

Effect of ATP-sensitive Potassium Channel Openers on Intraocular Pressure in Ocular Hypertensive Animal Models

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PURPOSE. To evaluate the effect of ATP-sensitive potassium channel openers cromakalim prodrug 1 (CKLP1) and diazoxide on IOP in three independent mouse models of ocular hypertension.

METHODS. Baseline IOP was measured in TGF β 2 overexpression, steroid-induced, and iris dispersion (DBA/2J) ocular hypertension mouse models, followed by once daily eyedrop administration with CKLP1 (5 mM) or diazoxide (5 mM). The IOP was measured in conscious animals with a handheld rebound tonometer. Aqueous humor dynamics were assessed by a constant perfusion method. Effect of treatment on ocular tissues was evaluated by transmission electron microscopy.

RESULTS. CKLP1 decreased the IOP by 20% in TGF β 2 overexpressing mice ($n = 6$; $P < 0.0001$), 24% in steroid-induced ocular hypertensive mice ($n = 8$; $P < 0.0001$), and 43% in DBA/2J mice ($n = 15$; $P < 0.0001$). Diazoxide decreased the IOP by 32% in mice with steroid-induced ocular hypertension ($n = 13$; $P < 0.0001$) and by 41% in DBA/2J mice ($n = 4$; $P = 0.005$). An analysis of the aqueous humor dynamics revealed that CKLP1 decreased the episcleral venous pressure by 29% in TGF β 2 overexpressing mice ($n = 13$; $P < 0.0001$) and by 72% in DBA/2J mice ($n = 4$ control, 3 treated; $P = 0.0002$). Diazoxide lowered episcleral venous pressure by 35% in steroid-induced ocular hypertensive mice ($n = 3$; $P = 0.03$). Tissue histology and cell morphology appeared normal when compared with controls. Accumulation of extracellular matrix was reduced in CKLP1- and diazoxide-treated eyes in the steroid-induced ocular hypertension model.

CONCLUSIONS. ATP-sensitive potassium channel openers CKLP1 and diazoxide effectively decreased the IOP in ocular hypertensive animal models by decreasing the episcleral venous pressure, supporting a potential therapeutic application of these agents in ocular hypertension and glaucoma.

Keywords: ocular hypertension, glaucoma, mouse models, K_{ATP} channels, diazoxide, CKLP1

Glaucoma is a progressive neurodegenerative disorder of the eye and is the primary cause of irreversible blindness worldwide. With an aging population and expanding demographics, the prevalence of the disease is expected to increase, with more than 140 million people affected by 2040.^{1,2} Currently, there is no cure for glaucoma, but treatments that decrease the IOP have been shown to slow down the disease progression.^{3,4}

The most common first-line treatment for glaucoma is the use of eyedrops containing ocular hypotensive medications. Unfortunately, all classes of ocular hypotensive agents are associated with mild to severe side effects.^{3,4} For example, topical medications like prostaglandin analogs (e.g., latanoprost), beta blockers (e.g., timolol), and carbonic anhydrase inhibitors (e.g., brinzolamide) can cause signif-

icant conjunctival hyperemia, iris pigmentation, hypertrichosis, and even systemic cardiac and respiratory side effects in susceptible individuals.²⁻⁵ Furthermore, topical medications may require multiple daily dosing, making it difficult for elderly patients to accurately instill the drops or to remember when to apply the drops. Both the side effect profiles and the inability to appropriately maintain the prescribed medication results in up to 50% patient noncompliance.^{6,7} Additionally, a high proportion of patients with glaucoma can become nonresponsive to frontline therapies requiring dosing with multiple drugs, further adding to treatment challenges. As a result, the development of therapeutics with little or no side effects can address a significant need in patients with glaucoma.

ATP-sensitive potassium (K_{ATP}) channel openers are hetero-octameric transmembrane proteins, that connect the metabolic and energetic states of cells by virtue of their sensitivity to micromolar concentrations of intracellular ATP.⁸ Our laboratory has shown that several pharmacologic openers of K_{ATP} channels (diazoxide, cromakalim, nicorandil, etc.) lower the IOP in ex vivo human eyes and in several normotensive animal models including mice, rats, rabbits, dogs, and nonhuman primates.^{9–18} Because commercially available K_{ATP} channel openers have limited aqueous solubility, we developed a novel, water-soluble, direct phosphate linked prodrug (cromakalim prodrug 1 [CKLP1]) based on the K_{ATP} channel opener levcromakalim. CKLP1 exhibits similar ocular hypotensive properties as levcromakalim and was found to decrease the IOP by directly lowering episcleral venous pressure with no observable side effects in normotensive mice.¹⁵ Owing to its unique site of action, K_{ATP} channel openers were shown to work in combination with existing glaucoma medications to lower IOP more than each treatment alone.¹⁵ Thus, K_{ATP} channel openers may be a novel therapeutic option for patients with glaucoma.

The effects of K_{ATP} channel openers on IOP in ocular hypertensive eyes are unknown. Based on their effects on normotensive animals, we hypothesized that K_{ATP} channel openers would similarly lower the IOP in animal models of ocular hypertension by decreasing the episcleral venous pressure. To test this, three separate animal models of ocular hypertension ($TGF\beta 2$ overexpression, steroid-induced, and iris dispersion [DBA/2J] mice) were treated with either CKLP1 or diazoxide followed by evaluation of the IOP, various parameters of aqueous humor dynamics, and tolerability.

METHODS

Reagents

CKLP1 (originally described as [3S,4R]-2) was synthesized as previously described and formulated by dissolving in PBS.¹² Diazoxide was purchased from MilliporeSigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO) to make a 100-mM stock solution. Stock solutions were diluted 20-fold in 10% Cremophor EL (MilliporeSigma) prepared in PBS, for a final working concentration of 5 mM.

A formulation of dexamethasone acetate as a suspension was prepared as previously described.¹⁹ All reagents required for suspension formulation were purchased from either MilliporeSigma or Spectrum Chemicals (New Brunswick, NJ). Briefly, sodium chloride, creatinine, EDTA, sodium bisulfite, polysorbate 80, benzyl alcohol, and carboxymethylcellulose were added sequentially, pH adjusted to 7.0 and brought to final volume with water (Table 1). Anhydrous, micronized dexamethasone powder

(Spectrum Chemicals) was added to the suspension formulation to a final concentration of 10 mg/mL. Stainless steel beads (5 mm) were added to the dexamethasone suspension formulation and vortexed for 5 minutes at room temperature. The dexamethasone suspension formulation was placed at 4°C, protected from light, and mixed overnight on a rotator. Following overnight rotation, the dexamethasone suspension formulation was isolated, aliquoted, and stored at 4°C for up to two weeks. Suspension formulation for vehicle was stored protected from light at 4°C for up to 90 days.¹⁹

Mouse Models of Ocular Hypertension

All animal experiments were preapproved by the respective Institutional Animal Care and Use Committees at Mayo Clinic, Rochester, Minnesota, or the North Texas Eye Research Institute, University of North Texas Health Science Center, Fort Worth, Texas. All animal experiments adhered strictly to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were housed with no more than 5 five total mice per cage and maintained in the animal facilities with daily 12-hour light/12-hour dark cycles (lights on at 6:00 AM) and unlimited access to standard rodent pellets and water.

