The Roles Played by FP/EP3 Receptors During Pressure-lowering in Mouse Eyes Mediated by a Dual FP/EP3 Receptor Agonist

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PURPOSE. We investigated the intraocular pressure (IOP)-lowering effect of topical sepetaprost (SPT), a dual agonist of the FP and EP3 receptors. We explored whether certain receptors mediated the hypotensive effect of SPT and outflow facility changes in C57BL/6 mice (wild-type [WT]) and FP and EP3 receptor-deficient mice (FPKO and EP3KO mice, respectively).

METHODS. IOP was measured using a microneedle. Outflow facility was measured using a two-level, constant-pressure perfusion method.

RESULTS. SPT significantly reduced IOP for 8 hours after administration to WT mice. The 2-hour IOP reductions afforded by latanoprost were 15.3 ± 2.5, 1.8 ± 2.0, and $12.3 \pm 2.4\%$ in WT, FPKO, and EP3KO mice, respectively; the SPT figures were 13.6 ± 2.1, 5.9 ± 2.7, and $6.6 \pm 2.6\%$, respectively. Latanoprost-mediated IOP reduction was significantly decreased in FPKO mice, and SPT-mediated IOP reduction was reduced in both FPKO and EP3KO mice. At 6 hours after administration, latanoprost did not significantly reduce the IOP in any tested mouse strain. SPT-mediated IOP reduction was reduced in both FPKO and EP3KO mice. IOP reduction at 6 hours was significantly higher after simultaneous administration of selective FP and EP3 receptor agonists, but IOP did not fall on administration of (only) a selective EP3 receptor agonist. SPT significantly increased outflow facility in WT mice, but less so in FPKO and EP3KO mice.

CONCLUSIONS. The IOP-lowering effect of SPT lasted longer than that of latanoprost. Our data imply that this may be attributable to augmented outflow facility mediated by the FP and EP3 receptors.

Keywords: FP/EP3 dual agonist, sepetaprost, IOP, FP receptor, EP3 receptor, prostanoid, mouse

Glaucoma is the second leading cause of blindness worldwide, associated with irreversible ganglion cell death and optic nerve degeneration.[1,2] Prostaglandin analogs (PGAs) are widely used as first-line drugs that reduce intraocular pressure (IOP). PGAs lower IOP via the FP receptor, principally by increasing aqueous outflow facility through the uveoscleral pathway.[3–7] There are eight types of prostanoid receptors.[8] Stimulation of the FP, EP2, and EP4 receptors reduces mouse IOP.[9] Endogenous PG production mediated by the FP receptor aids IOP-lowering via EP3 receptor stimulation.[10–14] Recently, new drugs targeting prostanoid receptors have been developed; the armamentarium today includes separate FP- and EP2-receptor agonists[15–17] and dual FP- and EP3-receptor agonists.[18–20]

Sepetaprost (SPT) is a novel dual EP3 and FP agonist that is hydrolyzed to an isopropyl ester of the biologically active free acid (ONO-AG-367) by corneal esterases.[21] The ONO-AG-367 half-maximal effective concentration (EC_{50}) values for the FP and EP3 receptors were 22.3 and 28.6 nM, respectively, as reflected by the intracellular calcium increases in Chem-1 and CHO cells expressing the receptors.[20] SPT

lowered IOP more than 0.005% (w/v) latanoprost in human and monkey eyes.[20,22] In the human eye, FP receptors are expressed in all tissues except the choroid, and EP3 receptors are distributed in tissues relevant to both the uveoscleral and conventional outflow facilities, including trabecular meshwork cells, Schlemm's canal endothelial cells, collecting tubes, aqueous humor veins, ciliary epithelial cells, ciliary muscles, and ciliary parenchymal cells.[23] In a preclinical trial, SPT was more potent than latanoprost or travoprost when used to reduce the IOP of monkey eyes,[20] and lowered IOP more than did latanoprost in the eyes of healthy human volunteers.[24] In a 28-day continuous-dose study on patients with open-angle glaucoma and ocular hypertension, SPT lowered IOP more than did latanoprost, in a dose-dependent manner.[22]

Thus, SPT may be stronger than existing PGAs; SPT simultaneously stimulates the FP and EP3 receptors to deliver sustained IOP-lowering effects. However, evidence that the FP and EP3 receptors are directly involved in the IOP-lowering effect of SPT is lacking. Therefore, we investigated the extent of involvement of these receptors in receptor-gene-deficient mice.

MATERIALS AND METHODS

Materials

Sepetaprost (FP and EP3 receptors dual agonist) and ONO-AE-248 (selective EP3 receptor agonist) were donated by ONO pharmaceutical (Osaka, Japan), and latanoprost (FP receptor agonist) was purchased from Pfizer (New York, NY, USA).

