Topical Ocular Sodium 4-Phenylbutyrate Rescues Glaucoma in a Myocilin Mouse Model of Primary Open-Angle Glaucoma

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Purpose. Mutations in the myocilin gene (*MYOC*) are the most common known genetic cause of primary open-angle glaucoma (POAG). The purpose of this study was to determine whether topical ocular sodium 4-phenylbutyrate (PBA) treatment rescues glaucoma phenotypes in a mouse model of myocilin-associated glaucoma (*Tg-MYOC*^{Y437H} mice).

Methods. *Tg-MYOC*^{Y437H} mice were treated with PBA eye drops ($n = 10$) or sterile PBS ($n = 8$) twice daily for 5 months. Long-term safety and effectiveness of topical PBA (0.2%) on glaucoma phenotypes were examined by measuring intraocular pressure (IOP) and pattern ERG (PERG), performing slit lamp evaluation of the anterior chamber, analyzing histologic sections of the anterior segment, and comparing myocilin levels in the aqueous humor and trabecular meshwork of *Tg-MYOC*^{Y437H} mice.

Results. *Tg-MYOC*^{Y437H} mice developed elevated IOP at 3 months of age when compared with wild-type (WT) littermates ($n = 24$; $P < 0.0001$). Topical PBA did not alter IOP in WT mice. However, it significantly reduced elevated IOP in *Tg-MYOC*^{Y437H} mice to the level of WT mice. Topical PBA-treated *Tg-MYOC*^{Y437H} mice also preserved PERG amplitudes compared with vehicle-treated *Tg-MYOC*^{Y437H} mice. No structural abnormalities were observed in the anterior chamber of PBA-treated WT and *Tg-MYOC*^{Y437H} mice. Analysis of the myocilin in the aqueous humor and TM revealed that PBA significantly improved the secretion of myocilin and reduced myocilin accumulation as well as endoplasmic reticulum (ER) stress in the TM of *Tg-MYOC*^{Y437H} mice. Furthermore, topical PBA reduced IOP elevated by induction of ER stress via tunicamycin injections in WT mice.

Conclusions. Topical ocular PBA reduces glaucomatous phenotypes in *Tg-MYOC*^{Y437H} mice, most likely by reducing myocilin accumulation and ER stress in the TM. Topical ocular PBA could become a novel treatment for POAG patients with myocilin mutations. (*Invest Ophthalmol Vis Sci.* 2012;53:1557–1565) DOI:10.1167/iovs.11-8837

Glaucoma is the second leading cause of visual impairments and blindness worldwide, affecting approximately 70 million people, and is the leading cause of blindness among African Americans in the United States.[1] Primary open-angle glaucoma (POAG) is the most common form, accounting for approximately 70% of all cases worldwide. It is characterized by progressive loss of retinal ganglion cell (RGC) axons and irreversible loss of vision,[2] which is often associated with elevated intraocular pressure (IOP). Although the exact mechanisms that lead to elevated IOP are poorly understood, an increased resistance to outflow of aqueous humor through the drainage structures in the iridocorneal angle (the trabecular meshwork and Schlemm's canal) is thought to be the major cause of IOP elevation in POAG.

Mutations in the myocilin gene (*MYOC*) are the most common known genetic causes of glaucoma.[3–5] These mutations, which cause elevated IOP, are responsible for approximately 4% of POAG, and most cases of autosomal dominant juvenile-onset open-angle glaucoma.[5,6] Myocilin is expressed in many ocular tissues including the TM cells.[5,7–9] The physiological function of myocilin is not completely understood, although recent reports suggested a possible role in cell–matrix interaction.[5,9–11] Myocilin knock-out and -in mouse studies have indicated that wild-type myocilin is not necessary for the physiological regulation of IOP and supports the hypothesis that glaucoma associated with the myocilin mutation is the result of an abnormal property of mutant myocilin.[7–9,12] In vitro studies suggest that glaucoma-causing myocilin mutants are misfolded and detergent resistant and do not degrade properly.[9,13] Several studies have demonstrated that disease-causing myocilin mutants are secretion incompetent,[14–16] accumulate in the endoplasmic reticulum (ER) and induce ER stress.[13,17–19] However, in vivo mechanistic studies in a myocilin mouse model that faithfully replicates the human disease have not been available. We have recently generated a transgenic mouse model (*Tg-MYOC*^{Y437H}) that expresses human *MYOC* containing the Y437H mutation under the control of the CMV promoter within relevant eye tissues including TM.[19] Of note, adult *Tg-MYOC*^{Y437H} mice have elevated IOP and show functional and structural loss of RGCs as well as optic nerve degeneration closely resembling those seen in POAG patients with myocilin mutations. Furthermore, we have demonstrated that ER stress plays a key role in the development of elevated IOP in *Tg-MYOC*^{Y437H} mice.[19]

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Sodium 4-phenylbutarate (PBA) is a small chemical chaperone approved for clinical use in patients with urea cycle disorders and has been shown to reduce protein mislocalization and ER stress in different diseases.[20–29] A well-known example of this mechanism is the restoration of the cell surface expression and function of the mutant cystic fibrosis transmembrane conductance regulator protein (CFTR) by PBA.[20,29] Several studies have suggested that myocilin-associated glaucoma is a protein-misfolding disease and that chemical chaperones can be used to correct myocilin misfolding. Consistent with this hypothesis, in another study, we demonstrated that systemic PBA prevents glaucoma in *Tg-MYOC^{Y437H}* mice.[19] Since PBA is approved by the U.S. Food and Drug Administration (FDA) for oral use in urea cycle disorders and has a good safety profile,[30,31] we sought to evaluate whether a topical form of this drug, administered as an eye drop could be used for treatment of glaucoma. The purpose of the present study was to determine whether topical ocular PBA treatment would reduce glaucomatous damage in *Tg-MYOC^{Y437H}* mice.

Methods and Materials

Mouse Husbandry

Mice were housed and bred at the University of Iowa Research Animal Facility. They were maintained on a 4% fat NIH 31 diet provided ad libitum and housed in cages containing dry bedding (SoftZorb Enrichment Blend; Northeastern Products, Warrensburg, NY). The environment was kept at 21°C with a 12:12-hour light:dark cycle. All animal procedures performed in the study complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the University of Iowa Animal Care and Use Committee.

