Telmisartan Reduces Axon Degeneration in Mice With Experimental Glaucoma

Ralph J. Hazlewood,[1] John Kuchtey,[1] Hang-Jing Wu,[1] and Rachel W. Kuchtey[1,2]

[1]Department of Ophthalmology and Visual Sciences, Vanderbilt University Medical Center, Nashville, Tennessee, United States
[2]Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee, United States

Correspondence: Rachel W. Kuchtey, Department of Ophthalmology and Visual Sciences, Vanderbilt University Medical Center, 2311 Pierce Avenue, Nashville, TN 37232, USA;
rachel.kuchtey@vumc.org.

RJH and JK contributed equally to this work.

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Purpose. The purpose of this study was to determine if treatment with telmisartan, an angiotensin II type 1 receptor blocker (ARB), protects against retinal ganglion cell (RGC) degeneration in a mouse glaucoma model with induced elevation of intraocular pressure (IOP).

Methods. IOP elevation was induced by injection of polystyrene microbeads into the anterior chamber of the right eye of 3-month-old C57BL/6J mice, with the left eye serving as contralateral control. Starting the day of microbead injection, mice were maintained on solid food pellets with or without incorporated telmisartan. IOP was measured by Tono Lab tonometry prior to and weekly after microbead injection. Twelve weeks postinjection, mice were euthanized to obtain optic nerves for analysis of RGC axons. The total numbers of optic nerve axons were determined manually and automatedly using AxonJ. Degenerating axons were counted manually.

Results. IOP elevation induced by microbead injection was similar in magnitude and duration in vehicle and telmisartan-fed mice, although IOP was reduced 5.8% in uninjected mice treated with telmisartan ($P = 0.0027$). Axon loss determined by manual and automated methods was greater in vehicle compared to telmisartan-treated mice (manual: 9.5% vs. 1.8%, $P = 0.044$; automated: 14.2% vs. 2.9%, $P = 0.0375$). An increase in the percent of axons undergoing degeneration was observed in nerves from microbead-injected eyes that was greater in vehicle-treated compared to telmisartan-treated mice (49.0% vs. –0.58%, $P = 0.0019$).

Conclusions. Elevation of IOP by microbead injection led to loss of RGC axons in vehicle-treated mice that was largely prevented by telmisartan treatment, suggesting a neuroprotective effect of telmisartan.

Keywords: glaucoma, angiotensin II type 1 receptor blocker, transforming growth factor beta, intraocular pressure, neuroprotection

Glaucoma is the leading cause of irreversible blindness worldwide,[1] characterized by progressive loss of peripheral vision.[2] Vision loss in glaucoma is caused by Wallerian-like degeneration of retinal ganglion cell (RGC) axons, which form the optic nerve, and ultimately death of RGCs.[3] Elevated intraocular pressure (IOP) is an important risk factor for glaucoma. Currently, the only available treatment is to lower IOP by pharmacological or surgical means, which has proven effective in slowing glaucoma progression. However, many patients experience intolerance or are not responsive to the available medications. In addition, glaucoma can develop in the absence of clinically detectable IOP elevation. Therefore, development of new IOP-lowering drugs and discovery of novel neuroprotective approaches to glaucoma, independent of IOP-lowering, is a critical unmet need for the treatment of patients with glaucoma.

Angiotensin II type 1 receptor blockers (ARBs) are drugs used to treat systemic hypertension[4] that have been investigated as potential treatments for glaucoma. ARBs are known to reduce transforming growth factor beta (TGFβ)-mediated signal transduction,[5–8] which is thought to play an important role in glaucoma pathogenesis.[9] Currently, there are eight ARBs in clinical use: losartan, azilsartan, candesartan, eprosartan, irbesartan, olmesartan, telmisartan, and valsartan.[4] Losartan has been shown to lower IOP in humans, with and without elevated IOP.[10] In animal models of glaucoma induced by experimentally raising IOP, olmesartan was shown to have IOP-lowering effects.[11,12] Losartan lowered experimentally elevated IOP in rabbits,[13] but not in mice.[14] Despite lack of IOP-lowering, losartan reduced RGC degeneration in mice with induced IOP elevation,[14] suggesting a direct neuroprotective effect. Similarly, candesartan did not affect IOP in a mouse model of IOP-independent glaucoma, and a rat model of induced IOP elevation, but protected against RGC loss, further suggesting a neuroprotective effect for ARBs, independent of lowering IOP.[15,16]

In a recent study, we compared the capabilities of losartan, irbesartan, and telmisartan to lower IOP and suppress TGFβ signaling in the retina of C57BL/6J mice.[17] In this study, mice received ARBs ad libitum incorporated into their

solid food pellets, a method that in our previous study resulted in physiologically relevant systemic concentrations, as evidenced by lowered blood pressure (BP),[17] a known effect of ARBs on mice.[14] ARBs crossed the blood/brain and blood/retinal barriers, suggesting that direct mechanisms of action in the eye are possible using this drug delivery method.[17] Telmisartan was an order of magnitude higher of concentration in plasma, brain, and eyes, compared to the other ARBs, likely due to its lipophilic nature.[4] We found that treatment with telmisartan or irbesartan lowered IOP, whereas losartan did not. In addition, telmisartan treatment suppressed TGFβ signaling in RGCs, whereas the other two ARBs did not. These findings suggested that telmisartan may have enhanced beneficial effects compared to other ARBs due to its dual capabilities of lowering IOP and suppressing TGFβ.

