The IOP-lowering effects and mechanism of action of tafluprost in prostanoid receptor-deficient mice

Running title: Effect of Tafluprost on IOP in EP and FPKO Mice

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ABSTRACT

Aims: To clarify the intraocular pressure (IOP)-lowering profile of tafluprost, a newly synthesized prostaglandin F₂α analog, in mice. Methods: C57BL/6J, and EP1, EP2, EP3 and FP receptor-deficient (WT, EP1KO, EP2KO, EP3KO, and FPKO, respectively) mice were bred and acclimatized under a 12-hour light (06:00 to 18:00)-dark cycle. To evaluate effects of tafluprost (0.0015%) on IOP at night, a single 3-µL drop of tafluprost solution was applied topically at 18:00 once into one eye in each mouse. IOP was measured 3 hours after the application with a microneedle method. To clarify whether endogenous prostaglandin is concerned with the tafluprost-induced IOP reduction, we applied 0.5% diclofenac Na, a cyclooxygenase inhibitor, or PBS 30 minutes before the application of tafluprost in WT and EP3KO mice and measured IOP 3 hours after the tafluprost application. We also determined whether animals responded predictably to 0.1% bunazosin HCl, a drug known to increase uveoscleral outflow.

Results: Three hours after the application of 0.0015% tafluprost, IOP reductions were 25.8 ± 2.1%, 26.3 ± 0.8%, 24.2 ± 1.4%, 16.5 ± 1.7% and -0.9 ± 1.5% in WT, EP1KO, EP2KO, EP3KO, and FPKO mice, respectively. IOP reductions in EP3KO and FPKO mice were significantly smaller than in WT mice. Pretreatment with diclofenac Na significantly attenuated the IOP lowering effect of tafluprost in WT mice but not in EP3KO mice. Bunazosin HCl lowered IOP significantly in all genotypes by the same amount. Conclusion: We conclude that tafluprost lowers IOP through the prostanoid FP receptor. A part of ocular hypotensive effect of tafluprost is attributed to FP receptor-mediated prostaglandin production acting through the prostanoid EP3 receptor.
1. Introduction

Prostanoid FP receptor agonists are used widely for treatment of glaucoma and ocular hypertension because they more effectively lower intraocular pressure (IOP) and have fewer systemic side effects than β-blockers. Latanoprost and travoprost are thought to lower IOP mainly via the FP-receptor because of their strong specificity for this receptor.[1] This hypothesis is supported by our recent reports that latanoprost had no IOP-lowering effect in FP receptor-deficient mice.[2, 3] We reported that bimatoprost and unoprostone, in addition to latanoprost and travoprost, also lower IOP mainly through the prostanoid FP receptor.[3]

Tafluprost, isopropyl (5Z)-7-[(1R,2R,3R,5S)-2-[(1E)-3,3-difluoro-4-phenoxybut-1-enyl]-3,5-dihydroxycyclopentyl]hept-5-enoate, is a newly synthesized prostaglandin (PG) F\textsubscript{2α} analog under development as an ocular hypotensive drug. It is a pro-drug ester that facilitates corneal penetration and allows delivery of the active carboxylic acid form to the aqueous humor. Tafluprost has higher affinity for the prostanoid FP receptor than latanoprost and a stronger effect on IOP than latanoprost in monkey;[4, 5] and it has a stronger effect than latanoprost, bimatoprost and unoprostone and similar or stronger effect than travoprost in mice.[6] However, the prostanoid receptor-mediated mechanism of action of tafluprost has not been fully clarified. Transgenic mice or gene knock-out mice can facilitate us to elucidate the molecular mechanism of specific gene function. In this study, we examined the effect of tafluprost on IOP in wild-type mice and prostanoid EP1, EP2, EP3 and FP receptor-deficient mice.

2. Methods

2.1. Animals

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The mouse genes encoding EP1, EP2 or EP3 receptors were disrupted by gene knockout methods using homologous recombination, as reported previously [7, 8] and EP1, EP2 or EP3-homozygous knockout (EP1KO, EP2KO or EP3KO, respectively) mice were obtained. FP receptor-deficient mice (FPKO) were also generated by gene knockout methods using homologous recombination,[9] the murine genotype was determined by PCR,[2] and FP-receptor homozygous knockout mice were used. C57BL/6 mice (Japan SLC; Hamamatsu, Japan), which is the background species of the EP1-3 and FP knockout mice, were used as the wild-type control (WT). Mice were bred and housed in clear cages loosely covered with
air filters, and white chip bedding was provided. The environment was kept at 21°C with a 12-hour light (06:00 to 18:00) and 12-hour dark cycle. All mice were fed ad libitum and acclimatized to the environment for at least two weeks prior to experiments. We used mice older than 8 weeks in our study.