Tg-MYOC^{Y437H} Mice

A detailed characterization of *Tg-MYOC^{Y437H}* mice has been published recently.[19] C57BL/6J mice were crossed with F2 and later generations of intercrossed mice B6SJL;Tg(CMV-MYOCY437H)/Vcs (abbreviated throughout as *Tg-MYOC^{Y437H}*). The mice were genotyped by PCR with primers specific to human MYOC, as described in that publication.

PBA Treatment

To examine whether topical PBA reduces elevated IOP in *Tg-MYOC^{Y437H}* mice, we allowed elevated IOP to develop in the *Tg-MYOC^{Y437H}* mice for 1 month or 5 months and then initiated treatment with topical PBA eye drops. Pharmaceutical-grade 4-PBA (4-phenylbutyric acid sodium salt) was purchased from Scandinavian Formulas (Scandinavian Formulas Inc., Sellersville, PA). PBA (0.2%) solution was made in sterile phosphate-buffered saline. Fresh solution was made every week and kept at room temperature. Baseline nocturnal IOP was measured in 3-month-old WT (n = 14) and *Tg-MYOC^{Y437H}* mice (n = 24), as we reported.[19] One month later, *Tg-MYOC^{Y437H}* mice were randomly divided into two groups: The first group received topical ocular PBA twice daily in both eyes, and the second group received topical ocular vehicle control solution (sterile PBS) twice daily. For long-term treatment of topical PBA eye drops, we treated both eyes because of concern about the potential systemic effect of PBA in the fellow eye. Typical eye drops contain up to 50 μL of PBA solution, and mice have a tendency to lick them. In addition, PBA has been shown to have high tissue penetrance. Thus, long-term treatment with PBA eye drops twice daily may result in a systemic therapeutic concentration in the untreated eye, potentially causing confounding effects. Intraocular pressure was monitored every month for a total of 5 months. To determine whether PBA reduces elevated IOP in older mice, we used 9-month-old *Tg-MYOC^{Y437H}* mice, which had ocular hypertension for 6 months. The left eyes were treated with PBA, and the contralateral right eyes were kept untreated. Nocturnal IOP was measured after 1 week of treatment.

IOP Measurements

Nocturnal IOP was measured with a rebound tonometer (TonoLab; Icare Finland, Helsinki, Finland), as in our prior study.[19] Briefly, IOP was measured in the isoflurane-anesthetized mice at night (between 11 PM and 1 AM).

Pattern-Evoked Electroretinography

Pattern-evoked electroretinography (PERG) was used to objectively measure the function of RGCs by recording amplitudes and latency of the N35-P50 and P50-N95 PERG waveforms as described previously.[19] Briefly, mice were initially anesthetized with a mixture of 75% O_2, 25% NO, and 3.5% halothane, and after 3 minutes, the concentration of halothane was decreased to 1.5%. PERG responses were evoked using alternating, reversing, black-and-white, vertical stimuli delivered on a CRT monitor with a commercial ERG system (Roland Consult, Brandenburg, Germany). Each animal was placed at the same fixed position in front of the monitor to prevent recording variability due to animal placement. Stimuli (9° full-field pattern, 1 Hz frequency, 200 averaged signals with a filter frequency cutoff of 1–30 Hz) were delivered in photopic conditions, since slow stimulation speed in mesopic and scotopic conditions can elicit rod-mediated, full-field ERG responses, which can completely conceal the PERG responses in rodents. PERG responses were evaluated by measuring amplitudes (N35-P50 and P50-N95) and corresponding implicit times (latencies). Implicit times were calculated for N35, P50, and N95 markers, in addition to the implicit times for N35-P50 and P50-N95 components.

Mouse Slit Lamp Examination

Anterior chamber phenotypes were assayed with a slit lamp (SL-D7; Topcon, Tokyo, Japan) and photodocumented with a digital camera (D100; Nikon, Tokyo, Japan), as published elsewhere.[32]

Histologic Analysis of Iridocorneal Angle

Eyes were enucleated and fixed by immersion in a solution of 4% paraformaldehyde. After 2 to 4 hours of fixation, anterior segments were dissected and washed three times in PBS followed by infiltration and embedding in acrylamide. Cryostat sections were collected at a thickness of 10 μm, and stored at 4°C until use. Gross morphology of the iridocorneal angle was evaluated with hematoxylin/eosin staining. Photomicrographs were taken with a fluorescence microscope (BX-41; Olympus, Tokyo, Japan) equipped with a digital camera (SPOT-RT; Diagnostic Instruments, Sterling Heights, MI).

Bioavailability of PBA in the Aqueous and Vitreous Humor of Mice

Five WT mice were given topical 0.2% PBA eye drops in both eyes (n = 10). Thirty minutes later, the mice were euthanized, and the aqueous and vitreous humors were obtained, as described previously.[19] The samples were analyzed for the presence of PBA by using gas chromatograph mass spectrometry by a commercially available service provided by Toxicology Associates, Inc. (Columbus, OH).

Western Blot Analysis of Myocilin in the Aqueous Humor

Mice were anesthetized with ketamine (73 mg/kg) and xylazine (1.8 mg/kg). Approximately 2 μL of aqueous humor was collected from each eye with a glass capillary and added to lysis buffer. For each sample, an equal amount of aqueous humor was loaded and separated on denaturing polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. Western blot analysis was conducted as described previously.[33] To ensure equal protein loading, the same blot was stained with Coomassie blue. A representative band at 65 kDa showed equal loading (see Fig. 4A). Each lane in Figure 4A represents aqueous humor from a different mouse. Quantitation was performed with Image J software (developed by Wayne Rasband, National Insti-