TGF $\beta 2$ Ocular Hypertension Model. TGF $\beta 2$ cDNA (NM_003238) was obtained from Origene (Rockville, MD) in pCMV6-XL5 vector. Nucleotides 1086 and 1991 were changed from G to C to convert amino acids 226 and 228 of the human TGF $\beta 2$ protein from cysteines to serines. The cDNA was subcloned into pacAd5.CMV.KN.pA shuttle vector (Gene Transfer Vector Core, University of Iowa) using EcoRI/Xba1 restriction for making Ad.hTGF $\beta 2^{226/228}$ as described previously.²⁰ Before injection, C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with ketamine/xylazine/acepromazine (73/7.8/1.8 mg/kg), pupils were dilated with one to two drops of cyclopentolate (Mydracil; Alcon, Fort Worth, TX), and eyes were topically anesthetized with two drops of 0.5% tropicamide (Alcaine; Alcon). A 2- μ L suspension of Ad.hTGF $\beta 2^{226/228}$ (5×10^7 pfu) was injected intravitreally with a Hamilton 33G needle containing a 10° bevel and a glass microsyringe (Hamilton, Reno, NV).

Steroid-induced Model of Ocular Hypertension. Six-month-old C57BL/6J mice (Jackson Laboratory) were anesthetized with ketamine, xylazine, and acepromazine (90, 10, and 1 mg/kg body weight). A 20- μ L volume of dexamethasone acetate (10 mg/mL) or suspension formulation (vehicle) was loaded into a 32G Hamilton glass microsyringe. For injection, the beveled end of the needle (facing up) was inserted into the conjunctival fornix and the syringe volume expelled slowly over 15 seconds. For each animal, one eye received dexamethasone acetate and the fellow eye received vehicle. Injections were performed once a week until the end of the experiment.

DBA/2J Mice. DBA/2J mice were obtained from Jackson Laboratory at approximately 4 months of age. Mice were housed in the Mayo Clinic animal facilities until they were 8 to 10 months old to ensure the development of ocular hypertension.²¹

IOP Measurements and Treatments

IOP was measured using a handheld rebound tonometer (Icare Tonolab, Colonial Medical Supply, Franconia, NH) using previously described methods.^{10,15} Briefly, animals

TABLE 1. Reagents for Dexamethasone Acetate Formulation

Reagent	Volume/100 mL	Source
Sodium chloride	0.667 g	Millipore Sigma
Creatinine	0.5 g	Spectrum Chemical
Edetate disodium dihydrate	0.05 g	Millipore Sigma
Carboxymethylcellulose sodium	0.5 g	Millipore Sigma
Polysorbate 80	0.1 mL	Millipore Sigma
Benzyl alcohol	0.9 mL	Millipore Sigma
Sodium bisulfite	0.1 g	Spectrum Chemical
Sodium hydroxide 1%	To adjust pH to 7	Millipore Sigma
Dexamethasone acetate	10 mg/mL	Spectrum Chemical

were acclimatized to the handling through sham IOP measurements for up to 5 days. After acclimation, daily IOP measurements were taken at three separate time points corresponding with 1, 4, and 23 hours after treatment (approximately 10:00 AM, 2:00 PM, and the following morning at 9:00 AM), averaged, and recorded as the daily IOP. For all experiments, baseline values were determined by measuring and averaging IOPs for 3 consecutive days before treatment. For TGF β 2 overexpression and DBA/2J experiments, the IOPs were measured three times daily corresponding with 1, 4, and 23 hours after treatment until the termination of the experiment. For the steroid-induced ocular hypertension model, after the first injection, IOP was measured once weekly, 2 days after each injection (three timepoints corresponding with 1, 4, and 23 hours after treatment) until the end of the experiment. CKLP1 or diazoxide was instilled topically in a 5- μ L bolus to one eye of each animal while the contralateral eye received vehicle (10% Cremophor EL in PBS for diazoxide; PBS for CKLP1). In mice injected with dexamethasone acetate in one eye and the vehicle (suspension formulation) in the contralateral eye, one group was treated with either diazoxide or CKLP1 in both eyes, whereas the other group received the respective vehicle (DMSO in 10% Cremophor for diazoxide; PBS for CKLP1). All drugs were added once daily at a final working concentration of 5 mM^{10,11,13} for the entire duration of the treatment period.

Evaluation of Aqueous Humor Dynamics In Vivo by Constant Flow Infusion

All parameters related to aqueous humor dynamics (outflow facility, aqueous flow rate, episcleral venous pressure, uveoscleral outflow) were determined by constant flow infusion as described previously.^{15,22,23} Briefly, mice were anesthetized with ketamine/xylazine (100/10 mg/kg body weight). A 32G needle connected to a calibrated variable height manometer and a BLPR-2 pressure transducer (World Precision Instruments, Sarasota, FL) was inserted into the anterior chamber. The transducer was attached to a SP101i microdialysis infusion pump (World Precision Instruments), a TBM4M Bridge Amplifier, and a Lab-Trax analog to digital converter (World Precision Instruments) via a three-way valve. Once pressure was stabilized, data were recorded and analyzed using the LabScribe4 software (World Precision Instruments). Among the various aqueous humor dynamic parameters, the outflow facility and episcleral venous pressures were directly measured, and the uveoscleral and aqueous flow rate were calculated based on a modified Goldmann equation, as previously described.^{22,23}

Histology

After completion of the experiments, animals were euthanized, and eyes were enucleated and fixed in 10% neutral buffered formalin (Thermo Fisher Scientific, Pittsburgh, PA). Eyes were removed from 10% buffered formalin and post-fixed in 2% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA). Eyes were dehydrated in ascending alcohol concentrations (70%–100%) and cleared with acetone (Fisher Chemical, Fair Lawn, NJ). Whole eyes were embedded in epoxy resin and thin sectioned with an ultramicrotome (Leica Microsystems, Buffalo Grove, IL). Sections were stained with 2% uranyl acetate (Electron Microscopy Sciences) and lead citrate (Mager Scientific, Dexter, MI) for

evaluation of cell and tissue morphology using a JEOL 1400 transmission electron microscope (JEOL USA, Peabody, MA).

Statistics

All values are expressed as mean \pm standard deviation. Means from treated and control eyes within the same population were compared using Student's two-tailed paired *t*-test and independent two-tailed *t*-tests were used for comparing means from separate cohorts. Differences were considered significant when $P < 0.05$. Statistical analyses were performed with Microsoft Excel and its data analysis add-on feature.