2.2. Preparation and instillation of ophthalmic solution

Tafluprost (0.0015%) and bunazosin HCl (0.1%) ophthalmic solutions were provided by Santen Pharmaceuticals Co., Ltd. (Osaka, Japan). Diclofenac Na (0.1%), a cyclooxygenase inhibitor, was purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan) and dissolved in phosphate buffered saline (PBS). The doses of tafluprost and diclofenac Na were determined according to the clinical dose. The dose of bunazosin HCl was determined according to the previous studies.[10-12] With a micropipette, 3 µL of a drug solution was applied topically in a masked manner to a randomly selected eye, and the untreated contralateral eye served as the control.

2.3. IOP measurement

IOP was measured in mice anesthetized with ketamine and xylazine with a microneedle method as described previously.[13] Briefly, a borosilicate glass microneedle (100 µm tip diameter and 1.0 mm outer diameter; World Precision Instruments (WPI), Sarasota, FL) was connected to a pressure transducer (Model BLPR, WPI). The pressure detected by the transducer was recorded by a data acquisition and analysis system (PowerLab, ADInstruments, Colorado Springs, CO). The microneedle was placed in the anterior chamber. The conducted pressure was recorded in both eyes during a four- to seven-minute time window after anesthesia. Until the mouse was placed on the table for IOP measurement, room lighting was maintained similarly to that in the vivaria. In previous studies,[3, 6] we found stronger IOP reduction induced by PG-analogs at night; therefore, in this study we performed all IOP measurement to estimate the efficacy of tafluprost and other drugs at night. For measurements at night (dark phase), all procedures were performed under red light illumination to eliminate the effect of lighting on IOP.[14, 15] The effect of each drug was calculated as the percent IOP reduction, defined as 100 x (IOP of treated eye – IOP of contralateral untreated eye)/(IOP of contralateral untreated eye) in each mouse.

2.4. Effect of tafluprost on IOP in WT, EP1KO, EP2KO, EP3KO, and FPKO mice

Three microliters of tafluprost (0.0015%) was administered topically to one randomly chosen eye at 18:00. Three investigators, blinded to the drugs they
administered, instilled eye drops to WT, EP1KO, EP2KO, EP3KO, or FPKO mice. A fourth investigator, also masked to the treatment, measured IOP 3 hours after drug instillation. Thus, all measurements were performed under masked conditions. The time of IOP measurement was determined according to our previous study.[3, 16] In our previous study, we observed the maximum IOP reduction at 3 hr after the application of latanoprost and IOP measurement were performed at 3 hrs in subsequent experiments.

2.5. Effect of diclofenac Na (0.1%) on tafluprost-induced IOP reduction in WT and EP3KO mice

Three microliters of diclofenac Na (0.1%) or PBS was applied topically to one randomly chosen eye in WT or EP3KO mice 30 minutes before the tafluprost (0.0015%) instillation. At 18:00, 3 µL of tafluprost was applied topically to the same eye. The instillation and IOP measurement were performed under the masked conditions described above. Three hours after the tafluprost instillation, IOP was measured.

2.6. IOP lowering effect of bunazosin HCl

To confirm the functional presence of uveoscleral outflow in each prostanoid receptor knock out mouse, we examined the IOP-lowering effect of bunazosin HCl (0.1%), which lowers IOP by increasing uveoscleral outflow.[10, 12] Three microliters of bunazosin HCl (bunazosin) (0.1%) was applied topically to one randomly chosen eye in WT, EP1KO, EP2KO, EP3KO, or FPKO mice at 18:00. The instillation and IOP measurement were performed under the masked conditions described above. Three hours after drug instillation, IOP was measured.

2.7. Statistical analysis

The Wilcoxon signed-rank test was used for comparison of IOP between the treated eye and the contralateral untreated eye. A Kruskal-Wallis test followed by Steel's test was used for multiple comparisons of IOP reduction. Mann-Whitney’s U-test was used for comparison of IOP reduction between PBS plus tafluprost-treated and diclofenac Na plus tafluprost-treated groups in WT and EP3KO mice. P < 0.05 was considered statistically significant. All data are shown as mean ± SEM.

3. Results

3.1. Effect of tafluprost on WT, EP1KO, EP2KO, EP3KO, and FPKO mice

Baseline IOP showed no significant difference among WT, EP1KO, EP2KO,
EP3KO, and FPKO mice ranging between 17.0 and 20.9 mmHg. In WT, EP1KO, EP2KO, and EP3KO mice, tafluprost significantly lowered IOP by 25.8 ± 2.1% (n=8, P=0.0039), 26.3 ± 0.8% (n=11, P=0.0010), 24.2 ± 1.4% (n=10, P=0.0020), and 16.5 ± 1.7% (n=10, P=0.0020), respectively; whereas the -0.9 ± 1.5% IOP reduction in FPKO mice was not significant (n=10, P=0.2754) (Fig.1.). IOP reductions induced by tafluprost in EP3KO and FPKO mice were significantly smaller (P=0.0387 and 0.0009, respectively) than in WT mice. The IOP reductions in EP1KO and EP2KO mice were statistically indistinguishable from the reduction in WT mice.