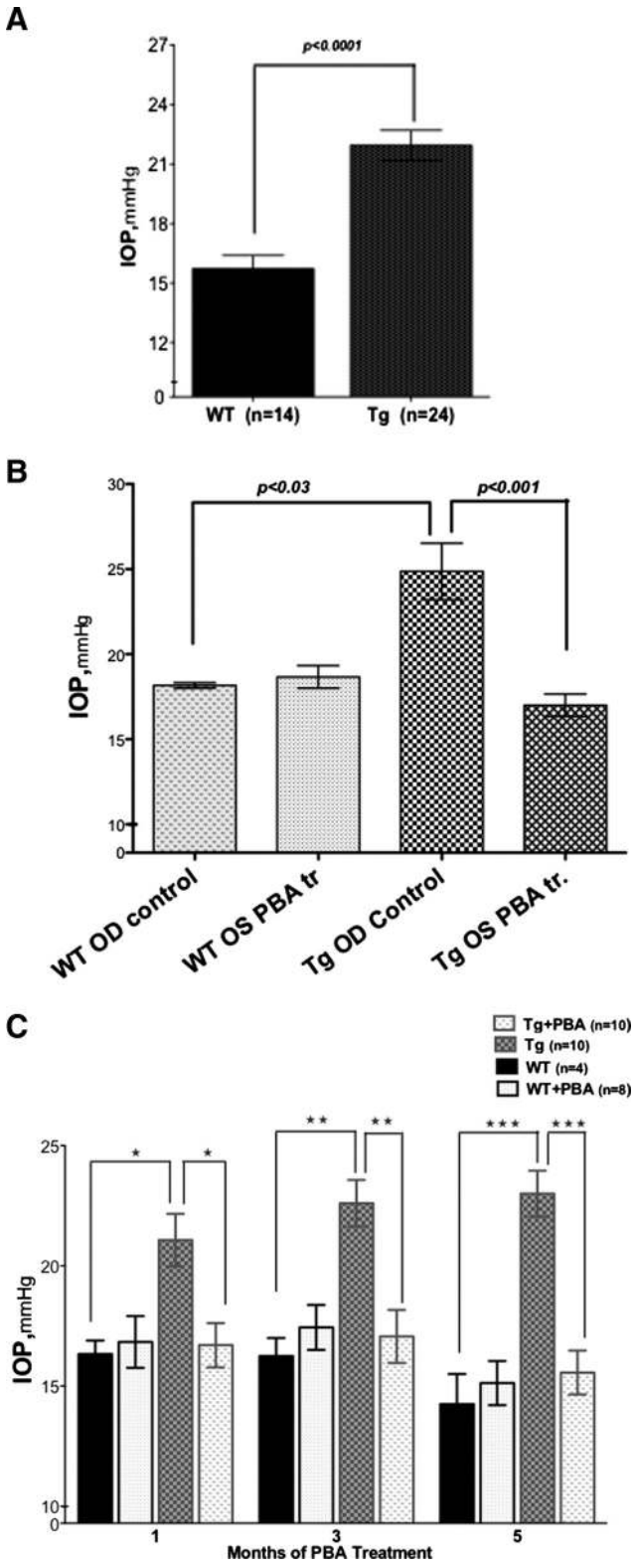

FIGURE 1. Topical ocular PBA rescues ocular hypertension in *Tg-MYOC*^{Y437H} mice. (**A**) IOP measurements of 3-month-old WT and *Tg-MYOC*^{Y437H} mice. *Tg-MYOC*^{Y437H} mice developed elevated IOP compared with WT littermates. (**B**) Topical ocular PBA reduced IOP in the left eye compared with the contralateral control eye in 9-month-old *Tg-MYOC*^{Y437H} mice. Topical ocular PBA was applied to the left eye of *Tg-MYOC*^{Y437H} mice, and the right eye served as the control. One week after PBA treatment, IOP in the left eye was significantly reduced compared with the contralateral eye. $n = 3$ WT and $n = 5$ *Tg-MYOC*^{Y437H} mice. (**C**) IOP measurements of vehicle or PBA-treated WT and *Tg-MYOC*^{Y437H} mice for 5 months. WT and *Tg-MYOC*^{Y437H} mice (3 months old) were divided into two groups: the first received topical ocular PBA (0.2%) twice daily, and the second was given sterile PBS (vehicle) twice daily. The IOP of these mice was measured every month. Vehicle-treated *Tg-MYOC*^{Y437H} mice showed elevated IOP compared with that of the WT littermates; however, PBA treatment normalized the IOP of the *Tg-MYOC*^{Y437H} mice to WT levels. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$, versus vehicle-treated *Tg-MYOC*^{Y437H} mice. Data are the mean ± SEM.

tutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/index.html), as described previously.[34] The densitometric analysis represents average myocilin secretion from this Western blot. Figure 4B presents data from three vehicle-treated WT mice, three PBA-treated WT mice, five untreated *Tg-MYOC*^{Y437H} mice, and four PBA-treated *Tg-MYOC*^{Y437H} mice.

Immunohistochemistry

Mouse anterior segment tissues were fixed in 4% formaldehyde and embedded in sucrose. The sections were then blocked with 5% normal goat serum. Slides were incubated overnight with primary antibody in 1.5% (vol/vol) normal goat serum, washed three times with PBS, and incubated for 2 hours in the appropriate secondary AlexaFluor antibodies (1:200; Invitrogen, Carlsbad, CA). Sections were subsequently incubated with DAPI for 30 minutes to stain the nuclei and then washed and mounted. Images were captured with a confocal imaging system (model 710; Carl Zeiss Meditec, Dublin, CA) at the University of Iowa Central Microscopy Research Facility. The KDEL antibody, which predominantly recognizes GRP78 and GRP94, was purchased from Abcam (Cambridge, MA).

Tunicamycin Injections

WT mice (C57BL6) were anesthetized as described previously.[19] Subconjunctival periocular injections of tunicamycin (2 μl, 0.2 μg/eye) were performed in both eyes ($n = 14$). Similar control periocular injections of vehicle were performed in both eyes ($n = 6$). We had demonstrated that tunicamycin injections elevate IOP 1 week after injection of tunicamycin.[19] IOP was measured for 1 week, to ensure that mice developed ocular hypertension. Topical 1% PBA eye drops were applied twice daily in both eyes of tunicamycin-injected mice, and IOP was measured every week afterward, to evaluate whether PBA reduces IOP. An additional group of WT mice was injected with periocular tunicamycin; however, this group was not treated with PBA, to ensure that tunicamycin-injected mice would have elevated IOP during the course of the study (not shown).

SD-OCT Imaging

The cornea and other anterior segment structures were visualized with a spectral-domain optical coherence tomographer (SD-OCT; Bioptigen, Inc., Research Triangle Park, NC). Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), wrapped in gauze, and secured in a small-animal imaging cassette. Balanced salt solution (BSS; Alcon Laboratories, Fort Worth, TX) was applied to the eye to maintain a consistent tear film. A 12-mm telecentric bore with a reference arm position of 979 was used to image the anterior segment of each eye. The bore was positioned so that the pupil of the eye was centered in the volume intensity projection. Scan parameters were as follows: radial volume scans 3.0 mm in diameter, 1000 A-scans/B-scan, 25 B-scans/volume, 3 frames/B-scan, and 1 volume. Central corneal thickness (CCT) was measured in each eye with vertical, angle-locked, B-scan calipers.