To investigate the therapeutic potential of telmisartan as a treatment for glaucoma, we used the same method of drug delivery as in our previous study[17] and induced IOP elevation in C57BL/6J mice by injection of microbeads into the anterior chamber of one eye, while the other eye was left uninjected to serve as contralateral control.[18] For mice treated with vehicle, microbead injection resulted in degeneration and loss of RGC axons in the optic nerve, whereas there was no significant loss in mice treated with telmisartan. Consistent with our previous study,[17] we found that IOP in the uninjected eye was lowered in mice treated with telmisartan, although IOP elevation was similar in the microbead-injected eyes of telmisartan and vehicle-treated mice. Our findings with this animal model of glaucoma suggest a neuroprotective effect of telmisartan treatment, independent of lowering IOP.

Materials and Methods

Mice

Male and female C57BL/6J mice were used for experiments approved by the Institutional Animal Care and Use Committee of Vanderbilt University and conducted in accordance with the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research. Mice were housed in a facility managed by Vanderbilt University Division of Animal Care and Use, with 12 hours of light/dark cycle and ad libitum access to mouse chow and water.

Microbead Injections

Elevated IOP was induced unilaterally by injection of microbeads into the anterior chamber of the right eye of 3-month-old mice using a protocol based on Sappington et al.[19] and Cone et al.[20] Briefly, after dilation of the right eye with 1% atropine (Akorn, Lake Forest, IL, USA), mice were anesthetized by inhalation of 2.5% isoflurane in oxygen (Vet Equip, Livermore, CA, USA). Using a glass micropipette pulled from borosilicate capillaries with a final diameter of approximately 100 μm and a microsyringe pump, as previously described,[19] 2 µl of 6 µm polystyrene microbeads (Polybead Polystyrene Violet Dyed Microspheres, Polysciences, Warrington, PA, USA), followed by 1 µl of sodium hyaluronate[18] were injected into the anterior chamber of the dilated eye.[20] Prior to injection, beads were sterilized with ethanol, washed, and pelleted, as previously described.[21] Refluxing was avoided by holding the glass micropipette in place for 2 minutes before removing. No manipulations were done to the contralateral eye, which served as intra-animal control.

Telmisartan Treatment

Treatment with telmisartan (n = 18) or vehicle control (n = 19) commenced on the day of microbead injection and continued for the duration of the experiment (12 weeks). Telmisartan (AK Scientific, Union City, CA, USA) was incorporated into 5001 base rodent chow by Envigo, Teklad Diets (Madison, WI, USA) at 2 g/kg, the concentration we previously used and found to lower IOP and reduce TGFβ signaling in the RGC layer of the retina as well as lowering BP in C57BL/6J mice.[17] Mice were grouped in cages housing 3 to 5 mice, each cage was assigned to receive either telmisartan-containing or normal chow (vehicle) placed in the standard food receptacle in the roof of the cage, available ad libitum. There were approximately equal numbers of males and females in each treatment group (8 females and 11 males in the vehicle group; and 7 females and 11 males in the telmisartan group), with five cages of telmisartan-treated and vehicle-treated mice.

Body weight was monitored by weighing mice at each IOP measurement time point. To determine the rate of chow consumption, an aliquot of chow was weighed and added to the food receptacle of each cage and the weights of the mice determined. Chow was replenished and weighed as needed and the weights of the mice were again determined. Consumption rate was calculated for each cage as the amount of chow consumed divided by the number of mice in the cage, divided by the number of days of consumption, and normalized to the average weight of the mice in the cage over the consumption period.

IOP Measurement and Quantitation

IOPs of both eyes of the mice under isoflurane anesthesia were measured by an operator masked to treatment status using a TonoLab tonometer (iCare, Finland) following manufacturer's recommendations, and as previously described.[22] IOP measurements were collected prior to and subsequently continued on a weekly basis for 11 weeks following microbead injection and initiation of drug treatment. Mean IOP was calculated at each measurement time point for each of four groups of eyes (uninjected and bead-injected eyes from vehicle-treated mice and uninjected and bead-injected eyes from telmisartan-treated mice). Mean IOP over the experimental period was calculated for individual eyes from each group.