Animals

All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the dictates of our local Animal Use Committee at the University of Tokyo. Male C57BL/6J (wild-type [WT]) mice were purchased from Japan Tokyo Laboratory Animals Science Co. Ltd. Mouse genes encoding the FP or EP3 receptor were disrupted by gene knockout methods using homologous recombination, as reported previously,[25,26] and FP, or EP3-homozygous knockout (FPKO or EP3KO) mice were used. The strains of EP3KO mice were maintained in homozygous knockout mice and FPKO mice were maintained in heterozygous KO mice because generations of FP-/- mice had reduced fertility. C57BL/6 was the background strain of all KO and WT mice. The number of generations of backcrossing was unknown. Mice were bred and housed in clear cages covered loosely with air filters. The cages contained white-chip bedding. The temperature was maintained at 21°C with a 12-hour light (6:00 AM–6:00 PM) and 12-hour dark cycle. After purchase, all mice had access to food and water ad libitum in a conventional animal room in our laboratory for at least 1 week before the scheduled experimental date; the FP- and EP3-receptor KO mice were bred in the same animal room after completion of their quarantine and acclimation periods. For all experiments, 8 to 10 week old male mice (body weight range was from 18 to 24 g) were used.

Preparation and Instillation of Ophthalmic Solution

Latanoprost (0.005% w/v) and SPT (0.003 and 0.01% w/v) were stored at 4°C. ONO-AE-248 was stored at -20°C in 100% dimethyl sulfoxide and diluted with phosphate buffered saline just before use to a 5% (v/v) DMSO concentration. Using a micropipette, 3 µL of each drug solution was topically applied (in a masked manner) to one eye selected at random; the other (nontreated) eye served as the control. When topical administration was "continuous," drops were delivered at 5-minute intervals.

Mouse IOP Measurement

IOP was measured using a microneedle, as previously described.[27] In brief, the mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/kg, Ketalar; Daiichi Sankyo Company, Tokyo, Japan) and xylazine (9 mg/kg; Seractal, Bayer, Berlin, Germany), prepared at room temperature. Anesthesia was administered with a 29-gauge needle. A timer was started immediately after the injection and IOP measurement was performed within 4 to 5 minutes after administration of anesthesia in all animals because of the effect of anesthesia. The borosilicate glass microneedle (75–100 µm in tip diameter and 1.0 mm in outer diameter, 25 degrees angle bevel) was connected to a pressure transducer and the data were sent to a data acquisition and analysis system. The microneedle was placed in the anterior chambers of both eyes with the mice under anesthesia. All measurements were performed between 9 and 11 PM.

Mouse Outflow Facility Measurement

To clarify the mechanism by which SPT reduced IOP, outflow facility was measured 2 hours after latanoprost or 4 hours after SPT or carrier solution injection. As previously discussed,[9] latanoprost served as the positive outflow facility control. The measurement times were those of the maximum IOP reductions afforded. Outflow facility was measured using a two-level, constant-pressure perfusion method, and was reported as a C value.[4,9,27] Briefly, an infusion needle (the same type of glass needle that was used for the IOP measurement) inserted into the anterior chamber was connected to a reservoir filled with artificial aqueous humor via a pressure transducer. The liquid surface height in the chamber was maintained at 25 or 35 mm Hg for 10 minutes in the steady state (H_{25} or H_{35}, respectively). The inner cross-sectional area of the reservoir (S) was calculated from the inner diameter. The volumes of artificial aqueous humor infused/min at 25 or 35 mm Hg were termed V_{25} and V_{35}. The total outflow facility (C_{total}, µL/min/mm Hg) was calculated as: $C_{total} = (V_{35} - V_{25})/10 = [S(H_{35} - H_{25})]/100$. The C_{total} was the sum of C_{conv} and C_{uveo}, where C_{conv} was the outflow facility of the pressure-dependent system and C_{uveo} that of the uveoscleral system (which was assumed to be less pressure-dependent).

Data Analysis and Statistics

The effect of each drug was calculated as 100 × (the IOP of the contralateral eye minus the IOP of the drug-treated eye)/the IOP of the contralateral eye (%) for each mouse and was termed the "% reduction." The data were subjected to area under the curve (AUC; percentage/hour) analysis, following the trapezoidal rule. All results are presented as means ± standard deviations (SDs). Statistical analysis was performed with the aid of JMP Pro version 11 software. All data are the means of those of at least three independent experiments. A difference was considered statistically significant at a P value < 0.05 as calculated by ANOVA, the Student t-test, or the Dunnett test.

RESULTS

The Time-Dependent SPT IOP-Lowering Effect in WT Mice

The IOP reductions in WT mice were measured at 2, 4, 6, 8, and 12 hours after drug administration ($n = 6$–8/time point). ANOVA and the Student t-test were used to compare saline- and drug-treated mice. The IOP reductions at 2 hours after administration of saline, latanoprost, 0.003% (w/v) SPT, and 0.01% (w/v) SPT were −1.1 ± 1.9, 16.5 ± 2.2, 10.8 ± 2.3, and 7.3 ± 2.1%, respectively. Compared to the saline-treated group, the IOP reductions afforded by latanoprost, and 0.003 and 0.01% (w/v) SPT, were significantly greater ($P < 0.01$ or 0.05). The 4-hour figures were 0.5 ± 0.9, 11.6 ± 2.4, 16.2 ± 1.9, and 16.4 ± 2.1%, respectively. Again, compared to the saline-treated group, the IOP reductions