Statistics

For comparisons between two groups, the Student's *t*-test was used. For comparisons among three or more groups, one-way ANOVA with a Bonferroni post hoc test was applied. $P < 0.05$ denoted statistical significance.

RESULTS

Topical Ocular PBA Reduces Ocular Hypertension in *Tg-MYOC^{Y437H}* Mice

Consistent with our previous study,[19] nocturnal IOP measurements demonstrated that the *Tg-MYOC^{Y437H}* mice developed significant elevation of IOP at 3 months of age, compared with the WT mice (Fig. 1A). We first examined whether topical ocular PBA reduces elevated IOP in the 9-month-old *Tg-MYOC^{Y437H}* mice by treating the left eye with PBA while leaving the contralateral right eye untreated (Fig. 1B). Topical PBA did not affect IOP in the WT mice. However, 1 week of topical PBA treatment of the *Tg-MYOC^{Y437H}* mice reduced IOP in the left eye, whereas the untreated contralateral right eye had elevated IOP (17 mm Hg in PBA-treated eyes of the *Tg-MYOC^{Y437H}* mice versus 25 mm Hg in the untreated eyes of the *Tg-MYOC^{Y437H}* mice).

We next sought to examine whether prolonged topical ocular PBA treatment would reduce elevated IOP in *Tg-MYOC^{Y437H}* mice. Topical PBA application to 4-month-old *Tg-MYOC^{Y437H}* mice significantly reduced IOP over a period of 5 months (Fig. 1C). In addition, PBA did not alter IOP of the WT mice compared with vehicle-treated WT mice (16 mm Hg in the vehicle-treated WT versus 16.6 mm Hg in the PBA-treated WT mice). These data indicate that topical ocular PBA is capable of reducing IOP for a sustained period in *Tg-MYOC^{Y437H}* mice.

Topical Ocular PBA Treatment Prevents RCG Functional Deficits in *Tg-MYOC^{Y437H}* Mice

We have shown that *Tg-MYOC^{Y437H}* mice lose RGC function, as measured by PERG amplitudes (~50% reduction compared with that of WT littermates at 9 months of age).[19] In the present study, we examined whether prolonged topical ocular PBA treatment, which reduced elevated IOP in *Tg-MYOC^{Y437H}* mice for 5 months, would also prevent PERG deficits in *Tg-MYOC^{Y437H}* mice. Our results showed that vehicle-treated *Tg-MYOC^{Y437H}* mice developed significant PERG amplitude deficits compared to WT littermates (Fig. 2). The WT mice treated with PBA did not have a significant change in PERG amplitudes when compared with amplitudes in the vehicle-treated WT mice, which is suggestive of a good retinal safety profile associated with long-term PBA topical use. Treatment of *Tg-MYOC^{Y437H}* mice with topical PBA eye drops caused significant improvement in RGC function when compared with vehicle-treated *Tg-MYOC^{Y437H}* mice (Fig. 2). In the 9-month-old PBA-treated *Tg-MYOC^{Y437H}* mice, RGC function was not significantly different from that of the control WT mice.

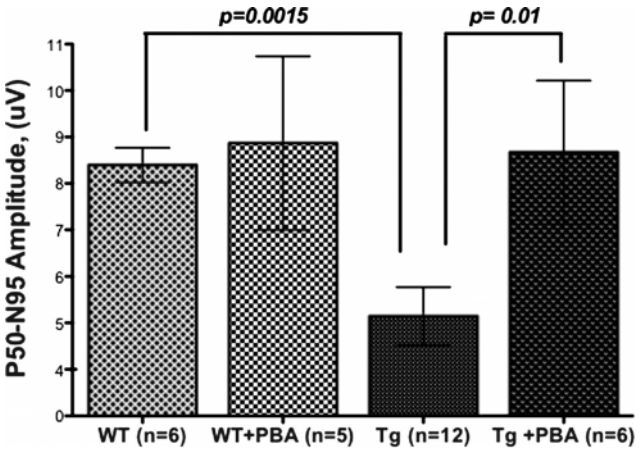

FIGURE 2. Topical ocular PBA treatment prevents retinal ganglion cells functional deficits in *Tg-MYOC^{Y437H}* mice. PERG amplitudes (P50–N95, μV) were measured to evaluate the functional deficit in the RGCs of *Tg-MYOC^{Y437H}* mice treated with topical PBA. PBA treatment of *Tg-MYOC^{Y437H}* mice for 5 months prevented a reduction in PERG amplitudes compared with those in vehicle-treated *Tg-MYOC^{Y437H}* mice (~50% loss in PERG amplitudes). $n = 6$ vehicle-treated WT, $n = 5$ PBA treated WT, $n = 12$ vehicle-treated *Tg-MYOC^{Y437H}*, and $n = 6$ PBA-treated *Tg-MYOC^{Y437H}* mice. Data are the mean ± SEM.

Topical Ocular PBA Does Not Cause Abnormalities to the Anterior Segment Structures in WT or *Tg-MYOC^{Y437H}* Mice

We next examined whether topical ocular PBA causes any anterior segment abnormalities by using slit lamp examination (Figs. 3A, 3B), histologic staining (Figs. 3C, 3D), and OCT of the cornea (Figs. 3E–G). Slit lamp examination of PBA-treated WT (Fig. 3A, $n = 6$) and *Tg-MYOC^{Y437H}* littermates (Fig. 3B, $n = 10$) demonstrated that PBA treatment for 5 months did not cause any eye abnormalities in WT or *Tg-MYOC^{Y437H}* littermates. The iris, cornea (including cornea thickness by OCT; Figs. 3E–G), anterior chamber, and lens of PBA-treated mice appeared indistinguishable from those of vehicle-treated mice. In addition, H&E staining of WT and *Tg-MYOC^{Y437H}* mice treated with PBA demonstrated that PBA treatment did not affect cornea or iridocorneal angle tissues compared with vehicle-treated mice (Figs. 3C, 3D).