RESULTS

Effect of CKLP1 on IOP in TGF β 2 Overexpressing Ocular Hypertensive Mouse Model

To evaluate the effect of CKLP1 during high IOP, one eye of C57BL/6J mice was injected intravitreally with adenoviral vector encoding the bioactive form of TGF β 2.²⁰ At 5 days after the injection, when elevated IOP reached a plateau (OS 21.2 \pm 0.62 mm Hg and OD 20.8 \pm 0.2 mm Hg, compared with control baseline IOPs of OS 12.7 \pm 0.4 mm Hg and OD 12.6 \pm 0.5 mm Hg; $n = 6$, in a separate group of mice treated with PBS only), the left eye of each mouse was started on a once daily ocular topical regimen of 5 mM CKLP1 for 5 consecutive days and the contralateral eye received vehicle. The IOP progressively decreased to 16.4 \pm 0.4 mm Hg in the TGF β 2 overexpressing eyes reaching a 23% decrease (compared with the average baseline IOP of the TGF β 2-treated eye before start of CKLP1 treatment) on day 5 of treatment (Fig. 1). When the IOP was averaged over the entire 5 days of treatment, the IOP was lowered in CKLP1-treated TGF β 2-overexpressing eyes by 20% (vehicle control, 21.4 \pm 0.7 mm Hg; CKLP1 treated, 17.8 \pm 2.0 mm Hg; $n = 6$; $P < 0.0001$). After the cessation of treatment, the IOP returned to levels similar to the vehicle-treated eye within 3 days (vehicle control, 21.4 \pm 0.7 mm Hg; CKLP1 treated, 20.4 \pm 1.3 mm Hg; $n = 6$; $P = 0.1$) (Fig. 1).

Effect of CKLP1 and Diazoxide on IOP in Steroid-induced Ocular Hypertensive Mice

To further validate the hypotensive effects of K_{ATP} channel openers during elevated IOP, we used the steroid-induced ocular hypertensive mouse model, where the IOP is increased through weekly dexamethasone acetate injections into the conjunctival fornix of one eye, while the contralateral eye received vehicle. After IOP elevation in the dexamethasone acetate injected eye, both eyes of each animal were treated with topical drops of 5 mM CKLP1 dissolved in PBS (once daily along with continued weekly injections of dexamethasone). After three weekly injections of dexamethasone acetate, IOP increased from an average baseline value of 16.5 \pm 0.2 mm Hg to 21.3 \pm 1.4 mm Hg ($n = 8$; $P < 0.0001$), while no change was seen in the vehicle treated contralateral eye (baseline, 16.4 \pm 0.1 mm Hg; after vehicle treatment, 16.4 \pm 0.1 mm Hg; $n = 8$; $P = 0.7$) (Fig. 2A). After subsequent once daily treatment with CKLP1 (along with continued weekly injections of dexamethasone acetate), the average IOP in eyes treated with dexamethasone acetate was decreased to 16.2 \pm 0.3 mm Hg, corresponding with a 24% decrease ($P < 0.0001$, $n = 8$). In

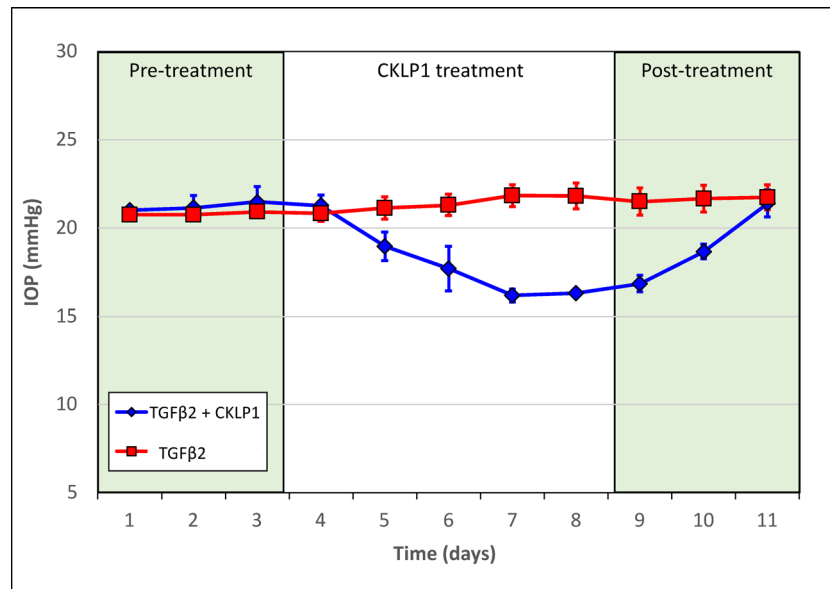


FIGURE 1. Effect of CKLP1 on TGF β 2 ocular hypertensive mice. Once daily treatment with 5 mM CKLP1 significantly lowered IOP in TGF β 2 overexpressing mice by 20% ($n = 6$; $P < 0.0001$) when compared with the vehicle treated contralateral eye. IOP returned to baseline within 72 hours of cessation of treatment ($n = 6$; $P = 0.1$). Pretreatment refers to IOP measured before treating with CKLP1, but after injection of Ad.TGF β 2^{226/228} or control in the mice eyes.

conformation with our previous reports of IOP reduction in normotensive eyes,^{12,14–18} CKLP1 also decreased the IOP in the contralateral eyes that received vehicle (suspension formulation) by 23% ($P < 0.0001$) (Fig. 2A, solid lines). For controls, a separate group of mice ($n = 5$) was similarly injected with dexamethasone acetate in one eye and the suspension formulation in the contralateral eye. Both eyes in this group were treated with PBS (vehicle for CKLP1). In these mice, dexamethasone acetate increased the IOP from 16.0 ± 0.1 mm Hg to 20.4 ± 1.1 mm Hg ($P = 0.0008$), whereas no change was seen following treatment with PBS (22.8 ± 2.7 mm Hg; $P = 0.12$) (Fig. 2A, dotted lines).

After termination of CKLP1 treatment, ultrastructural evaluation of the trabecular meshwork showed evidence of extracellular matrix (ECM) deposition in the dexamethasone acetate treated eyes (Fig. 2B) in the trabecular meshwork just below Schlemm's canal (i.e., juxtacanalicular region), consistent with a previous report.¹⁹ In contrast, little or no excess ECM was observed in the trabecular meshwork from eyes that were injected with dexamethasone acetate and subsequently treated with CKLP1 (Fig. 2B). No other changes were observed in eyes from the vehicle-treated groups. Overall, the trabecular meshwork showed intact beams and healthy cells, with an uninterrupted inner and outer wall of Schlemm's canal (Fig. 2B).

To determine if the IOP-lowering effect of CKLP1 is also true for other K_{ATP} channel openers, we treated a separate cohort of dexamethasone acetate-induced ocular hypertensive mice with 5 mM diazoxide. In this group, injection with dexamethasone acetate increased the IOP from 16.8 ± 0.7 mm Hg at baseline to 21.9 ± 1.5 mm Hg ($n = 13$; $P < 0.0001$) (Fig. 3A). Using a treatment strategy with diazoxide similar to that described above with CKLP1, IOP in the steroid-induced eye decreased to 14.9 ± 0.7 mm Hg ($P < 0.0001$), corresponding with a 32% decrease in the IOP. In five of the mice, diazoxide treatment was stopped but dexamethasone acetate injection and IOP measurements

continued for one additional week. IOP in these animals increased to 21.9 ± 0.7 , similar to prediazoxide treatment levels ($P = 0.99$), suggesting reversibility and specificity of diazoxide as an ocular hypotensive agent. In the eyes that were injected with the suspension formula only, no change in the IOP was noted when compared with baseline (16.3 ± 0.1 mm Hg vs. 16.2 ± 0.3 mm Hg, $n = 13$; $P = 0.2$). However, after treatment with diazoxide, the IOP was decreased by 22% ($P < 0.0001$) (Fig. 3, solid lines). In a separate group, when the dexamethasone acetate injected animals were treated with the vehicle for diazoxide (DMSO in 10% Cremophor), there was a slight increase in the IOP (dexamethasone acetate, 20.9 ± 1.0 mm Hg; vehicle, 22.3 ± 0.8 mm Hg; $n = 9$; $P = 0.0001$). When the ultrastructure was analyzed after the termination of treatment, eyes that received dexamethasone acetate followed by diazoxide treatment looked normal, with healthy looking trabecular meshwork cells on beams and no interruptions in Schlemm's canal inner and outer wall endothelial cell layer (Fig. 3B). In eyes that were injected with dexamethasone acetate but were not treated with diazoxide, sporadic ECM buildup could be seen in the juxtacanalicular region of the trabecular meshwork just below Schlemm's canal. No histological changes were observed in the trabecular meshworks from the vehicle-treated mice.