A

B
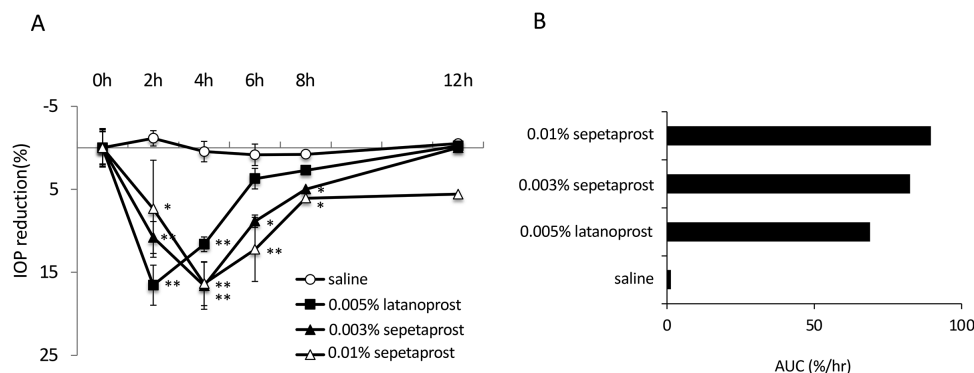
Figure 1. Time courses of IOP reductions induced by 0.005% (w/v) latanoprost, 0.003% (w/v) SPT, and 0.01% (w/v) SPT in WT mice. Data are expressed as means $\pm$ SDs ($n = 6$–8). *$P < 0.05$ for treated versus saline-treated eyes. (**A**) The time courses of the IOP reductions. (**B**) The ocular hypotensive effects of the four drugs as indicated by the AUCs (percentages/hour). ANOVA and the Student t-test were used to compare the saline-treated group to the drug-treated groups.

afforded by latanoprost, and 0.003 and 0.01% (w/v) SPT, were significantly greater. The 6-hour figures were 0.8 $\pm$ 1.2, 3.7 $\pm$ 0.9, 8.8 $\pm$ 2.8, and 12.2 $\pm$ 1.1%, respectively. Compared to the saline-treated group, the IOP reductions afforded by 0.003 and 0.01% (w/v) SPT were significantly greater ($P < 0.01$ or 0.05); latanoprost did not reduce the IOP. The 8-hour figures were 0.8 $\pm$ 1.3, 2.7 $\pm$ 1.2, 5.0 $\pm$ 0.7, and 6.1 $\pm$ 1.4%, respectively. Again, compared to the saline-treated group, the IOP reductions afforded by 0.003 and 0.01% (w/v) SPT were significantly greater. The 12-hour figures were –0.5 $\pm$ 1.1, –0.2 $\pm$ 0.6, 0.0 $\pm$ 1.2, and 5.6 $\pm$ 2.4%, respectively, thus not significantly different (Fig. 1A). The peak reductions afforded by latanoprost and SPT were observed at 2 and 4 hours, respectively; SPT was more efficacious than latanoprost and the effects lasted longer. In terms of the hypotensive effects, the AUCs (% * hours) of the saline, latanoprost, and 0.003 and 0.01% (w/v) SPT groups were 1.37, 68.9, 82.5, and 89.6, respectively. SPT was more effective than latanoprost at either concentration (see Fig. 1B).

IOP Reductions Afforded by 0.003% (w/v) SPT in FPKO and EP3KO Mice

We measured the IOPs of FPKO and EP3KO mice at 2 and 6 hours after topical drug/saline administration ($n = 6$–8/strain); latanoprost again served as the FP receptor-positive control. As shown in Figure 1, we investigated 0.003% and 0.01% SPT. The 0.003% SPT showed the sufficient IOP lowering effect and there was no significant difference between the two concentrations. Because 0.003% was the concentration used in clinical trials for SPT, we used 0.003% only in subsequent IOP measurement. At 2 hours after administration, the IOP reductions afforded by saline in the WT, FPKO, and EP3KO groups were 0.7 $\pm$ 0.9, 0.1 $\pm$ 1.2, and –0.8 $\pm$ 2.1%, respectively, thus not significantly different. The latanoprost values were 15.3 $\pm$ 2.5, 1.8 $\pm$ 2.0, and 12.3 $\pm$ 2.4%, respectively. The IOP reduction was significantly less in the FPKO group than in the latanoprost-treated WT group ($P < 0.01$), but not in the EP3KO group, implying that latanoprost targets principally the FP receptor. In contrast, the SPT values were 13.6 $\pm$ 2.1, 5.9 $\pm$ 2.7, and 6.6 $\pm$ 2.6%, respectively. The IOP reductions were significantly less in the FPKO and EP3KO groups than in the WT group, implying that SPT targeted both receptors (Fig. 2).
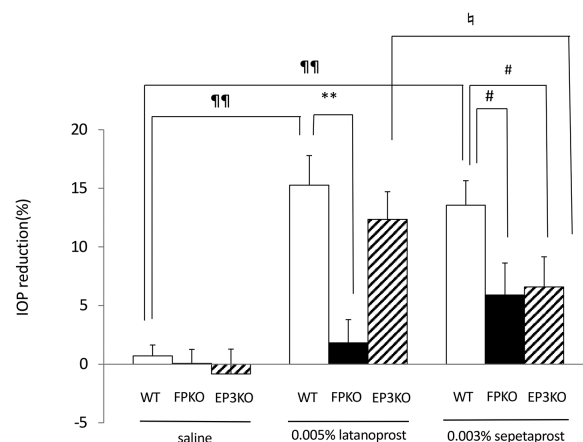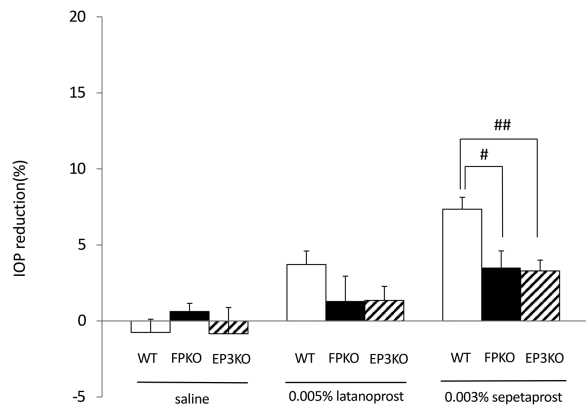