We further examined whether PBA is present in the aqueous and vitreous humor of WT mice treated with topical PBA eye drops. Aqueous and vitreous humors were obtained 30 minutes after application of topical PBA eye drops. Analysis of PBA in these samples demonstrated that 10 μM (1.83 μg/mL) PBA was present in the aqueous humor. Remarkably, 5 μM (0.93 μg/mL) PBA was also present in the vitreous humor of these mice. These data suggest that topical PBA efficiently penetrates the cornea and is present in the aqueous and vitreous humor in a concentration, which could have a therapeutic effect on the posterior segment (retinal ganglion cells).

Topical Ocular PBA Improves Myocilin Secretion in the Aqueous Humor of *Tg-MYOC^{Y437H}* Mice

We and others have shown that secretion of mutant myocilin into the aqueous humor is reduced.[13–16,19] We investigated whether topical ocular PBA would restore myocilin secretion in the aqueous humor of *Tg-MYOC^{Y437H}* mice (Fig. 4). Consistent with previous studies, Western blot analysis demonstrated little or no myocilin secretion in the aqueous humor of vehicle-treated *Tg-MYOC^{Y437H}* mice. However, PBA-treated *Tg-MYOC^{Y437H}* littermates showed a significant amount of myocilin secretion in the aqueous humor compared with that in vehicle-treated *Tg-MYOC^{Y437H}* mice. Myocilin protein is secreted in both glycosylated and nonglycosylated form, which is seen as a doublet in Western blot. Although a myocilin doublet seemed to be present only in PBA-treated mice in the representative blot in Figure 4A, a myocilin doublet that is not shown was also found to be present in other WT mice.

Topical Ocular PBA Reduces Intracellular Accumulation of Myocilin and ER Stress in the TM of *Tg-MYOC^{Y437H}* Mice

Consistent with improved secretion of myocilin in the aqueous humor, we determined whether topical PBA reduces myocilin accumulation and ER stress in the TM. To examine myocilin levels and ER stress in the TM, we performed immunostaining with myocilin antibody and KDEL antibody against the ER stress markers in vehicle or PBA-treated iridocorneal angle tissues. The KDEL antibody recognizes the ER stress markers

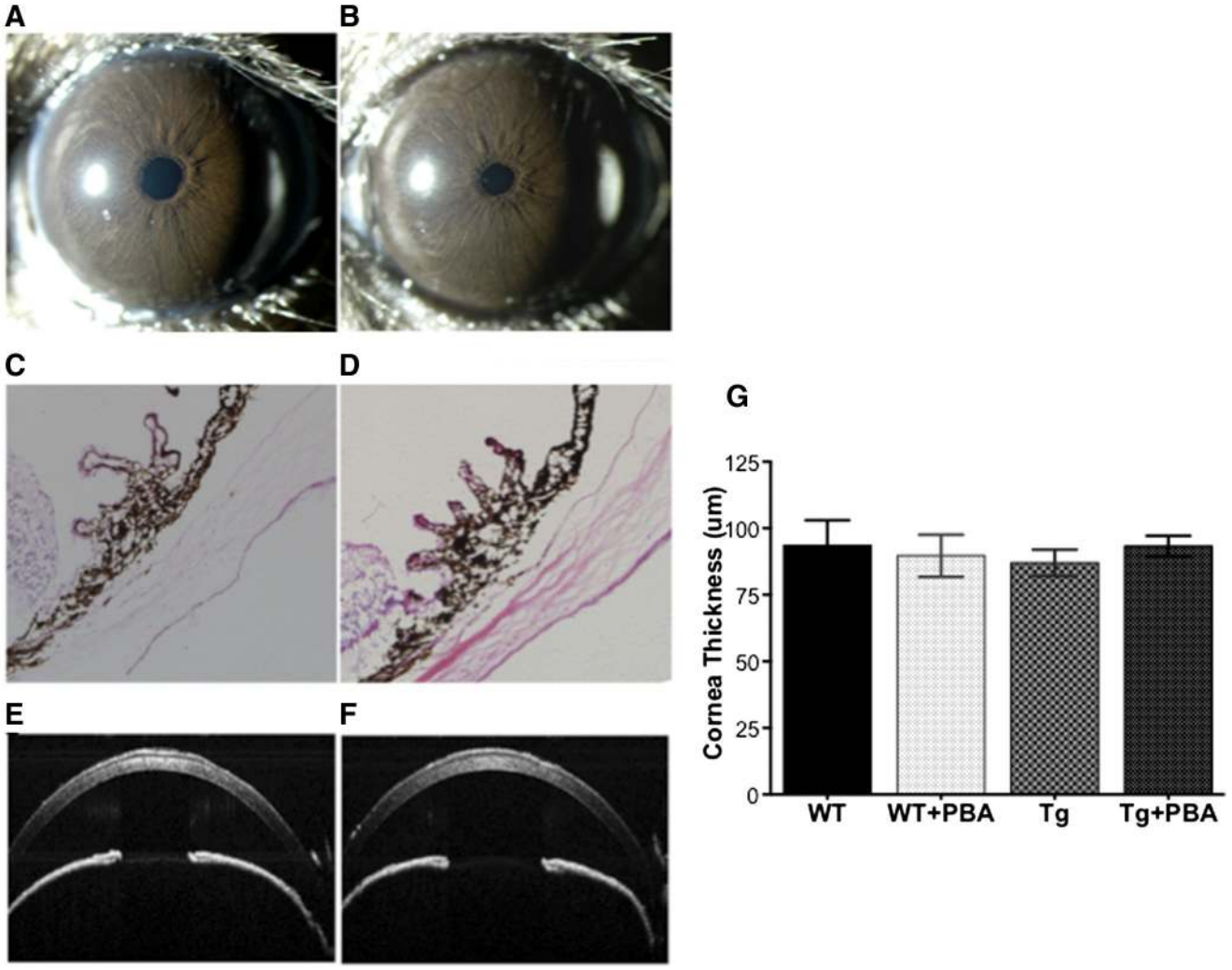

Figure 3. Topical ocular PBA does not cause abnormalities to anterior segment structures in WT or *Tg-MYOC*^{Y437H} mice. Slit lamp examination of PBA-treated WT (**A**) and *Tg-MYOC*^{Y437H} (**B**) mice revealed no abnormalities in the anterior segment structures (iris, pupil, lens, and cornea). H&E staining of PBA-treated WT (**C**) and *Tg-MYOC*^{Y437H} (D) mice. Optical coherence tomography (OCT) shows no abnormalities in cornea of PBA-treated *Tg-MYOC*^{Y437H} mice (**F**) compared with WT littermates (**E**). Cornea thickness measurements are shown in (**G**). $n = 4$ WT and *Tg-MYOC*^{Y437H} treated with PBA or vehicle. Data are the mean ± SEM.