Optic Nerve Processing and Quantification of Axons

Twelve weeks after microbead injection, mice were euthanized by CO_2 inhalation and cardiac perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Preparation of optic nerves was carried out as previously described.[22] Briefly, eyes were enucleated, and optic nerves cut approximately 1.5 mm from the globe and post-fixed in 1% glutaraldehyde/4% PFA in PBS. Optic nerves were transferred to 2% osmium tetroxide in PBS for 1 hour before dehydration and embedding in Epon, as previously described.[22] Using an ultramicrotome (Leica EM UC7, Wetzlar, Germany), 1-µm-thick cross-sections of optic nerves were cut, stained with paraphenylenediamine (PPD), which darkly stains axoplasm of degenerating axons in light microscopy,[23] and mounted with Permount

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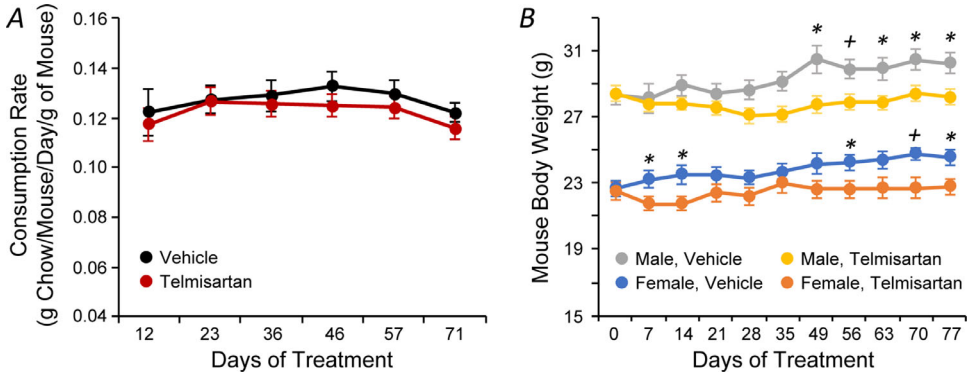

Figure 1. Chow consumption. The rate of chow consumption normalized to mouse body weight was determined at 12, 23, 36, 46, 57, and 77 days after starting administration of telmisartan-containing or normal (vehicle) chow (**A**). At each time point, there was no significant difference in chow consumption between vehicle and telmisartan-treated mice ($P > 0.3$, Student's *t*-test, $n = 19$ and 18, respectively). The body weights of male (gray and yellow lines) and female (blue and orange lines) fed telmisartan (yellow and orange lines) or vehicle (gray and blue lines) were determined weekly (**B**). Mice fed telmisartan tended to have lower body weight compared to vehicle-treated mice (1.3–8.4% lower), with differences reaching statistical significance at time points indicated in the figure ($^{*}P < 0.05$, $^{+}P < 0.01$, Student's *t*-test performed at each time point). Symbols represent group averages at each time point, with error bars indicating +/− SEM. Numbers of mice in each group were 11 males and 8 females treated with vehicle; and 11 males and 7 females treated with telmisartan.

Mounting Medium (Thermo Fisher Scientific, Waltham, MA, USA). Some samples were not successfully obtained during tissue collection and processing (vehicle-treated $n = 6$, telmisartan-treated $n = 1$). Axon data include all bead-injected mice for which optic nerve processing was successful.

Stained optic nerve cross-sections were imaged with an oil immersion 100× objective on a Nikon inverted light microscope equipped with an SLR DS-Ri2 camera (Nikon, Melville, NY, USA). Images were assembled into montages covering the entire nerve cross-section using the NIS-elements software (Nikon). For manual axon counts, a mask consisting of 24 boxes, each 310 μm^2, at central, medial, and peripheral locations was overlaid on the optic nerve images. Placement of the counting boxes was the same for all nerves. The combined area of the counting boxes was approximately 10% that of the optic nerve area. Normal and degenerating axons within the boxes were counted to determine axon densities. Degenerating axons were identified as those with darkly stained axoplasm, inclusion bodies within the axoplasm or with a thickened myelin sheath.[24–27] Manual counters were blinded as to treatment status of the nerves. Optic nerve area, excluding the pia mater, was determined by drawing a polygon around the nerve using ImageJ. The total numbers of normal and degenerating axons in the optic nerve were calculated by multiplying the mean axon density by the area of the nerve. Automated axon counting of all axons in the optic nerve, except those with darkly stained axoplasm, was performed using the AxonJ plugin with ImageJ created by Zarei et al.[28] Based on our previous study in which axon sizes were determined manually,[22] the size range of objects to be counted by AxonJ was set to 0.0294 to 15.51 μm^2, corresponding to 34.3 to 18,092 pixels.[2] To correct for undercounting by AxonJ compared to manual counting, a correction factor was derived by plotting the ratio of AxonJ counts to manual counts (Y) versus axon density (X), calculated as manual counts divided by the nerve area, to obtain the best-fit line, $Y = mX + b$ where m and b are the slope and y-intercept, which were -1.5786 $\times 10^6$ and 1.5107, respectively. The correction factor, f, was calculated as $f = mX + b$ for each nerve. AxonJ counts were corrected by dividing AxonJ count by the correction factor.