Effect of CKLP1 and Diazoxide on Elevated IOP in DBA/2J Mice

Because the TGF β 2 overexpression and steroid-induced animal models represent acute ocular hypertension models, we wanted to evaluate a model with chronic elevated IOP. We selected DBA/2J mice, because these mice develop an elevated IOP around 6 months of age owing to the resistance of aqueous humor removal from the anterior chamber. The accumulation of dispersed iris pigment in this model is

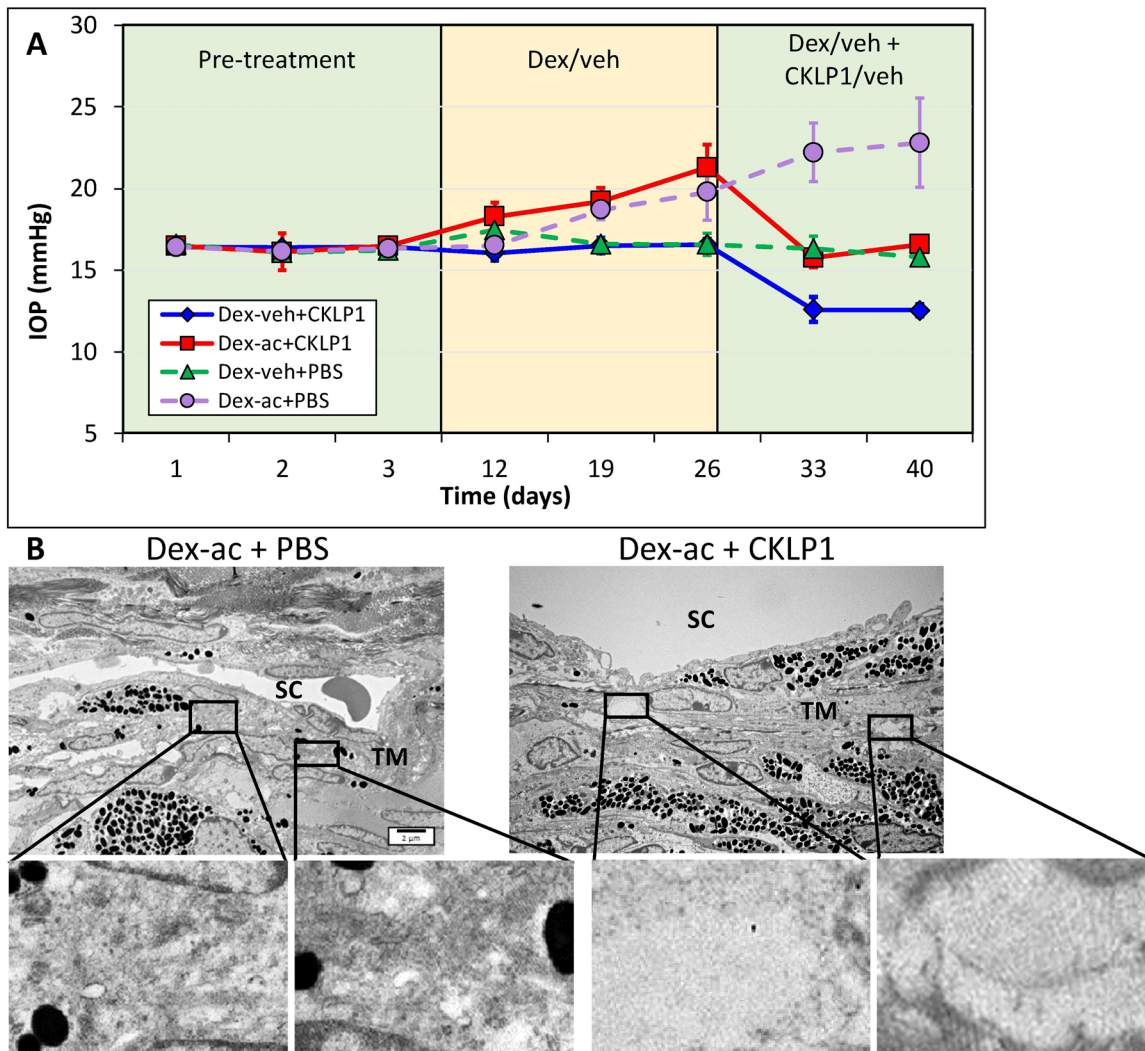


FIGURE 2. Effect of CKLP1 treatment on steroid-induced ocular hypertensive mice. **(A)** Once weekly injection of dexamethasone acetate (dex-ac) in the conjunctival fornix of C57BL/6J mice increased the IOP from 16.5 ± 0.2 to 21.3 ± 1.4 ($n = 8$; $P < 0.0001$). After treatment with once daily CKLP1 (5 mM), the IOP was decreased by 24% ($P < 0.0001$, $n = 8$) (solid red line). The contralateral eye, injected with only vehicle (veh) (suspension formulation without dexamethasone acetate) did not show any increase in IOP, but after CKLP1 treatment showed a 23% reduction in the IOP ($P < 0.0001$) (solid dark blue line). When eyes injected with dexamethasone acetate or vehicle were treated with PBS (vehicle for CKLP1), no changes in IOP were observed compared with baseline (dotted lines on graph). **(B)** Representative transmission electron microscopy images of the conventional outflow pathway of dexamethasone acetate/vehicle (dex-ac + PBS) and dexamethasone acetate/CKLP1 (dex-ac + CKLP1) treated eyes. In eyes treated with dexamethasone acetate and vehicle, excess ECM was found in the trabecular meshwork (TM) consistent with a previous report in this model (insets).¹⁹ However, normal ECM was observed in the trabecular meshwork of eyes treated with dexamethasone acetate and CKLP1 (insets). The remaining cell and tissue structures in the conventional outflow pathway appeared normal in both CKLP1 and vehicle treated eyes. SC, Schlemm's canal.

caused by mutations in the tyrosine-related protein (*Tyrp1^b*) and transmembrane glycoprotein (*Gpnmbr^{R150X}*) genes.^{24–28} We selected 8 to 10-month-old mice with an average baseline IOP of 21.4 ± 0.7 mm Hg OD and 21.3 ± 0.6 mm Hg OS. CKLP1 was added to OS once daily for 5 days, and the OD received vehicle (PBS). At the end of treatment, the IOP in the treated eye was decreased to 12.2 ± 0.7 mm Hg, equivalent to a 43% decrease ($P < 0.0001$, $n = 15$), whereas no change was observed in the contralateral vehicle-treated eye (baseline, 21.4 ± 0.7 mm Hg; vehicle treatment, 21.4 ± 1.2 ; $P = 0.9$, $n = 15$) (Fig. 4A). Within 72 hours after termination of CKLP1 treatment, IOP in the treated eye returned to levels similar to the vehicle treated eyes (OD [vehicle treated], 21.5 ± 1.3 mm Hg; OS [previously treated with CKLP1], 21.3 ± 0.9 ; $P = 0.8$; $n = 5$), indi-

cating the specific and reversible nature of the drug effects. Histological assessment of the trabecular outflow ultrastructure revealed pigment dispersion-related findings, including aberrant ECM formation as well as pigment ingesting macrophage like cells. However, no effects of CKLP1 treatment were apparent from the histology (Fig. 4B).