GRP78 and GRP94. In vehicle-treated WT mice, myocilin was localized to the TM and the ciliary body (CB) (Fig. 5A). PBA treatment of WT mice did not alter myocilin and ER stress marker staining compared with that in vehicle-treated WT mice (Fig. 5B). In vehicle-treated *Tg-MYOC*^{Y437H} mice, myocilin accumulation and ER stress markers were increased in the TM compared with levels in WT mice (Fig. 5C). However, PBA treatment reduced total myocilin accumulation in the TM (Fig. 5D). In addition, levels of ER stress markers decreased in the TM of PBA-treated mice compared with vehicle-treated *Tg-MYOC*^{Y437H} littermates. These data indicate that topical PBA reduces myocilin accumulation and ER stress in the TM of *Tg-MYOC*^{Y437H} mice.

Topical PBA Reduces IOP Elevated by Tunicamycin Injections in WT Mice

We next examined whether topical PBA reduces IOP in another model of IOP elevation. Tunicamycin, an antibiotic that inhibits N-glycosylation of proteins in the ER, is a commonly used chemical that induces ER stress in vitro and in vivo.[35] We showed in a prior study that induction of ER stress by tunicamycin injections significantly elevates IOP in WT mice in a dose- and time-dependent manner.[19] In the present study, we tested whether topical PBA reduces IOP elevated by tunicamycin injections. We performed periocular subconjunctival injections of 0.2 μm/eye tunicamycin in both eyes of WT mice (Fig. 6). Consistent with our earlier findings, tunicamycin injections significantly elevated IOP 1 week after injection (~24 mm Hg in the tunicamycin-injected mice compared with ~18 mm Hg in the controls). PBA eye drops were applied to both eyes of tunicamycin-injected mice, and IOP was measured every week. Two weeks of topical PBA treatment reduced elevated IOP, and the reduction was significant after 3 weeks of treatment (~22 mm Hg in the PBA-treated mice compared with ~25 mm Hg in the untreated tunicamycin-injected mice). These data suggest that topical PBA reduces IOP elevated by specific induction of ER stress with injections.

Discussion

In the present study, we investigated whether topical ocular PBA treatment would rescue glaucoma phenotypes in *Tg-MYOC*^{Y437H} mice. Topical ocular PBA reduced elevated IOP in *Tg-MYOC*^{Y437H} mice. Topical ocular PBA did not alter IOP of

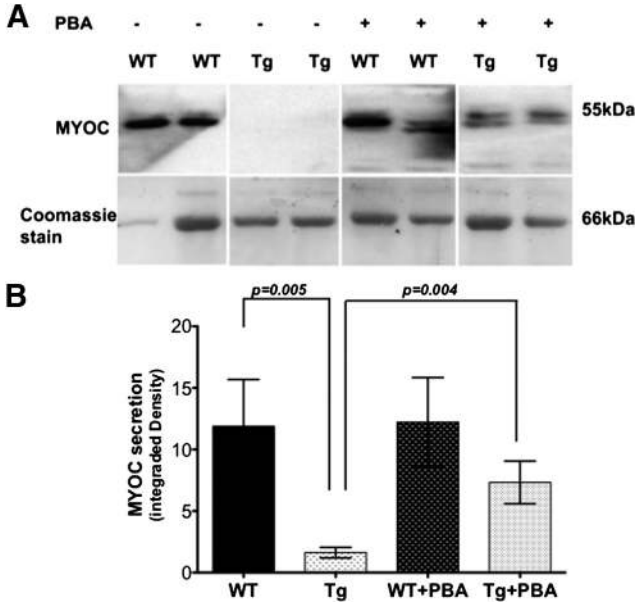

FIGURE 4. Topical ocular PBA reverses inhibition of myocilin secretion in the aqueous humor of *Tg-MYOC*^{Y437H} mice. (**A**) Western blot analysis of myocilin in the aqueous humor samples from 9-month-old PBA-treated WT and *Tg-MYOC*^{Y437H} mice is compared with vehicle-treated WT and *Tg-MYOC*^{Y437H} mice. Coomassie stain was performed to ensure equal loading of aqueous humor. (**B**) Densitometric analysis of myocilin secretion normalized to a loading control demonstrated a significant reduction in myocilin secretion in the *Tg-MYOC*^{Y437H} mice compared with that in WT littermates; however, PBA treatment of *Tg-MYOC*^{Y437H} mice significantly enhanced myocilin secretion in the aqueous humor. n = 3 WT, n =3 PBA-treated WT, n =5 *Tg-MYOC*^{Y437H}, and n =4 PBA-treated *Tg-MYOC*^{Y437H} mice.

WT mice and did not cause any abnormalities in anterior segment tissues. However, topical ocular PBA prevented RGC functional deficits in the *Tg-MYOC*^{Y437H} mice. Furthermore, it reduced glaucomatous phenotypes in the *Tg-MYOC*^{Y437H} mice, most likely by improving the secretion of myocilin in the aqueous humor and reducing the myocilin accumulation as well as ER stress in the TM. Topical PBA was also able to reduce IOP elevated by induction of ER stress using tunicamycin injections. These data identify topical ocular PBA as a potential treatment for POAG patients with myocilin mutations.

It is thought that myocilin-associated glaucoma belongs to the family of protein conformational disorders. Consistent with other misfolded proteins in other protein conformational disorders, mutant myocilin is misfolded, accumulates in the ER, induces ER stress, and activates unfolded protein response (UPR).[13,14,16,17,19] Chronic ER stress induced by mutant myocilin is associated with the loss of TM cells and elevation of IOP.[19] Recently, chemical chaperones that stabilize protein folding, have become attractive candidates for repairing the trafficking-defective proteins involved in protein conformational disorders.[36,37] Several attempts have been made to correct misfolded myocilin in vitro. For example, culturing TM cells expressing mutant myocilin at lower temperature (30°C), which is known to facilitate correct protein folding, enhanced the secretion of myocilin in the medium and improved cell viability.[13] Burns et al.,[38] demonstrated that various glaucoma-causing mutants of myocilin are thermally unstable and can be corrected by the use of chemical chaperones. Other studies have used several chemical chaperones including sodium 4-phenylbutyrate (PBA) to correct mutant myocilin protein folding.[39]