Statistical Analysis

Data were analyzed with GraphPad version 8.0.2 for Windows (GraphPad Software, La Jolla, CA, USA) and Excel (Microsoft, Redwood, WA, USA). Two-tailed paired *t*-tests were used to compare microbead-injected and contralateral uninjected eyes for IOP at each time-point (Fig. 2A) axon counts (Figs. 3B, 4D) and percent degenerating axons (Fig. 5A). Two-tailed Student's *t*-tests were used to compare vehicle-treated and telmisartan-treated mice in food consumption rates and body weights at each time-point (Fig. 1), IOP averaged over the experimental time-course (Figs. 2B, 2C), axon loss (Figs. 3C, 4E), and percent increase in the percent of axons that were degenerating (Fig. 5B). The *P* value threshold for statistical significance was set at $P < 0.05$. Numbers of samples are indicated in the figure legends.

Results

Tolerability of Chronic Telmisartan Treatment

Chronic administration of telmisartan during the 12-week study appeared to be well-tolerated, with no obvious adverse events. The rate of chow consumption was not significantly different between the vehicle-treated and telmisartan-treated groups ($P > 0.31$), with the average consumption over the course of the experiment of 0.127 +/− 0.0062 for vehicle and 0.122 +/−0.0052 g chow/mouse/day/g body weight (mean +/− SEM) for telmisartan-treated (Fig. 1A). However, body weight was lower in telmisartan-treated animals (Fig. 1B), with females ranging from 2.9% to 8.4% lower than vehicle-treated, reaching statistical significance on days 7, 14, 56, 70, and 77 and males ranging from 1.3% to 9.1% lower than vehicle-treated, reaching significance on days 35 to 77 after initiation of drug treatment (Student's *t*-tests).

IOP Elevation After Injection of Microbeads Into the Anterior Chamber

Although there was considerable heterogeneity between mice (see Supplementary Fig.), injection of microbeads into the anterior chamber of 3-month-old C57BL/6J mice, using a protocol adapted from Cone et al. and Sappington et al.,[19–21]

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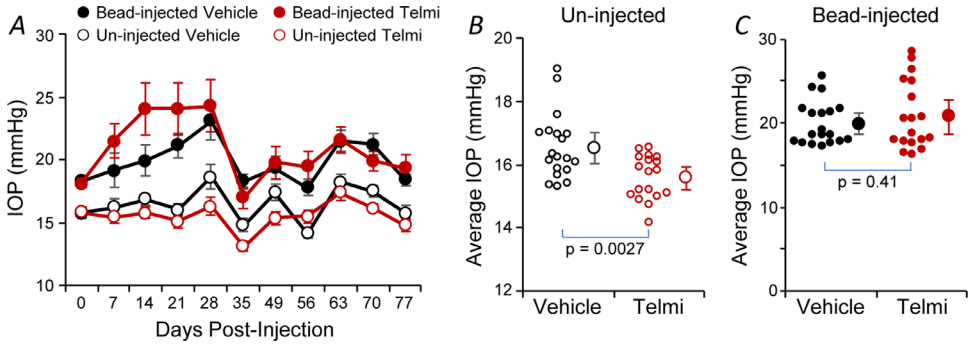

Figure 2. IOP responses to microbead injection. Microbeads were injected into the anterior chamber of the right eye (filled symbols) of mice treated with telmisartan (red symbols, $n = 18$) or vehicle (black symbols, $n = 19$), while the left eye was not injected (open symbols), resulting in similar elevations of IOP in microbead-injected compared to uninjected eyes (**A**). Average IOP for individual mice over the 77-day period showed that compared to vehicle, telmisartan treatment reduced IOP in uninjected eyes (**B**, $P = 0.0027$, Student's *t*-test) but did not reduce the magnitude of the IOP response in microbead-injected eyes (**C**, $p = 0.41$, Student's *t*-test). Symbols represent either group means at each time point +/− SEM **A** or means for individual eyes over the study period, with larger symbols to the right of each group representing mean with error bars representing the 95% CI **B** and **C**. Vehicle: eyes from mice treated with vehicle; Telmi: eyes from mice treated with telmisartan.

resulted in IOP elevation compared to uninjected contralateral eyes that on average developed within 7 days, peaked between 14 and 28 days and declined to a lower level that continued to the last measurement at 77 days postinjection (Fig. 2A). For both telmisartan-treated and vehicle-treated mice, IOP was significantly elevated in microbead-injected eyes compared to contralateral uninjected controls at each time point (*P* value range 2×10^{-5} −0.04, paired *t*-tests), except for vehicle-treated at day 49. The difference between IOP of injected eyes and their contralateral controls averaged over the course of the experiment was an increase of 3.8 +/− 2.81 mm Hg for vehicle-treated and 5.5 +/− 4.21 mm Hg for telmisartan-treated mice (mean +/− SD), but this difference did not reach statistical significance (except at day 14, $P = 0.02$, Student's *t*-test). These findings suggest that microbead injection was an effective means to induce chronic, moderate IOP elevation, the magnitude of which was similar between telmisartan-treated and vehicle-treated mice.