A separate set of DBA/2J mice ($n = 4$) was treated similar to the regimen described above, but with diazoxide instead of CKLP1. In this set of animals, diazoxide lowered IOP from a baseline value of 19.8 ± 1.8 mm Hg to 11.7 ± 1.5 mm Hg, corresponding with a 41% decrease ($P = 0.0002$, $n = 4$). No significant changes were noted in the IOP of vehicle-treated eyes when compared with baseline ($P = 0.6$, $n = 4$) (Fig. 5A). An ultrastructure analysis showed evidence of pigment dispersion, which is the hall-

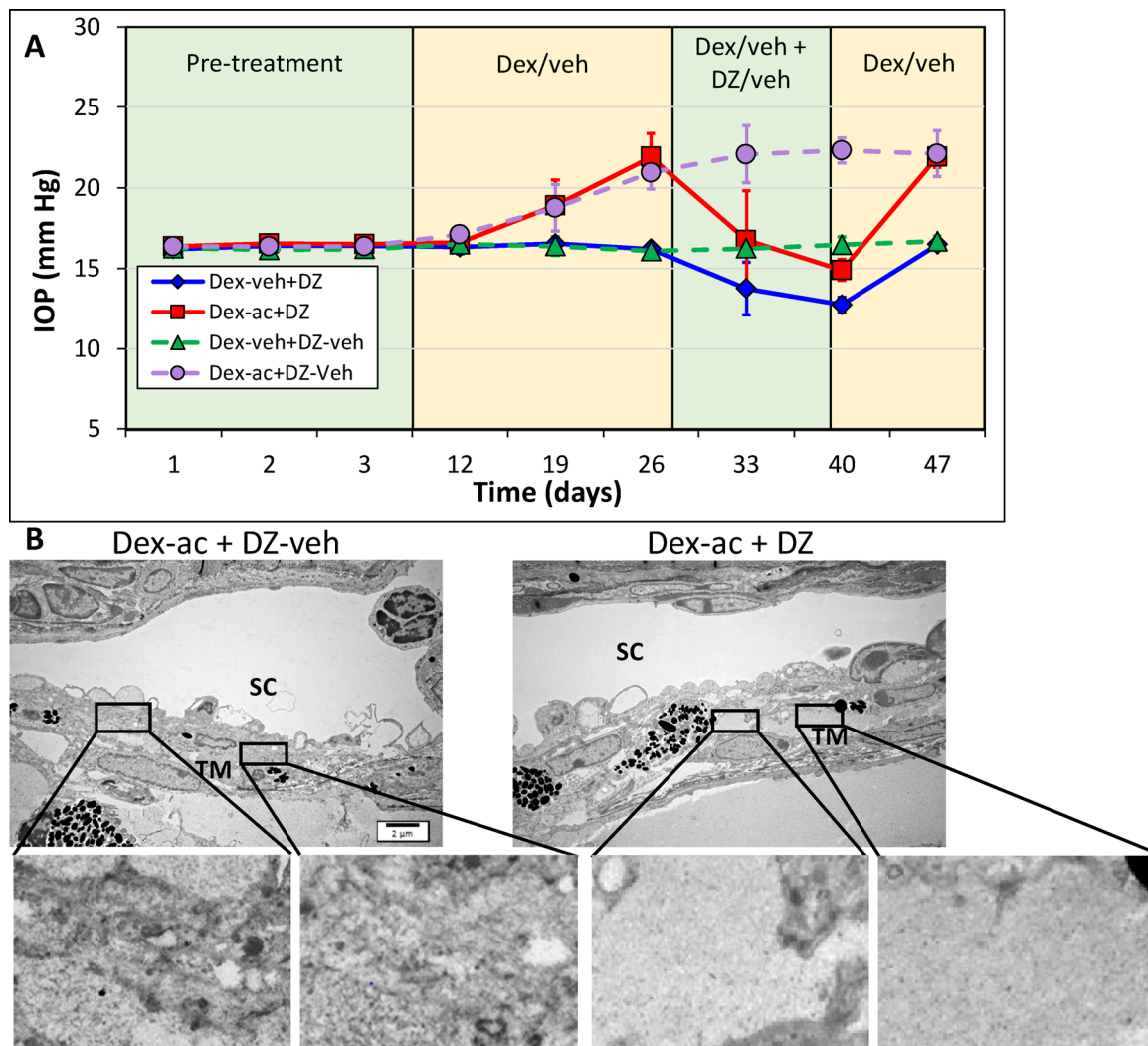


FIGURE 3. Effect of diazoxide on steroid-induced ocular hypertensive mice. **(A)** Dexamethasone acetate (dex-ac)-induced elevated IOP was abrogated by once daily treatment with diazoxide (5 mM). Diazoxide decreased the IOP by 32% ($n = 13$; $P < 0.0001$) compared with baseline (solid red line). Once treatment with diazoxide was stopped, IOP returned to pretreatment levels within 1 week ($P = 0.99$). Eyes treated with vehicle (veh) (suspension formulation without dexamethasone acetate) alone showed no increase in IOP, but after treatment with diazoxide, showed a 22% decrease in the IOP ($P < 0.0001$) compared with baseline (solid dark blue line). In a separate group of mouse eyes, similarly injected with dexamethasone acetate or the suspension formulation, but treated only with the vehicle for diazoxide (DZ-veh, DMSO in 10% Cremophor), no decrease in the IOP was noted in comparison with baseline (dotted lines on graph). **(B)** Histological analysis of the conventional outflow pathway ultrastructure by transmission electron microscope showed healthy looking trabecular meshwork (TM) cells and an intact inner and outer wall of Schlemm's canal. In some eyes, ECM deposition could be seen in the trabecular meshwork after treatment with dexamethasone acetate (insets). However, diazoxide treated eyes showed little or no ECM deposition (insets). SC, Schlemm's canal.

mark of this mouse model. Similar to DBA/2J mice treated with CKLP1, no adverse effects of diazoxide treatment were evident in the ultrastructural analysis of the trabecular meshwork region from CKLP1-treated and vehicle-treated control eyes (Fig. 5B).