Consistent with these studies, our prior studies have shown that systemic treatment with PBA prevents elevation of IOP in *Tg-MYOC*^{Y437H} mice. In the present study, we sought to determine whether a topical form of PBA would reduce elevated IOP in *Tg-MYOC*^{Y437H} mice. The systemic use of PBA is FDA approved for urea cycle disorders, and PBA is currently being tested in clinical trials for various diseases.[23,40,41] The basic pharmacology of PBA is well established for its oral use in patients with urea cycle disorders.[41] It is converted/oxidized in vivo into phenylacetate by β-oxidation, which is then eliminated in the urine.[42] Phenyl butyrate is relatively stable and has high tissue penetration.[41] In this project, a significant amount of PBA was found in the aqueous (10 μM) and vitreous (5 μM) humors of WT mice treated with topical PBA. These data suggest that topical PBA administration results in efficient corneal penetration and therapeutic drug doses in the anterior and posterior segments. Considering that previous human clinical studies have shown that PBA is relatively safe and well tolerated in a dose of up to 15 g/d after chronic systemic administration, it is likely that a similar (or better) safety profile will be present with topical PBA administration in dramatically smaller concentrations. Future studies will be conducted to test the safety and stability of a topical form of PBA.

There are several new insights that were gained from the present topical PBA study that enhance the findings in our systemic PBA study.[19] First, in the systemic PBA treatment study, we examined whether PBA prevents glaucoma (IOP and PERG) in *Tg-MYOC*^{Y437H} mice when given to mice before they develop elevated IOP (2 months of age).[19] In the present work, we examined whether the topical form of PBA would reduce elevated IOP in older *Tg-MYOC*^{Y437H} mice. The effects of PBA were examined in 4- and 9-month-old *Tg-MYOC*^{Y437H} mice, which had developed ocular hypertension of 1 month's and 5 months' duration, respectively (Fig. 1). An important result of this study was that topical PBA treatment of 9-month-old *Tg-MYOC*^{Y437H} mice completely normalized IOP to the levels in WT mice. Second, topical PBA reduced elevated IOP for a sustained period (5 months) in *Tg-MYOC*^{Y437H} mice, compared with the systemic PBA study, in which we evaluated the effect of PBA for 4 weeks. Third, we also wanted to know whether topical PBA prevents loss of RGCs function in older *Tg-MYOC*^{Y437H} mice (Fig. 2). Remarkably, after 5 months of treatment, PBA treatment of 9-month-old *Tg-MYOC*^{Y437H} mice completely normalized PERG function similar to that in WT mice, compared with a 50% reduction in untreated *Tg-MYOC*^{Y437H} mice. Fourth, topical PBA treatment for 5 months did not cause any structural abnormalities in the anterior chamber of the eye (Fig. 3), which is an important safety result of this study that was not addressed in prior work. Fifth, we tested whether topical PBA reduces IOP elevated by induction of ER stress with tunicamycin injection in WT mice. In our earlier study, tunicamycin elevated IOP significantly in a dose- and time-dependent manner by inducing ER stress in WT mice.[19] Consistent with that finding, in the present work, tunicamycin elevated IOP in WT mice after 1 week of injection. An important result of these experiments, which is new and was not addressed in our prior publication, is that topical PBA eye drops significantly reduced the IOP elevation caused by tunicamycin.

It is interesting to note that PERG amplitudes in PBA-treated 9-month-old *Tg-MYOC*^{Y437H} mice were similar to the normal PERG amplitudes in WT mice, compared with the 50% reduced PERG amplitudes in vehicle-treated *Tg-MYOC*^{Y437H} mice. It is conceivable that topical PBA relieves IOP-dependent stress on RGCs and reduces the ER stress associated with RGC death, thus improving the overall function of the remaining RGCs in *Tg-MYOC*^{Y437H} mice. Thus, in addition to lowering elevated IOP, PBA may also prevent functional loss of RGCs in *Tg-MYOC*^{Y437H} mice by reducing