Previously, we found that 3 days of telmisartan treatment reduced IOP in C57BL/6J mice.[17] Similarly, analysis of the average IOP over the experimental time course for individual mice in this study showed that telmisartan reduced IOP of uninjected eyes by 5.8%, as compared to vehicle-treated mice (Fig. 2B: mean [95% confidence interval {CI}], vehicle: 16.5 [16.0 to 17.0]; telmisartan: 15.6 [15.2 to 15.9] mm Hg; $P = 0.0027$, Student's *t*-test). However, for microbead-injected eyes, the average IOP over the experimental time-course was not different between vehicle-treated and telmisartan-treated mice (mean [95% CI], vehicle: 19.9 [18.6 to 21.1]; telmisartan 20.8 [18.8 to 22.8] mm Hg; $P = 0.41$, Student's *t*-test), suggesting that telmisartan did not reduce IOP responses to microbead-injection (Fig. 2C).

Effect of Telmisartan on Optic Nerve Axons in Experimental Glaucoma

To investigate possible protective effects of telmisartan against optic nerve axon degeneration induced by elevated IOP, mice were euthanized after 12 weeks of treatment to obtain optic nerves for analysis. The total number, including normal and degenerating axons, was determined in PPD-stained optic nerve cross-sections using the gold standard method of manual counting, and by an automated method using AxonJ,[28] a plug-in module for National Institutes of Health (NIH) ImageJ.

In the manual counting method, average axon density is determined by counting axons in boxed regions encompassing approximately 10% of the area of the optic nerve (Fig. 3A). The total number of axons is then calculated by multiplying the average axon density by the area of the nerve. Analysis comparing manual axons counts in nerves from bead-injected eyes to those from their contralateral uninjected controls eyes (Fig. 3C) showed a statistically significant difference in vehicle-treated mice ($P = 0.0012$, paired *t*-test), with 11 of 13 mice showing declines in axon number in the microbead-injected eye, consistent with pressure-induced axon loss. However, there was no significant difference between microbead-injected and control eyes in telmisartan-treated mice ($P = 0.39$, paired *t*-test), with only 9 of 17 mice showing reduced axon numbers in microbead-injected eyes. As shown in Fig. 3D, axon loss in nerves from microbead-injected eyes was significantly greater in vehicle-treated compared to telmisartan-treated mice (mean [95% CI]: vehicle: 9.5 [4.5 to 14.4]; telmisartan: 1.8 [−3.6 to 7.2] % axon loss, $P = 0.0396$), suggesting a neuroprotective effect of telmisartan treatment.

The automated method using AxonJ avoids experimenter bias in axon identification and does not rely on determination of nerve area since all axons within the nerve are counted. However, plotting AxonJ counts versus manual counts, we found that with our optic nerves, AxonJ tended to undercount axons compared to the manual method, with undercounting increasing with increasing axon number (Fig. 4A). Because we know from manual counting that delineating axon boundaries at higher densities becomes more challenging, we hypothesized that undercounting by AxonJ is related to axon density. To investigate this possibility, we plotted the ratio of AxonJ counts to manual counts versus axon density, calculated as the total number of axons determined manually divided by the nerve area. As shown in Fig. 4B, AxonJ undercounting was strongly correlated with axon density ($r^2 = 0.71$, $n = 60$, $P < 0.0001$), supporting axon density as a determining factor in the observed undercounting by AxonJ. Using the equation of the best fit line