Effect of K_{ATP} Channel Openers on Aqueous Humor Dynamics in Mouse Models of Ocular Hypertension

We have shown previously that, in normotensive animal models, K_{ATP} channel openers decrease the IOP by lowering episcleral venous pressure through the modulation of the region distal to Schlemm's canal. Given that the K_{ATP}

channel openers CKLP1 and diazoxide both decreased the IOP in ocular hypertensive mouse models, we reasoned that these drugs would also decrease the episcleral venous pressure in these animals. To test this hypothesis, we analyzed aqueous humor dynamics using a constant flow perfusion system^{15,22,23,29} in groups of TGF β 2-overexpressing, steroid-induced, and DBA/2J ocular hypertensive mice after treatment with either CKLP1 or diazoxide, because both of these drugs have similar mechanism of actions.^{10–13,15} In all three ocular hypertension models, treatment with CKLP1 or diazoxide significantly lowered IOP via reduction of the episcleral venous pressure (Table 2). In TGF β 2 mice, CKLP1 treatment lowered episcleral venous pressure by 29% (vehicle control, 10.22 ± 0.18 mm Hg; CKLP1, 7.15 ± 0.30 mm Hg; $P < 0.001$, $n = 13$). When mice with steroid-

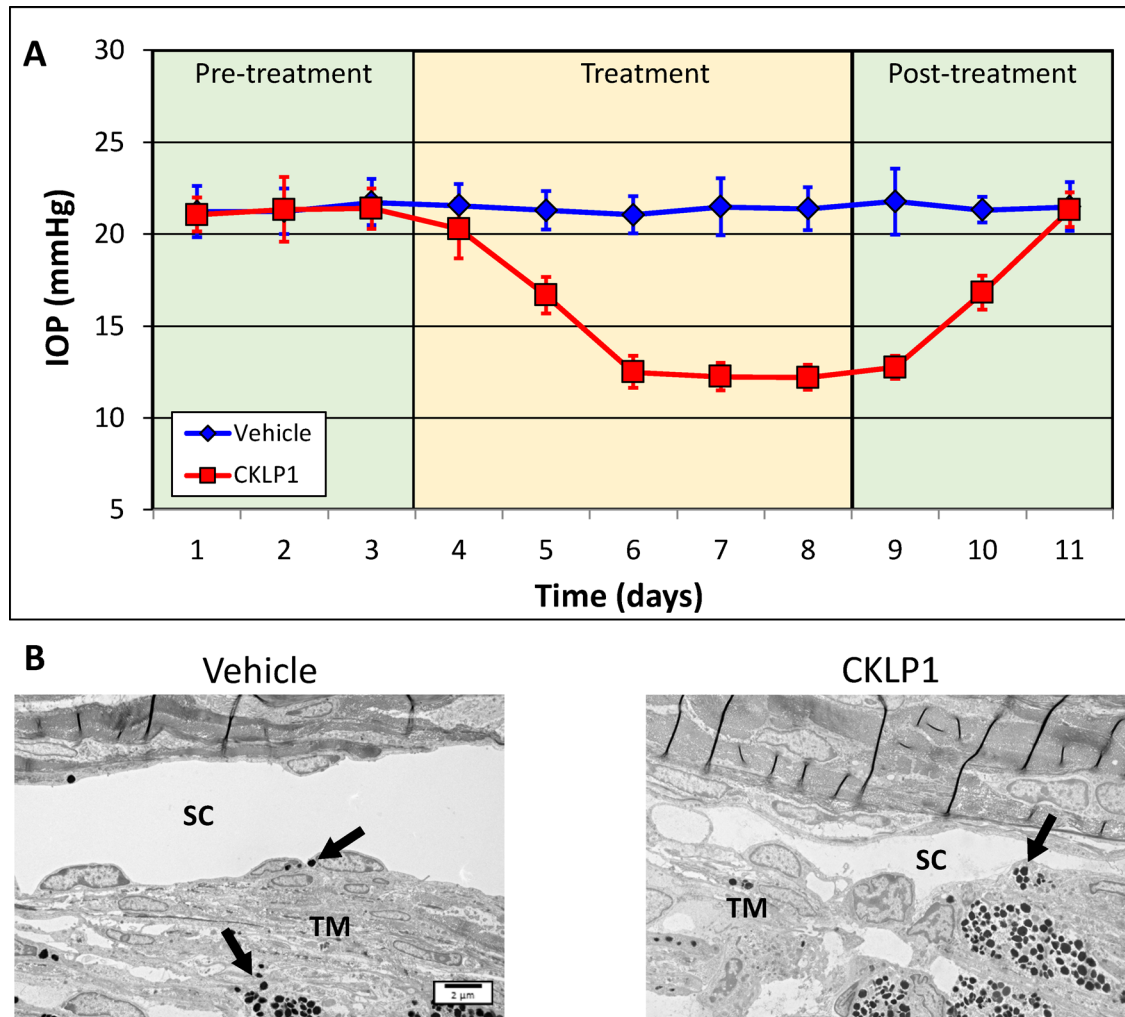


FIGURE 4. Effect of CKLP1 treatment in DBA/2J mice. **(A)** DBA/2J mice (8–10 months old) treated with CKLP1 once daily for five consecutive days showed a 43% decrease in the IOP compared with baseline ($P < 0.0001$; $n = 15$). The IOP in the treated eyes returned to baseline levels within 72 hours after termination of treatment. **(B)** Histological analysis of the trabecular meshwork (TM) shows pigment dispersion-related anomalies, which are characteristics associated with this mouse strain (black arrows). No effects associated with CKLP1 treatment were noted. SC, Schlemm's canal.

induced elevated IOP were treated with diazoxide, the episcleral venous pressure was decreased by 35% (vehicle control, 8.33 ± 0.42 mm Hg; diazoxide, 5.39 ± 1.53 mm Hg; $P = 0.03$, $n = 3$). Last, in DBA/2J mice, the episcleral venous pressure was lowered by 72% after CKLP1 treatment (vehicle control, 12.02 ± 1.53 mm Hg, $n = 3$; CKLP1, 3.40 ± 0.82 mm Hg; $P < 0.001$, $n = 4$). The aqueous flow rate was also decreased in DBA/2J mice treated with CKLP1 by 44% (vehicle, 0.34 ± 0.11 $\mu\text{L}/\text{min}$ [$n = 3$]; CKLP1, 0.19 ± 0.04 $\mu\text{L}/\text{min}$ [$n = 4$]; $P = 0.04$) (Table 2). The aqueous outflow facility and uveoscleral outflow showed no change in any of the model systems between drug- and vehicle-treated eyes. The aqueous flow rate was not affected by CKLP1 or diazoxide in TGF β 2 overexpression or steroid-induced models of ocular hypertension (Table 2).