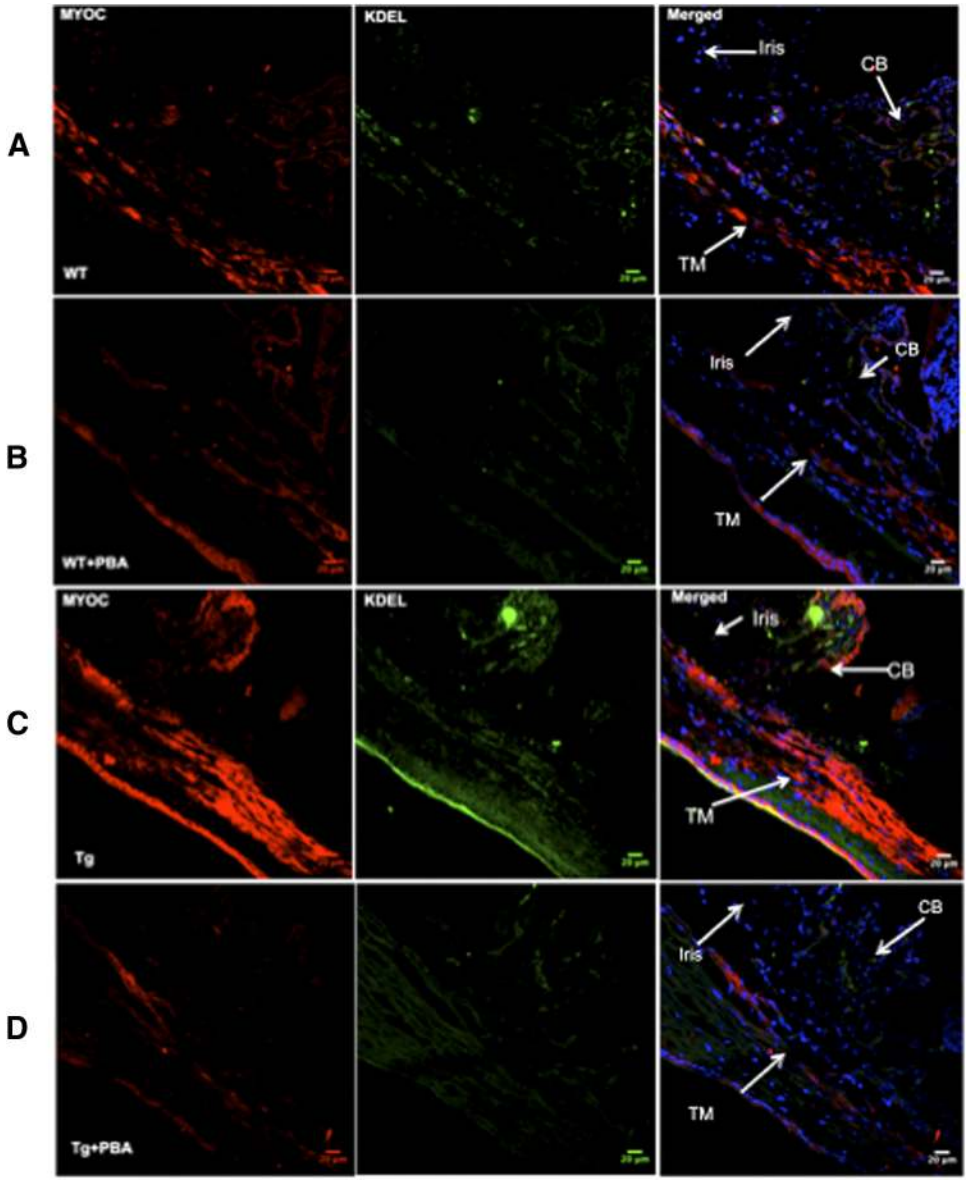

FIGURE 5. Topical ocular PBA reduces intracellular accumulation of myocilin and ER stress in the TM of *Tg-MYOC*^{Y437H} mice. Myocilin levels and ER stress markers in the iridocorneal angle of PBA-treated WT (**B**) and *Tg-MYOC*^{Y437H} (**D**) mice were compared with those in vehicle-treated WT (**A**) and *Tg-MYOC*^{Y437H} mice (**C**) by immunostaining and confocal imaging. *Arrows*: TM, CB, and iris. Vehicle-treated *Tg-MYOC*^{Y437H} mice demonstrated increased myocilin staining and ER stress markers (the KDEL antibody recognizes GRP78 and GRP94) in the TM and CB compared with that in the WT mice. Of note, PBA-treated *Tg-MYOC*^{Y437H} mice showed reduction of myocilin staining in the TM and CB compared with vehicle-treated *Tg-MYOC*^{Y437H} mice. $n = 3$ vehicle-treated WT, $n = 5$ vehicle-treated *Tg-MYOC*^{Y437H}, $n = 3$ PBA-treated WT, and $n = 4$ PBA-treated *Tg-MYOC*^{Y437H} mice. Scale bar, 20 μm.

ER stress on RGCs and the TM. Studies have shown that ER stress may play an important role in RGC death.[43–45] The recent study by Carbone et al.,[45] suggests that genes involved in the unfolded protein response pathway may harbor alleles that are associated with an increased risk for POAG. In future studies, we will seek to understand the role of ER stress in RGC dysfunction and possible therapeutic intervention in the ER stress pathway by PBA to prevent RGC dysfunction and cell death.

It is not entirely clear how PBA reduces elevated IOP in *Tg-MYOC*^{Y437H} mice. It is possible that PBA directly interacts with mutant myocilin and induces conformational changes, allowing efficient processing and secretion in the aqueous humor. Mutant myocilin is thermolabile, and its abnormal conformation can be corrected by culturing TM cells that express mutant myocilin at 30°C.[13] Thus, it is plausible that PBA thermally stabilizes mutant myocilin, allowing it to fold protein properly. Alternatively, PBA can transcriptionally regulate expression of certain genes that aid in the folding of mutant myocilin. PBA is a histone deacetylase inhibitor and has been shown to be a transcriptional regulator.[30,31,46] PBA can alter the expression of chaperones that enhance the processing of the mutant proteins. For example, upregulation of chaperone proteins, including heat shock proteins 90 and 70, was observed in microarray analyses of the transcript levels in PBA-treated bronchial epithelial cells.[47] However, in our earlier study, we did not observe changes in ER chaperones with PBA treatment.[19] Yam et al.,[39] demonstrated that secretion of mutant MYOC occurs as early as 30 minutes after PBA treatment, suggesting that PBA's effects may not be directed through transcriptional regulation of genes. These data suggest possible effects of PBA on restoring correct folding of mutant myocilin. Thus, we hypothesized that the chemical chaperone PBA promotes proper folding of mutant myocilin, thus allowing mutant myocilin to be secreted into the aqueous humor and reducing its intracellular accumulation in the TM, an effect that reduces the ER stress associated with the misfolding of myocilin. These results suggest that PBA eye drops are an effective strategy for clinical management of disease in POAG patients with myocilin mutations. Future studies are being directed toward assessing the effects of topical PBA in humans who have both myocilin-associated and other forms of POAG.

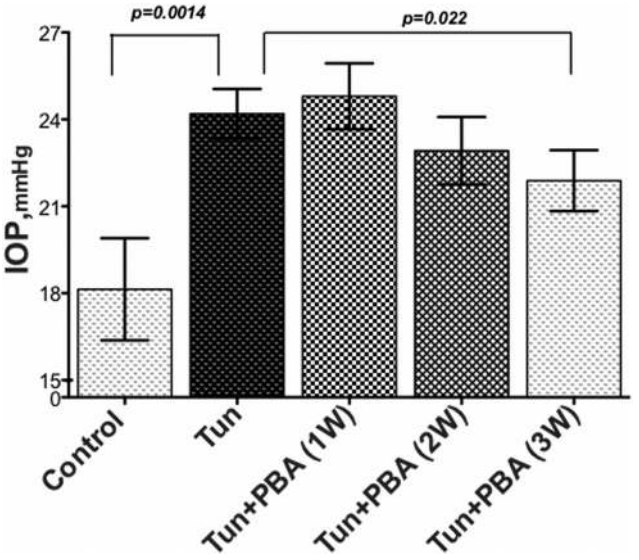

FIGURE 6. Topical PBA reduces IOP that has been elevated by tunicamycin in WT mice. WT mice ($n = 14$) injected with 0.2 μg/eye of tunicamycin had elevated IOP 1 week after injection, compared with IOP in WT mice with control vehicle injection ($n = 6$). Tunicamycin-injected mice that showed elevated IOP were treated with topical PBA eye drops (1%) twice daily afterward, and IOP was measured every week. A 3-week treatment with PBA significantly reduced the elevated IOP in the WT mice. Data are the mean ± SEM.

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