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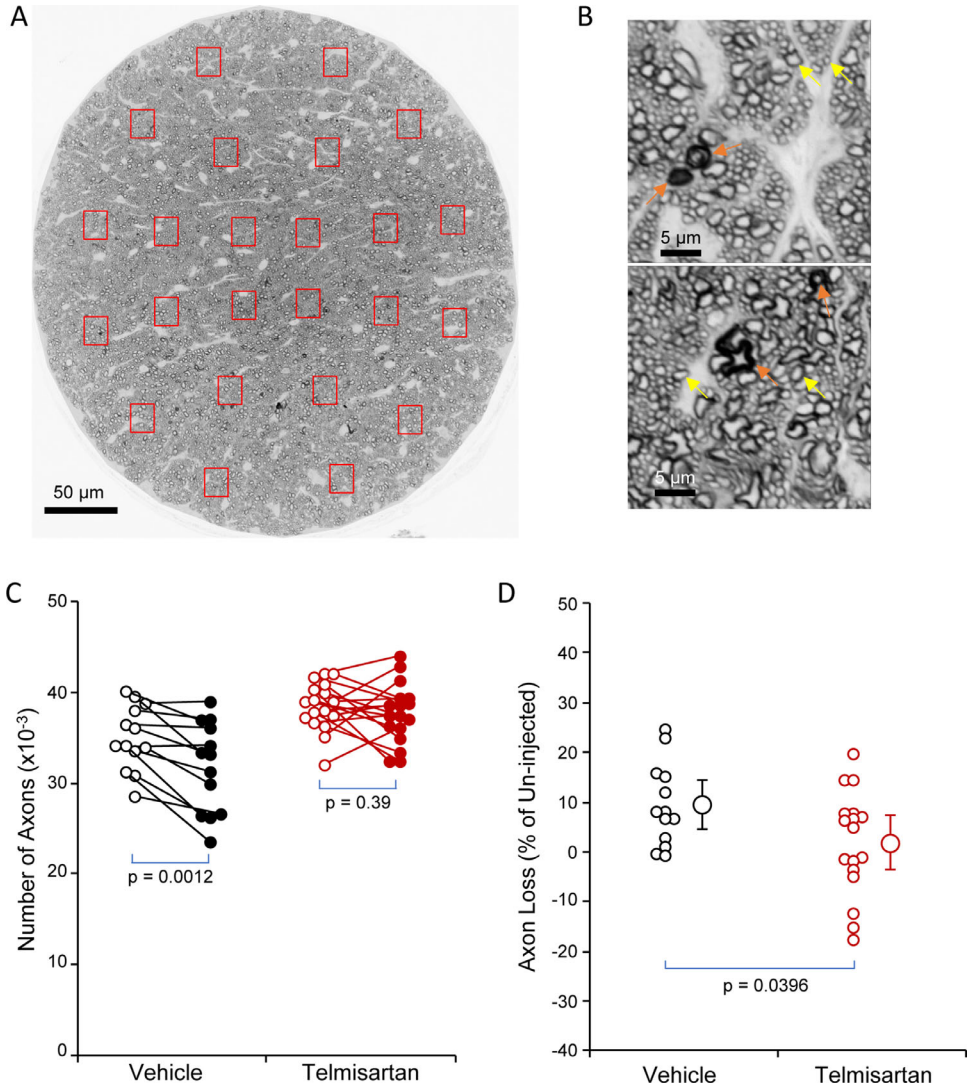

Figure 3. Manual axon counting. Images of PPD-stained optic nerve cross-sections were overlaid with a counting masks consisting of 24 boxed regions (**A**, red boxes) and both normal and degenerating axons were counted in each box to determine axon density, which was multiplied by nerve area to obtain the total number of axons. Examples of normal and degenerating axons are indicated by yellow and orange arrows, respectively in zoomed-in images (**B**). Manual axon counts of nerves (**C**) from vehicle-treated (black symbols) and telmisartan-treated mice (red symbols) revealed a significant decrease in the number of axons from microbead-injected eyes (closed symbols) as compared to their contralateral uninjected eyes (open symbols) for vehicle-treated ($P = 0.0012$, paired *t*-test), but not for telmisartan-treated mice ($P = 0.39$, paired *t*-test, lines connect paired eyes from individual mice). The percentage axon loss (**D**) in microbead-injected eyes compared to contralateral control eyes was significantly greater for vehicle-treated mice (black symbols) as compared to telmisartan-treated mice (red symbols, $P = 0.0396$), with larger symbols with error bars representing mean and 95% CI. Vehicle-treated: $n = 13$, telmisartan-treated, $n = 17$.

of the AxonJ/manual count ratio versus axon density plot (Fig. 4B, orange dotted line) and manually determined axon density, a correction factor was applied to AxonJ counts to account for density-dependent undercounting. Plotting corrected AxonJ versus manual counts resulted in a best-fit line that overlaps with the equivalence line (Fig. 4C), indicating that corrected AxonJ counts are consistent with the gold standard method of manual counting. Similar to results from manual counting, analysis of corrected AxonJ counts (Fig. 4D) showed statistically significant axon loss for vehicle-treated mice ($P = 0.0027$, paired *t*-test), with 12 of 13 mice having reduced numbers of axons in nerves from microbead-injected compared to contralateral control eyes. In telmisartan-treated mice, only 10 of 17 showed axon loss, with no significant difference between microbead-injected and control eyes ($P = 0.26$, paired *t*-test). As shown in Figure 4E, axon loss in nerves from microbead-injected eyes was significantly greater in vehicle-treated as compared to telmisartan-treated mice (mean [95% CI]: vehicle: 14.2 [6.7 to 21.8]; telmisartan: 2.9 [−4.9 to 10.7] % axon loss, $P = 0.0375$, Student's *t*-test). These results with automated counting are consistent with those from manual counting and together suggest that telmisartan treatment protected against RGC axon degeneration in response to elevated IOP.