DISCUSSION

The K_{ATP} channel openers CKLP1 and diazoxide were both found to effectively lower IOP during conditions of ocular hypertension as evidence from the data in three indepen-

dent mouse models of elevated IOP. In all three models, the K_{ATP} channel openers lowered the IOP by decreasing the episcleral venous pressure, similar to findings in our previous studies in normotensive mice.^{14–16} Among existing glaucoma drugs, only the prostaglandin analog latanoprost has been reported to decrease the episcleral venous pressure in mice,²² while netarsudil (brand name Rhopressa) has been found to decrease the episcleral venous pressure in humans.³⁰ However, both latanoprost (uveoscleral) and netarsudil (trabecular outflow) also affect other aspects of aqueous humor dynamics and IOP. The K_{ATP} channel openers are unique in this respect; they have been shown to only target the episcleral venous pressure to effectively decrease the IOP.¹⁵

All mouse ocular hypertensive models used for this study are well-established and have been characterized widely. In general, the ocular anterior segment physiology of living mice is considered similar to human eyes owing to a negligible washout rate and a linear pressure flow relationship over a wide range of IOPs, a property leveraged in the continuous flow perfusion method to accurately measure

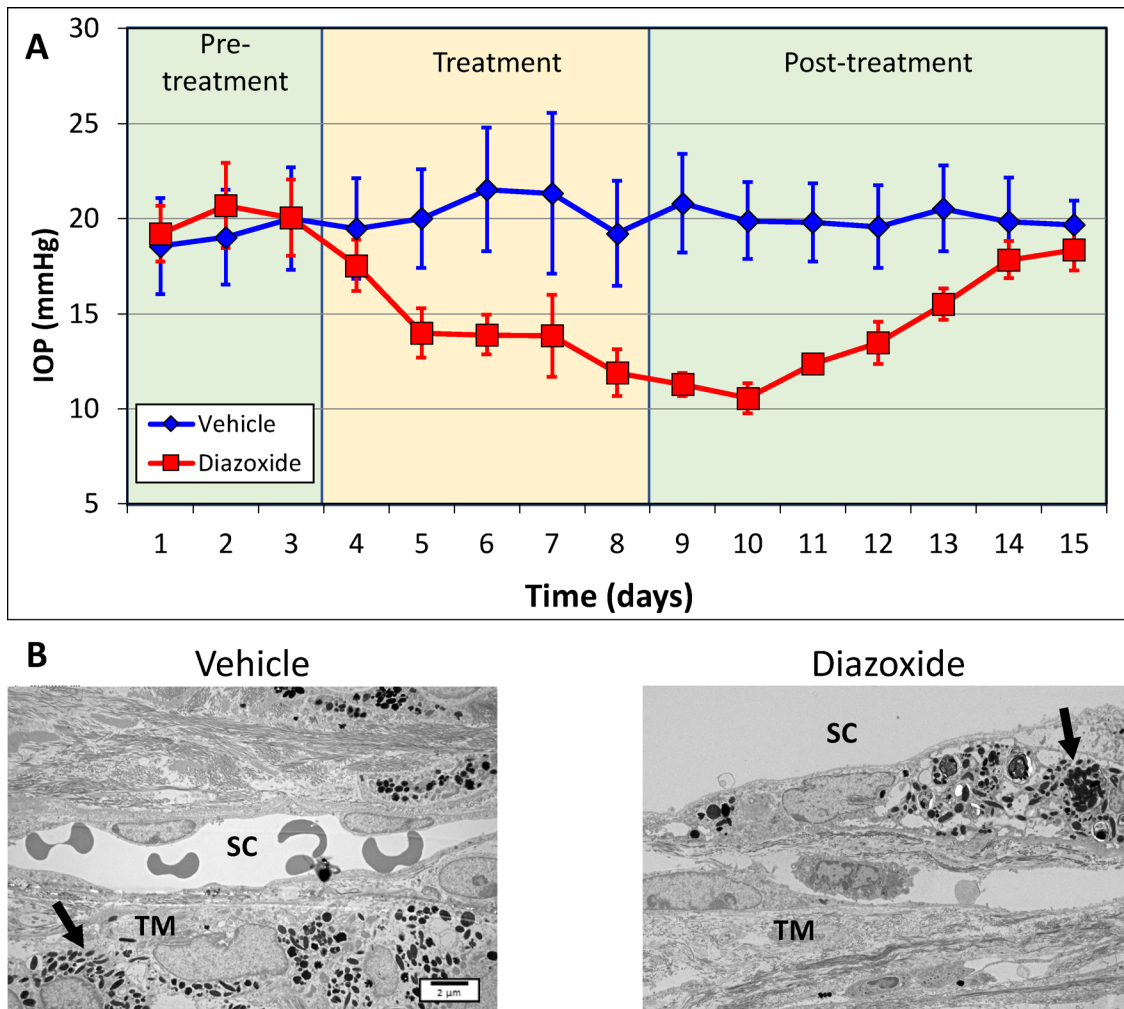


FIGURE 5. Effect of diazoxide treatment in DBA/2J mice. (A) Treatment with diazoxide (DZ) for 5 consecutive days in DBA/2J mice (8–10 months old) decreased the IOP by 41% ($P = 0.0002$; $n = 4$). After termination of treatment, the IOP returned to baseline levels within 6 days. (B) Histological analysis showed similar morphology and cell histology between treated and control samples indicating tissue tolerability of diazoxide treatment. Anomalous findings such as pigment dispersion and pigment filled vesicles (black arrows) are characteristics of this mouse model and were found in both treated and control eyes. SC, Schlemm's canal; TM, trabecular meshwork.

TABLE 2. Effect of K_{ATP} Channel Openers on Aqueous Humor Dynamics

AHD Parameters	Vehicle Control	Treated	P Value	IOP (mm Hg)	
				Baseline	After Treatment
TGFβ2 overexpressed mice + CKLP1 (n = 13)					
Aqueous outflow facility (μL/min/mm Hg)	0.012 ± 0.007	0.016 ± 0.010	0.27	23.3 ± 0.6	16.2 ± 0.4 ($P = 0.006$)
Uveoscleral outflow (μL/min)	0.018 ± 0.052	0.021 ± 0.047	0.90		
Aqueous flow rate (μL/min)	0.127 ± 0.073	0.125 ± 0.063	0.94		
Episcleral venous pressure (mm Hg)	10.217 ± 0.180	7.146 ± 0.300	<0.001		
Steroid-induced elevated IOP + diazoxide (n = 3)					
Aqueous outflow facility (μL min/mm Hg)	0.073 ± 0.044	0.040 ± 0.022	0.32	21.9 ± 1.5	14.9 ± 0.7 ($P < 0.0001$)
Uveoscleral outflow (μL/min)	0.015 ± 0.007	0.020 ± 0.015	0.72		
Aqueous flow rate (μL/min)	1.075 ± 0.969	1.000 ± 1.075	0.95		
Episcleral venous pressure (mm Hg)	8.333 ± 0.421	5.393 ± 1.530	0.03		
DBA/2J+CKLP1 (control, n = 3; treated, n = 4)					
Aqueous outflow facility (μL/min/mm Hg)	0.016 ± 0.005	0.015 ± 0.002	0.70	21.3 ± 0.6	12.2 ± 0.7 ($P < 0.0001$)
Uveoscleral outflow (μL/min)	0.077 ± 0.015	0.055 ± 0.038	0.40		
Aqueous flow rate (μL/min)	0.342 ± 0.110	0.186 ± 0.043	0.04		
Episcleral venous pressure (mm Hg)	12.015 ± 1.53	3.402 ± 0.816	<0.001		

the different parameters of aqueous humor dynamics.^{22,31,32} The mouse eye is also anatomically similar to humans with a well-defined trabecular meshwork containing three distinct regions (uveal, corneoscleral, and juxtacanalicular) and a true Schlemm's canal with inner and outer wall endothelium.^{33,34}

TGF β 2 has been reported widely as a key molecule involved in the development of glaucoma.^{35–38} When over-expressed in mouse eyes by intracameral injection of Ad.hTGF β 2^{226/228}, TGF β 2 has been reported to cause a rapid and sustained IOP increase between days 4 and 21, along with a decreased outflow facility.²⁰ The pathogenesis is believed to be through an increase in TGF β 2 levels in aqueous humor and subsequent upregulated expression of CTGF, PAI-1 and NOX4 in ocular anterior segment tissues.²⁰ It has been shown in TGF β 2-overexpressing mice that the extent of the IOP elevation is correlated directly with the level of TGF β 2 mRNA in the eye, suggesting that the model is directly based on TGF β 2 expression, a potent risk factor for glaucoma.²⁰ In light of this finding, the strong ocular hypotensive effects of CKLP1 on this model further adds confidence to its potential to decrease the IOP in glaucoma.