Figure 2. IOP reductions afforded by 0.005% (w/v) latanoprost and 0.003% (w/v) SPT in WT, FPKO, and EP3KO mice. IOP was measured at 2 hours after drug administration. Data are expressed as means $\pm$ SDs ($n = 6$–8). ¶¶ $P < 0.01$ for saline- versus drug-treated eyes of WT mice. ** $P < 0.01$ for latanoprost-treated WT mice versus other mice. # $P < 0.05$ for SPT-treated WT mice versus SPT-treated other mice. ANOVA and the Student t-test were used for comparisons. ♮ $P < 0.05$ for 0.005% (w/v) latanoprost-treated eyes versus 0.03% (w/v) SPT-treated eyes of EP3KO mice.

At 6 hours after administration, the IOP reduction afforded by saline or latanoprost did not differ among the groups. In contrast, SPT still afforded significant IOP reduction; the reductions were 7.3 $\pm$ 0.8, 3.5 $\pm$ 1.1, and 3.3 $\pm$ 0.7%, respectively. The IOP reductions were significantly lower in the FPKO and EP3KO groups than in the WT group (Fig. 3).

IOP Reduction by 0.1% (w/v) ONO-AE-248 (a Selective EP3 Agonist) in WT Mice

As SPT reduced IOP more potently and for longer than latanoprost, and as SPT may target both the FP and EP3 receptors, we next investigated IOP reduction on EP3 receptor stimulation. We measured IOP at 2 and 6 hours after selective EP3 receptor agonist administration ($n = 9$–11 mice/group). As shown in Figure 4A, at 2 hours, only latanoprost-treated eyes differed significantly from nontreated eyes. The IOP reductions in the DMSO-,

FIGURE 3. IOP reductions afforded by 0.005% (w/v) latanoprost and 0.003% (w/v) SPT in WT, FPKO, and EP3KO mice. IOP was measured at 6 hours after administration. Data are expressed as means ± SDs ($n = 6$–8). ANOVA and the Student t-test were used for comparisons. # $P < 0.05$ for SPT-treated WT mice versus other SPT-treated mice.

latanoprost-, and ONO-AE-248-treated groups were 0.8 ± 0.9, 17.3 ± 2.6, and $-0.3 \pm 1.8\%$, respectively, thus highest in the latanoprost-treated group (see Fig. 4B).

At 6 hours, the IOPs of control and DMSO-treated eyes were 20.4 ± 2.8 and 20.6 ± 2.5 mm Hg, respectively; 18.8 ± 1.7 and 18.6 ± 1.6 mm Hg in latanoprost-treated eyes, and 20.1 ± 1.3 and 19.9 ± 1.0 mm Hg in the ONO-AE-248-treated eyes. Control and drug-treated eyes did not differ (Fig. 5A). The IOP reductions in the DMSO-, latanoprost-, and ONO-AE-248-treated groups were 1.5 ± 1.2, 1.0 ± 0.2, and $3.4 \pm 1.8\%$, respectively. The IOP reduction tended to be higher in the latter group, but statistical significance was not attained (see Fig. 5B).

IOP Reduction on Simultaneous Stimulation of the FP and EP3 Receptors of WT Mice

Although mouse IOP was not significantly affected by a selective EP3 receptor agonist alone, we speculated that the EP3 receptor might prolong IOP-lowering. Therefore, we next examined IOP reduction on simultaneous stimulation of the FP and EP3 receptors ($n = 8$–10/group). At 2 hours, the latanoprost + DMSO- and latanoprost + ONO-

AE-248 treated eyes both differed significantly from the controls (Fig. 6A). In addition, the IOP reductions in the latanoprost + DMSO- and latanoprost + ONO-AE-248-treated groups differed significantly ($P < 0.01$) compared to the DMSO + DMSO-treated group, but those of the latanoprost + DMSO- and latanoprost + ONO-AE-248-treated groups did not (see Fig. 6B).