In addition to loss of axons, induction of RGC degeneration by elevation of IOP may result in increased numbers of axons undergoing active degeneration, which can be identified by thickened myelin sheaths and the presence of

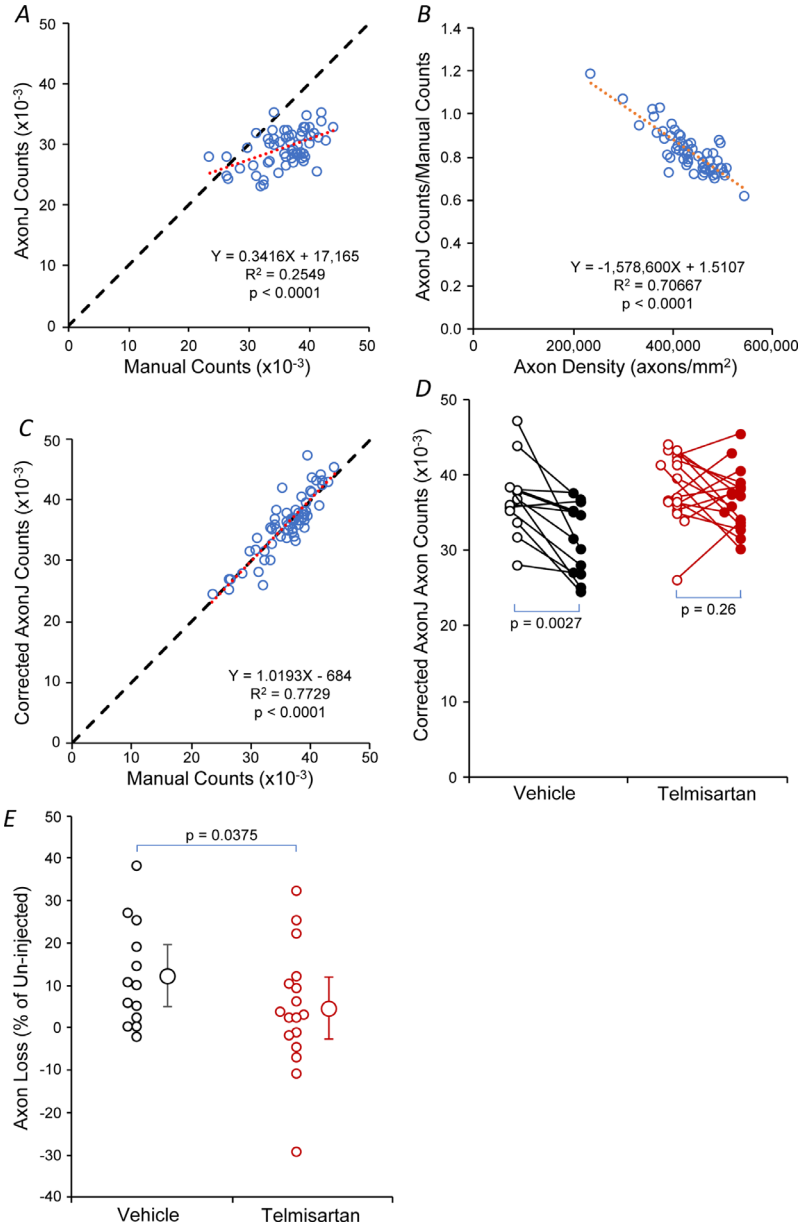

Figure 4. Automated axon counting. Plotting the number of axons determined by AxonJ on the vertical axis and the number determined manually on the horizontal axis for both eyes from vehicle-treated and telmisartan-treated mice (n = 60 eyes from 30 mice) revealed that AxonJ under-counted axons compared to the gold standard of manual counting, as indicated by data points below the dashed black equivalence line (**A**). Plotting the ratio of AxonJ counts to manual counts versus axon density for each nerve (**B**) shows that under-counting by AxonJ is correlated with axon density (R^2 = 0.70667, P < 0.0001). Adjusting AxonJ counts for axon density (**C**) aligned the best-fit line of the corrected AxonJ versus manual counts data (red dotted line) with the equivalence line (black dashed line). Using corrected AxonJ counts to evaluate axon loss (**D**) in nerves from mice treated with vehicle (black symbols) or telmisartan (red symbols) showed a significant decrease in microbead-injected eyes (closed symbols) as compared to their contralateral uninjected eyes (open symbols) for vehicle-treated mice (P = 0.0027, paired t-test, lines connect paired eyes from individual mice), but not for telmisartan-treated mice (P = 0.26, paired t-test). The percentage axon loss (**E**) in microbead-injected eyes compared to contralateral control eyes was significantly greater for vehicle-treated mice (black symbols) as compared to telmisartan-treated mice (red symbols, P = 0.0375), with larger symbols with error bars representing mean and 95% CI. Vehicle-treated: n = 13, telmisartan-treated, n = 17. Equations of the best-fit lines (red dotted line in **A** and **C**, orange dotted line in **B**) along with R^2 and P values are shown in each figure **A–C**.

inclusion bodies or dark staining in the axoplasm in PPD-stained optic nerve cross-sections (examples of degenerating axons shown in Fig. 3B). As a complimentary approach to total axon counts, the numbers of degenerating axons were counted manually to determine the percentage of degenerating axons in each nerve. Analysis of % degenerating axons (Fig. 5A) showed a statistically significant increase for vehicle-treated mice (P = 0.0044, paired t-test), with 11 of 13 mice having increased degeneration in nerves from microbead-injected compared to contralateral control eyes. In telmisartan-treated mice, only 6 of 17 showed increased axon degeneration, with no significant difference between microbead-injected and control eyes (P = 0.36, paired t-test). Furthermore, as shown in Figure 5B, the percentage