3.2. Effect of diclofenac Na (0.1%) on tafluprost-induced IOP reduction in WT and EP3KO mice

In WT mice treated first with PBS (control WT group) or diclofenac Na (diclofenac WT group), tafluprost lowered IOP significantly 3 hours after its instillation (25.4±1.5%, n=17, P<0.0001 and 15.6±1.5%, n=16, P=0.0002, respectively). However, the tafluprost-induced IOP reduction in the diclofenac WT group was attenuated significantly compared with that in the control WT group (p<0.0002). In EP3KO mice first treated with PBS (control EP3KO group) or with diclofenac Na (diclofenac EP3KO group), tafluprost lowered IOP significantly (17.4±1.8%, n=11, P=0.0010, and 13.9±1.4%, n=14, P=0.0015, respectively), but no significant difference was seen between the control EP3KO and diclofenac EP3KO groups (P=0.1889) (Fig. 2.).

3.3 IOP lowering effect of bunazosin HCl on WT, EP1KO, EP2KO, EP3KO, and FPKO mice

Three hours after the administration of bunazosin, IOP was significantly reduced by 20.9±1.3% (n=10, P=0.0020), 20.8±1.9% (n=9, P=0.0039), 19.9±1.3 (n=11, P=0.0010), 22.1±2.1% (n=11, P=0.0010), and 21.2±2.1% (n=9, P=0.0039) in WT, EP1KO, EP2KO, EP3KO, and FPKO mice, respectively (Fig. 3.). There were no significant differences in IOP reductions among WT, EP1KO, EP2KO, EP3KO, and FPKO mice (P=0.8569).

4. Discussion

In our previous work, we found that latanoprost, travoprost, bimatoprost, and unoprostone lowered IOP by prostanoid FP receptor activation.[3] In this study, we examined the mechanism of the IOP lowering effect of tafluprost, a novel prostanoid FP agonist, using WT, EP1KO, EP2KO, EP3KO, and FPKO mice. The baseline IOPs in
EP1KO, EP2KO, EP3KO, and FPKO mice in the present study are not significantly different from the baseline IOP in WT mice as previously reported.[2, 3, 17] We also found previously that the IOP lowering effect of latanoprost and other PG-related compounds is greater at night than during the day and that the maximum reduction in IOP occurs 3 hours after the latanoprost treatment in WT mice.[3, 6] Therefore, in this study we examined the IOP-lowering effect of tafluprost 3 hours after the treatment at night.

In WT (C57B6) mice, tafluprost lowered IOP significantly compared with the contralateral eye (25.8±2.1%) (Fig. 1). The degree of IOP reduction we observed is different from the reduction (17.6±1.7%) seen in our previous study using ddY mice.[6] These different responses of IOP to tafluprost may depend on the mouse strain, because baseline IOP is different between genetically distinct mice.[18, 19] Significant IOP reduction in different mouse strains and an IOP reduction similar to other clinically available PG-analogs indicate that tafluprost may have potential for use in human eyes.

The IOP reductions in EP1KO and EP2KO mice were not significantly different from that in WT mice (Fig. 1). These results are consistent with a previous study in which the affinities of tafluprost to prostanoid EP1 and EP2 receptors are very weak.[4] On the other hand, in FPKO mice, tafluprost had no obvious effect in this study confirming that tafluprost reduces the IOP through FP receptors. (Fig. 1). What is interesting was that, in EP3KO mice, the IOP-lowering effect of tafluprost was significantly smaller than the effect in WT mice (Fig. 1). Because of the relatively high affinity of tafluprost to prostanoid EP3 receptor (IC50=67 nM).[4] there is a possibility that tafluprost acts directly on the prostanoid EP3 receptor and lowers IOP,[20] but the absence of any effect of tafluprost in FPKO mice does not favor this possibility. To confirm whether tafluprost acts directly on prostanoid EP3 receptors or secondarily acts on EP3 receptors through endogenously produced PG, we examined the effect of pre-treatment with diclofenac Na on tafluprost-induced IOP reduction in WT and EP3KO mice (Fig. 2). Pre-treatment with diclofenac Na attenuated the IOP-lowering effect of tafluprost in WT mice but not in EP3KO. These results suggest that endogenous PGs produced by cyclooxygenase through prostanoid FP receptor activation lower IOP via the prostanoid EP3 receptor. It is reported that latanoprost and unoprostone induce endogenous PGE2, which is inhibited by NSAID.[21-23] and the IOP-lowering effect of latanoprost is attenuated by co-administration of NSAID in normal volunteers [24] and in rabbits.[23] Therefore, our present results suggest that tafluprost lowers IOP through the prostanoid FP receptor and a part of its ocular hypotensive effect is attributed to FP receptor-mediated prostaglandin production acting through the prostanoid EP3 receptor. This FP receptor-induced intracameral production of PGs should be clarified in a further
study.