At 6 hours, there were no significant differences between control and drug-treated eyes, but IOP tended to be lower in the latanoprost + ONO-AE-248-treated eyes ($P = 0.06$ compared to control eyes; Fig. 7A). The IOP reductions in DMSO + DMSO-, ONO-AE-248 + DMSO-, latanoprost + DMSO-, and latanoprost + ONO-AE-248-treated eyes were -0.6 ± 3.4, -0.5 ± 2.1, 1.3 ± 2.1, and $9.1 \pm 5.9\%$, respectively. Compared to the other groups, the latanoprost + ONO-AE-248-treated group differed significantly ($P < 0.01$), implying that EP3 receptor activation may prolong IOP lowering (see Fig. 7B).

The Effect of SPT on Outflow Facility in WT Mice

To clarify the IOP-lowering mechanism of SPT, we measured outflow facility ($n = 6$–11/group). The total outflow facilities (C_{total} values) in WT mice after administration of saline, latanoprost (at 2 hours later), 0.003% (w/v) SPT, and 0.01% (w/v) SPT (at 4 hours later) were 0.0027 ± 0.0007, 0.0036 ± 0.0008, 0.0034 ± 0.0006, and 0.0040 ± 0.0010 µL/min/mm Hg, respectively. All drug-treated groups differed significantly from the saline treated-group, but the drug-treated groups did not differ (Fig. 8).

Effect of SPT on Outflow Facility in Each Receptor-Deficient Mouse

Next, we measured outflow facility enhancement by SPT in WT, FPKO, and EP3KO mice ($n = 6$–11/group). The total C_{total} values in FPKO mice differed after administration of saline, latanoprost (measured 2 hours later), 0.003% (w/v) SPT, and 0.01% (w/v) SPT (measured 4 hours later) and were 0.0032 ± 0.0007, 0.0032 ± 0.0006, 0.0031 ± 0.0001, and 0.0030 ± 0.0002 µL/min/mm Hg, respectively. In FKPO mice, the drug-treated groups did not differ from the saline-treated group (see Fig. 8). In EP3KO mice, the C_{total} values were 0.0031 ± 0.0011, 0.0038 ± 0.0010, 0.0033 ± 0.0010,
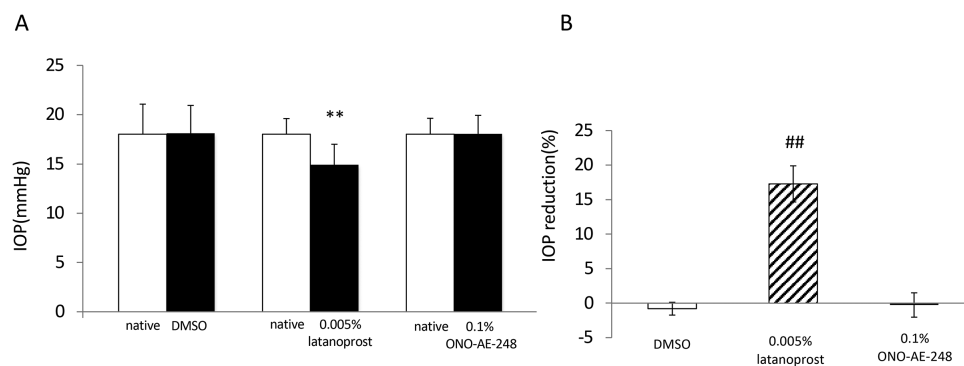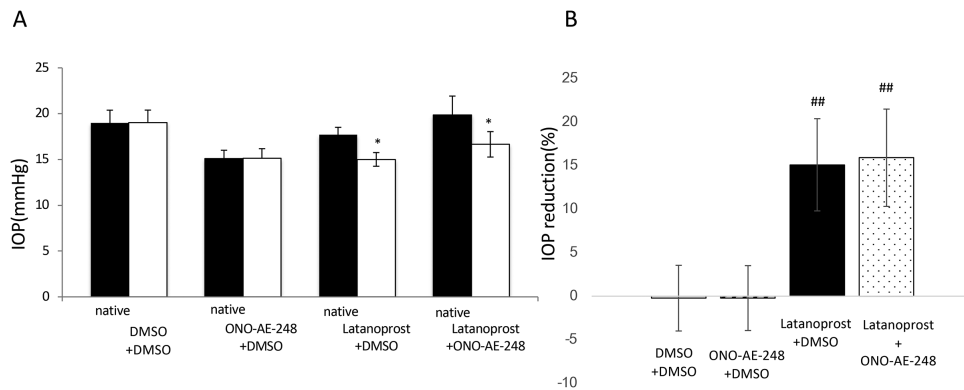

