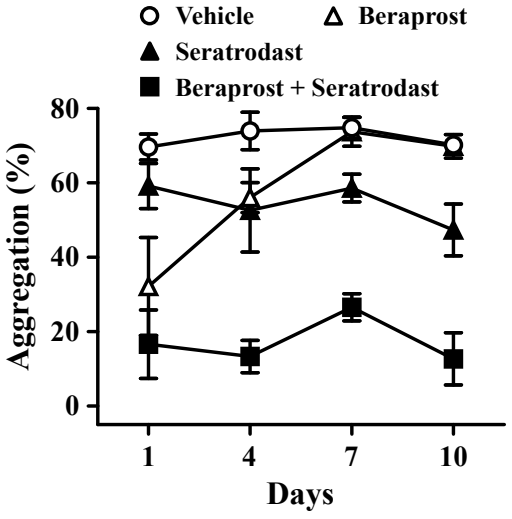

A
300
200
100
0
TXB2 level (pg/mL)
*
Vehicle
ONO-1301
Beraprost
Repeated administration
B
4
3
2
1
0
11-dehydro TXB2 level
(ng/mg creatinine)
*
Vehicle
ONO-1301
Beraprost
Repeated administration
C
*
*
*
300
200
100
0
TXB2 level (pg/mL)
Beraprost − + + +
Low-dose ozagrel − − + −
Low-dose aspirin − − − +
Repeated administration

Figure 9

A


Vehicle
Beraprost
Aggregation (%)
80
60
40
20
0
1
4
7
10
Days

B


Vehicle
Beraprost
Seratrodast
Beraprost + Seratrodast
Aggregation (%)
80
60
40
20
0
1
4
7
10
Days