The lack of IOP reduction by tafluprost in FPKO mice may be due to a dysfunction of the outflow pathway caused by the genetic manipulation. In all strains used in this study, the positive response of the IOP lowering effect of bunazosin HCl, which decreases IOP by increasing uveoscleral outflow \cite{10, 12} shows that functional uveoscleral outflow pathways may be present in WT, EP1KO, EP2KO, EP3KO, and FPKO mice (Fig. 3). It should be noted, however, that no report clarified whether bunazosin increase uveoscleral outflow in mice. Therefore there is a limitation that the result in this mouse study is extrapolated to that in human eyes.

Although how tafluprost affects aqueous humor dynamics in the mouse eye has not been established, its pharmacological property for prostanoid receptors is not greatly different from that of latanoprost and tafluprost also increased uveoscleral outflow in monkeys.\cite{4} We do know that total aqueous outflow is increased by latanoprost in mouse eyes.\cite{25} These facts indicate that tafluprost also improves the outflow pathway in mouse eyes through FP receptors.

In conclusion, tafluprost lowers IOP through the prostanoid FP receptor in mice, and a part of the IOP reduction induced by tafluprost may be through the EP3 receptor that is stimulated by endogenous PG produced by FP stimulation. Because tafluprost is more potent FP selective agonist than latanoprost, causing more IOP reduction in monkeys \cite{4} and mice \cite{6}, tafluprost may be also used as a test drug for further elucidation of ocular hypotensive mechanism through FP receptors.

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**FIGURE REGENDS**

**Fig. 1.** Effect of tafluprost (0.0015%) on wild type (WT), EP1, EP2, EP3 and FP receptor knockout (EP1KO, EP2KO, EP3KO, and FPKO, respectively) mice at night. Three microliters of tafluprost was applied topically at 18:00 and IOP was measured at 21:00 in anesthetized mice. The % reduction was defined as described in the method section. Data are expressed as mean ± SEM (n=8-11). *, P < 0.05 vs. WT (Steel's test).

**Fig. 2.** Effect of diclofenac Na (0.1%) on tafluprost-induced IOP reduction in wild type (WT) and EP3 receptor knockout (EP3KO) mice at night. Three microliters of diclofenac Na (0.1%) or PBS was applied topically 30 minutes before the tafluprost (0.0015%) application. Tafluprost was applied topically at 18:00 and IOP was measured at 21:00 in anesthetized mice. The % reduction was defined as described in the method section. Data are expressed as mean ± SEM (n=11-17). * P < 0.05 vs. PBS plus tafluprost-treated group; NS, no significant difference (Mann-Whitney's U-test).

**Fig. 3.** Effect of bunazosin HCl (0.1%) on wild type (WT), EP1, EP2, EP3 and FP receptor knockout (EP1KO, EP2KO, EP3KO, and FPKO, respectively) mice at night. Three microliters of bunazosin HCl (0.1%) was applied topically at 18:00 and IOP was measured at 21:00 in anesthetized mice. The % reduction was defined as described in the method section. Data are expressed as mean ± SEM (n=9-11). There was no significant difference among genotypes (Kruskal-Wallis test).
References


Ota et al. Figure 1

![Graph showing IOP (% reduction) for different genotypes: WT, EP1KO, EP2KO, EP3KO, FPKO. The graph indicates a significant reduction in IOP for EP1KO and EP2KO compared to WT and EP3KO, with asterisks (*) denoting statistical significance.](attachment:image.png)
Ota et al. Figure 2

![Graph showing IOP (% reduction) with different treatments and genotypes.](https://example.com/figure2.png)

- PBS
- Diclofenac
- Tafluprost

WT
- +
- +
- +

EP3KO
- +
- +
- +

IOP (% reduction)

- *NS*

- *NS*
Ota et al. Figure 3 ↑

![Graph showing IOP (% reduction) for different genotypes: WT, EP1KO, EP2KO, EP3KO, FPKO. The graph indicates that EP1KO, EP2KO, and EP3KO have a higher IOP reduction compared to WT and FPKO.]
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