FIGURE 4. IOP reductions afforded by 0.005% (w/v) latanoprost and 0.1% (w/v) ONO-AE-248 (an EP3 agonist) in WT mice. IOP was measured at 2 hours after administration. Data are expressed as means ± SDs ($n = 9$–11). ANOVA and the Student t-test were used for comparisons. * $P < 0.05$ for treated versus non-treated eyes. ## $P < 0.01$ for DMSO-treated versus drug-treated eyes. (**A**) IOPs (mm Hg). (**B**) IOP reductions (%).
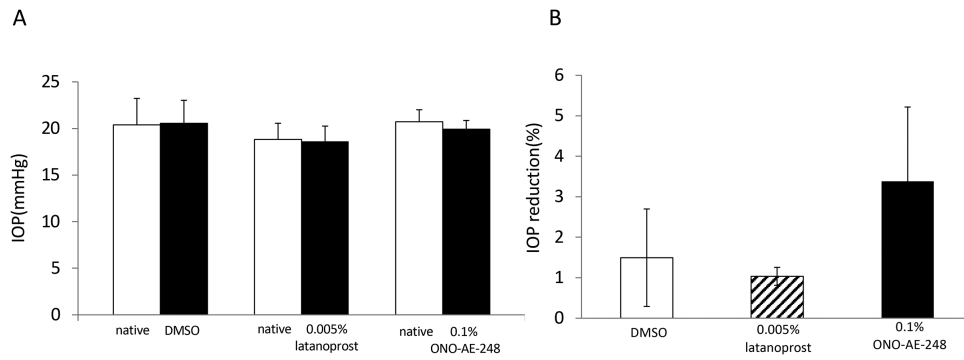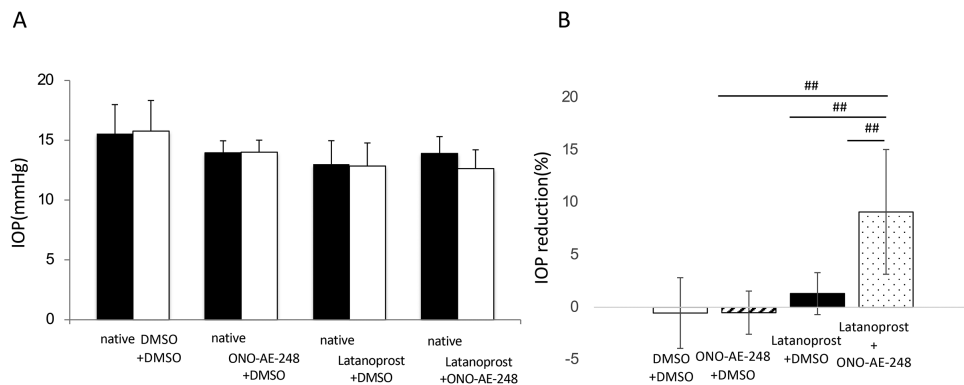
FIGURE 5. IOP reductions afforded by 0.005% (w/v) latanoprost and 0.1% (w/v) ONO-AE-248 (an EP3 agonist) in WT mice. IOP was measured at 6 hours after administration. Data are expressed as means ± SDs ($n = 9$–11). ANOVA and the Student t-test were used for comparisons. (**A**) IOPs (mm Hg). (**B**) IOP reductions (%).

FIGURE 6. IOP reductions afforded by a combined 0.1% (w/v) EP3 agonist and 0.005% (w/v) latanoprost in WT mice. IOP was measured at 2 hours after administration. The data are means ± SDs ($n = 8$–10). ANOVA and the Student t-test were used for comparisons. * $P < 0.05$ for treated versus non-treated eyes. ## $P < 0.01$ for the DMSO + DMSO group versus the drug-treated groups. (**A**) IOPs (mm Hg). (**B**) IOP reductions (%).

and 0.0032 ± 0.0010 µL/min/mm Hg, respectively, thus not significantly different, although latanoprost enhanced outflow facility to a similar extent in WT and EP3KO mice

($P = 0.096$; see Fig. 8). SPT was less effective in both EP3KO and FPKO mice, implying the involvement of both receptors.


FIGURE 7. IOP reduction afforded by a combined 0.1% (w/v) EP3 agonist and 0.005% (w/v) latanoprost in WT mice. IOP was measured at 6 hours after administration. The data are means ± SDs ($n = 8$–10). ANOVA and the Student t-test were used for evaluation. ## $P < 0.01$ for the DMSO + DMSO-, EP3 agonist + DMSO-, and latanoprost + DMSO-treated groups versus the latanoprost + EP3 agonist-treated group. (**A**) IOPs (mm Hg). (**B**) IOP reductions (%).
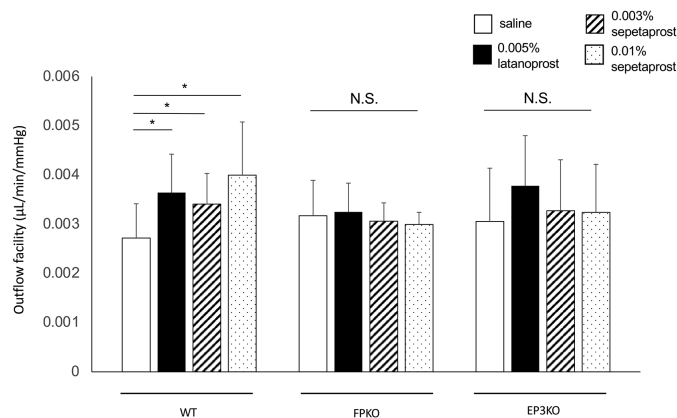
FIGURE 8. Outflow facility after treatment of WT, FPKO, and EP3KO mice with 0.005% (w/v) latanoprost and 0.003 and 0.01% (w/v) SPT. Outflow facilities were measured at the peak IOP-reduction times of each drug (at 2 or 4 hours after administration). * $P < 0.05$ for the saline- versus drug-treated eyes. The data are means ± SDs ($n = 6$–11). ANOVA and the Dunnett test were used for statistical evaluation.