The steroid-induced ocular hypertension mouse model closely mimics glucocorticoid-induced glaucoma in humans and also shows similar histological changes observed in the trabecular meshwork of glucocorticoid-treated, high IOP eyes.¹⁹ Common to this model is the buildup of ECM in the trabecular meshwork, in part owing to the involvement of the TGF β signaling pathway.³⁹ In our studies, it is interesting to note that treatment with CKLP1 and diazoxide both seemed to decrease the buildup of excess ECM caused by dexamethasone acetate. This finding is consistent with previous reports of K_{ATP} channel activation in nonocular tissues. Studies performed in the heart and pancreas have shown that diazoxide can directly affect Erk1/2 phosphorylation and matrix metalloproteinase activity.^{40,41} The Erk1/2 pathway is a strong modulator of matrix metalloproteinases, which have been shown to be necessary for ECM turnover in the trabecular meshwork and uveoscleral outflow pathway in association with IOP reduction.^{42,43} Additionally, the K_{ATP} channel opener iptakalim was shown to reduce accumulation of collagen IV and fibronectin in the renal vasculature of a rat hypertension model by inhibiting TGF β 1 expression and normalizing matrix metalloproteinase 9 and TIMP1 function.⁴⁴ We have previously shown that upregulation of Erk1/2 phosphorylation is necessary for IOP lowering properties of diazoxide.¹³ Given the potential association with K_{ATP} channel activation and ECM remodeling, it is interesting to note that we did not see an effect on outflow facility after treatment with CKLP1 or diazoxide. Although the result is consistent with studies we performed in normotensive mice,¹⁵ we cannot rule out the fact that evaluation of a larger sample size may show some alterations in outflow facility after treatment with K_{ATP} channel openers. Future studies designed specifically to understand the changes in ECM structure after treatment with K_{ATP} channel openers will be able to provide the required insights and possibly new target molecules for IOP regulation. Nevertheless, the ocular hypotensive effects of K_{ATP} channel openers in the steroid-induced ocular hypertensive model seem to validate the IOP-lowering abilities of CKLP1 and diazoxide during events of aberrant ECM deposition.

Mutations in the tyrosine-related protein 1 (*Tyrt1^b*) gene and a stop codon mutation in the transmembrane glycoprotein *nmb* (*Gpnmb^{R150X}*) gene in the DBA/2J mice result

in iris pigment dispersion.^{24,26–28} Additionally, melanosomal toxicity and abnormal immunity also contribute to increased pigment dispersion in these mice.^{25,45} These pigment granules enter and block the aqueous drainage systems of the anterior chamber, causing an increase in the IOP.^{24–27,46} Owing to the iris atrophy and concomitant anterior synechia, the DBA/2J mice develop secondary glaucoma, resulting in significant elevation of IOP starting at 6 months of age. For this reason, all animals for the current study were selected in the 8- to 10-month age range to provide sufficient time for initiation of ocular hypertension.^{21,47} The elevated IOP consequently causes progressive degenerative alterations of the retinal tissue and optic nerve head that are reminiscent of glaucomatous disease progression in humans.^{47–49} Both CKLP1 and diazoxide showed a significant decrease in the IOP in this model, decreasing the IOP by 43% and 41%, respectively. Given these findings, it will be interesting to assess the effect of long-term treatment with these K_{ATP} channel openers on retinal cell degeneration in this model.

One of the limitations with the DBA/2J model is the significant variability in the development of elevated IOP and glaucoma-like disease progression. This factor has mainly been attributed to the age of animals, differences in breeding colonies, and possible environmental factors.^{48,50–54} To overcome these issues, we sourced all our mice at a young age (4 months) from a specific breeding colony (Jackson Laboratory) and aged them in our animal facility until 8 to 10 months of age. The IOP of these mice were monitored weekly and animals demonstrating a similar level of elevated IOP were selected for treatment. Therefore, we used well-defined ocular hypertensive animals.

Of the various hypertensive models tested, all showed that a decrease in the episcleral venous pressure was the main aqueous humor parameter affected by treatment with CKLP1 and diazoxide. This finding is consistent with results that were previously reported in normotensive animals after treatment with CKLP1.¹⁵ We were surprised to see a significant effect on aqueous flow rate after treatment with CKLP1 in DBA/2J mice. This is the first time we have seen an effect of a K_{ATP} channel opener on an aqueous humor dynamic parameter other than episcleral venous pressure. Because this is also the first time DBA/2J mice were treated with K_{ATP} channel openers, it is possible that the aqueous suppression is unique to this particular model. Owing to the inherent ocular issues found in the DBA/2J mice, their eyes have a compromised blood aqueous barrier.^{45,55} According to the blood–aqueous barrier model proposed by Freddo,⁵⁶ an increase in pigmentary protein deposits along with a disrupted blood aqueous barrier can cause an increased production of aqueous humor to normalize the protein content in the fluid. Reports of increased protein concentration (flares) found after treatment with timolol in patients with an intact blood–aqueous barrier but low aqueous production indirectly supports this notion. According to Freddo's model, without the blood aqueous barrier, the flares would have been normalized through increased aqueous production.^{56–58} Because K_{ATP} channel openers have cell protective properties,^{59–63} it is feasible that treatment with CKLP1 may normalize the blood–aqueous barrier in DBA/2J mice. As a consequence, the rate of aqueous humor formation would be suppressed indirectly by K_{ATP} channel openers owing to improved functioning of the relevant ocular cells. Although speculative, this hypothesis will be interesting to evaluate in DBA/2J mice before and after the onset of iris pigment dispersion and with K_{ATP} channel opener treatment

in future studies. If it is found that K_{ATP} channel openers indeed have a protective effect on damaged cells of the anterior chamber, it would make K_{ATP} channel openers a promising drug class for other ocular hypertensive glaucomas like pseudoexfoliation or pigment dispersion, where in addition to lowering the IOP, the K_{ATP} channel openers can help to normalize the functions of the iris along with other cells and tissues of the anterior segment.

In summary, the K_{ATP} channel openers CKLP1 and diazoxide show a robust ocular hypotensive effect in three independent mouse models of ocular hypertension by lowering episcleral venous pressure. The fact that these K_{ATP} channel openers were able to successfully lower pressure in three rodent models of elevated IOP developed on different etiological aspects of glaucoma, provides confidence to the potential of this class of drugs being used as new ocular hypotensive agents that can lower IOP across various forms of glaucoma.

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