DISCUSSION

Thus, SPT, a dual FP/EP3 receptor agonist, significantly reduced the IOP of mouse eyes more potently and for longer than did latanoprost. Such SPT effects were attenuated in EP3KO mice, implying that the EP3 receptor may reduce late IOP. However, few reports have explored any direct contribution made by the EP3 receptor to IOP reduction. This is the first report to clarify the role played by the EP3 receptor in IOP reduction mediated by a dual FP/EP3 receptor agonist.

We confirmed that SPT lowered IOP as effectively as did latanoprost (see Fig. 1). The IOP lowering effect by 0.003 and 0.01% SPT treatment was almost equivalent the effect of 0.005% latanoprost treatment. Although we cannot deny the differences of drug concentration may have effect on our results, we also assume that the major factor affecting our results is not only the concentration of the drug used, but also the pharmacokinetics and activity of each drug. In order to take these factors into consideration, in the present study, we decided to investigate the involvement of FP and EP3 receptors in IOP by comparing each drug under conditions of similar IOP reduction rates, and set the concentrations used in this study as 0.003 and 0.01% for SPT and 0.005% for latanoprost. The receptor-binding latanoprost Ki values were 98 nM for the FP and 7,519 nM for the EP3 receptors.[6] In contrast, the ONO-AG-367, the biologically active form of SPT, values were 0.727 nM for the FP and 25 nM for the EP3 receptors.[20] Therefore, SPT may exhibit a higher affinity for the FP receptor than does latanoprost. However, the peak IOP reduction afforded by SPT was comparable to that of latanoprost, but the effect was more durable over time. We speculate that this may reflect differences in FP receptor-binding affinities. To the best of our knowledge, no relationship between FP receptor-binding affinity and the strength/sustainability of IOP reduction has yet been reported, although several earlier studies suggested that FP-receptor affinity might reflect sustained IOP lowering but not peak IOP lowering. For example, tafluprost, which exhibits a 10-fold higher affinity for the FP and EP3 receptors than latanoprost,[28,29] similarly lowered the peak IOP in mice, but tafluprost evidenced longer-term effects than latanoprost; the AUC (% * hours) of tafluprost was almost two-fold that of latanoprost.[30] In addition, travoprost and another FP agonist

with a higher affinity for the FP receptor, reduced mouse eye IOP long-term.[6,30] The IOP-lowering effect of travoprost was higher than that of latanoprost at 14 days after drug instillation into human eyes.[31] Thus, we speculate that the sustained IOP-lowering effect of SPT may be because SPT may bind with high affinity to FP receptors. However, we lack direct evidence indicating that the drug-binding affinity to FP receptors explains the long-lasting IOP reductions. Further work is needed.

As expected, the 2-hour IOP reduction afforded by latanoprost was reduced in FPKO mice (see Fig. 2). In addition, SPT-mediated IOP reduction at 2 and 6 hours decreased in both FPKO and EP3KO (compared to WT) mice (see Figs. 2, 3). Thus, both the FP and EP3 receptors play roles in SPT-mediated IOP-reduction. In previous reports, IOP reduction by latanoprost was attenuated in patients taking nonsteroidal anti-inflammatory drugs (NSAIDs).[10,12,13,32] We previously reported that the PGA-mediated IOP reductions in mouse eyes were attenuated by concomitant administration of NSAIDs, and that the PGA-mediated reductions were weaker in EP3KO than in WT mice.[14,33] We suggested that EP3 receptor stimulation by endogenous PGE2 (after FP receptor stimulation) explained the IOP reduction afforded by PGAs. Here, we show that the maximal and sustained IOP reductions afforded by SPT were attenuated in FPKO and EP3KO mice (see Figs. 2, 3), and that sustained IOP reduction reflected simultaneous stimulation of the FP and EP3 receptors by a receptor-specific agonist (see Fig. 7). Thus, both the FP and EP3 receptors play crucial roles in IOP reduction by SPT, especially in terms of sustainability.

This is the first report indicating that EP3 is directly involved in IOP reduction. However, the 2- and 6-hour IOPs did not change after administration of a selective EP3 agonist, implying that (only) EP3 receptor stimulation may not affect the IOP (see Figs. 4, 5). The simultaneous stimulation described above may ensure sustained IOP reduction via stimulation of the EP3 receptor.

We investigated the effect of SPT on outflow facility. SPT enhanced outflow facility (as did latanoprost) in WT mice, but less so in FPKO or EP3KO mice (see Fig. 8). This is the first report exploring the roles played by the FP and EP3 receptors in outflow facility. To the best of our knowledge, few reports have explored the effects of FP and EP3 receptors on mouse aqueous humor dynamics, because the

receptor locations are not well known. EP3 receptor expression has varied by the method used to evaluate such expression. For example, it was reported that the EP3 receptor was not expressed in ciliary muscles in a study using in situ hybridization,[34] but some expression was immunochemically evident in non-pigmented ciliary epithelial cells.[35] In immunohistochemical human studies, EP3-receptor-specific staining was prominent in the corneal endothelium, keratocytes, trabecular cells, the ciliary epithelium, and conjunctival and iridal stroma cells,[23] although the gene-expression levels were lower than those of the EP2-encoding gene.[36] In addition, we used homozygous EP3KO mice for breeding to maintain these strains in this study. However, because homozygotes could accumulate compensatory mutations, we thought that maintaining the strain as heterozygotes and using homologous controls would be necessary in future studies for a more rigorous approach. Thus, the influence of the EP3 receptor on aqueous humor dynamics requires further study.

We found that IOP reduction was augmented by simultaneous stimulation of the FP and EP3 receptors, but not via stimulation of the EP3 receptor alone. This may reflect a unique mode of action of the EP3 receptor. Three EP3 receptor isoforms (α, β, and γ) are found in mice, and four (I to IV) in humans. The isoforms share the same amino acid sequences in the extracellular ligand-binding domain but their C-terminal splicing patterns differ,[37–40] and they are coupled to different G-proteins, thus Gi/G12 for EP3α and β and Gi/Gs for EP3γ.[41] The isoforms exhibit similar ligand-binding properties but differ in terms of signal transduction.[42] FP receptor stimulation increases [Ca2+]i by activation of inositol triphosphate and diacylglycerol synthesis via coupling to the Gq protein. EP3 receptor stimulation suppresses cyclic AMP production via Gi coupling.[37] EP3α and EP3β activation suppress cyclic AMP production, thus increasing IP3/Ca2+ levels and activating Rho.[40,43] On the other hand, EP3γ, which is coupled to Gi/Gs, exhibits higher Gs and Gi activities than do EP3α and EP3β, thus suppressing cyclic AMP production and increasing IP3/Ca2+ levels via Gi coupling but also increasing cyclic AMP production via Gs coupling.[44] Cyclic AMP production was suppressed by forced expression of the EP3 receptor (only) in CHO cells,[37] but such production was enhanced by co-expression of the EP2 or EP4 receptor with the EP3 receptor (compared to EP2 or EP4 expression alone) in COS-7 and P-815 cells.[45,46] The EP2 and EP4 receptors become conjugated to Gs proteins and then activate cyclic AMP production. In summary, stimulation of the EP3 receptor suppresses cyclic AMP production via Gi coupling, but we speculate that an environmental change or an activation signal might modulate the Gs activity of the EP3 receptor. The human and animal EP3 receptor isoforms involved in IOP control remain unknown; it is not clear why IOP was lowered by simultaneous stimulation of the FP and EP3 receptors. The expression patterns of the EP3 receptor isoforms and their interactions with the FP receptor family require further study.

Our work had several limitations. First, we did not consider the distributions of EP3 receptor isoforms. As stated above, the EP3 receptor is reportedly expressed by (only) the ciliary non-pigmented epithelia of tissues that are likely to be involved in aqueous humor dynamics,[41] although the precise distributions of/contributions made by EP3 receptor isoforms remain unclear. EP3α and EP3β suppress adenylate cyclase activity by binding to Gi proteins. In contrast, EP3γ binds to both the Gi and Gs proteins and increases adenylate cyclase activity.[44] It is reasonable to speculate that cyclic AMP production is principally suppressed via EP3 receptor stimulation by the Gi protein, but the three isoforms may interact differently with Gi proteins depending on the EP3 receptor-bearing tissue under examination. As for latanoprost or other selective FP receptor agonists, it has been reported that they had little effect on an aqueous humor production in animals or humans.[47–49] However, some other literatures reported that prostaglandin promoted aqueous humor production,[50] so the effect of FP receptor agonist on aqueous humor production is not clear. In the present study, we observed IOP decrease around 2 to 2.5 mm Hg by FP and EP3 receptor dual agonist. This reduction of IOP was rather greater, concerning the increase of outflow facility observed in the present study, which could imply the involvement of the other effects on aqueous humor formation, uveoscleral outflow, or episcleral venous pressure, or some combination of these. We speculate that SPT may suppress aqueous humor production via the EP3 receptor by inhibiting cAMP production (as does a β-blocker) when the EP3 receptor binds to Gi. The distributions of the EP3 receptor isoforms, and the roles played by the isoforms in aqueous humor production, will be explored in a future study. In conclusion, SPT exhibited a stronger and longer lasting IOP-lowering effect compared to PGAs (FP receptor agonists). SPT stimulated both the FP and EP3 receptors. Such a dual agonist is very promising.

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