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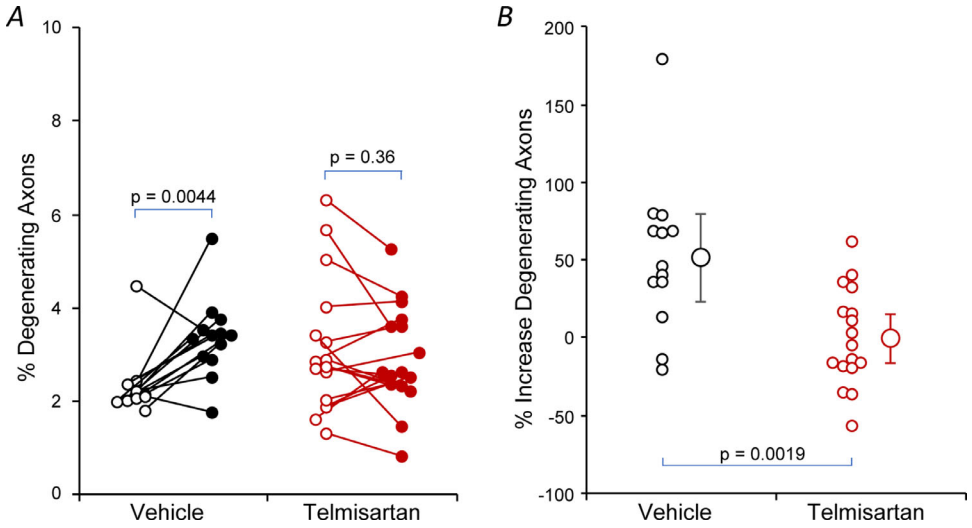

Figure 5. Degenerating axons. The percentage of axons undergoing degeneration was determined by manual counting (**A**) of nerves from vehicle-treated (black symbols) and telmisartan-treated mice (red symbols) and revealed a significant increase in the percentage of axons undergoing degeneration from microbead-injected eyes (closed symbols) as compared to their contralateral uninjected eyes (open symbols) for vehicle-treated mice ($P = 0.0044$, paired t-test), but not for telmisartan-treated mice ($P = 0.36$, paired t-test, lines connect paired eyes from individual mice). The percentage increase in % degenerating axons (**B**) in microbead-injected eyes compared to contralateral control eyes was significantly greater for vehicle-treated mice (black symbols) compared to telmisartan-treated mice (red symbols, $P = 0.0019$), with larger symbols with error bars representing mean and 95% CI. Vehicle-treated: $n = 13$, telmisartan-treated, $n = 17$.

increase in % degenerating axons in nerves from microbead-injected eyes was significantly greater in vehicle-treated as compared to telmisartan-treated mice (mean [95% CI]: vehicle: 49.0 [20.6 to 77.4]; telmisartan: −0.6 [−16.6 to 15.5] percent increase in % degenerating axons, $P = 0.0019$, Student's t-tests). The observed reduction in axon pathology in telmisartan-treated mice further supports a neuroprotective effect of telmisartan.

Discussion

Using a mouse model of glaucoma with sustained IOP elevation, this study provides evidence that telmisartan may be beneficial in treating glaucoma, as had been suggested by our previous study with normal mice.[17] We used the same drug delivery method as in our previous study in which we found reduced BP, indicating physiologically relevant systemic concentrations, as well as reduced IOP. Although BP was not measured in this study, we did find reduced IOP in telmisartan-treated mice, indicating efficacy of the drug delivery method. In the current study, we found that in response to experimentally induced IOP elevation, axon loss was significantly lower in mice treated with telmisartan compared to vehicle; 1.8% vs. 9.5% by manual counting and 2.9% vs. 14.2% by corrected AxonJ counts (Figs. 3C, 4E), suggesting that telmisartan protects against pressure-induced damage.

RGC axons undergo degeneration in response to IOP elevation, which initiates distally at their central synapses and slowly proceeds toward their cell bodies, resulting in persistent degenerating axons.[3] This retrograde degenerative process can increase the proportion of axons in the optic nerve that display pathology indicative of degeneration. As a complimentary approach to counting all axons, we determined the percent of axons that were undergoing degeneration. Counting degenerating axons entails identifying qualitative features indicative of degeneration in PPD-stained post-laminar cross-sections as used in this study, such as inclusion bodies in the axoplasm, dark staining of the axoplasm, and thickened myelin sheaths. To compensate for the subjective nature of this method, the counter was blinded as to the treatment status of the nerves. Microbead injection resulted in a 49.0% increase in the percentage of degenerating axons in vehicle-treated mice, which was prevented in telmisartan-treated animals that had a mean increase of -0.58% (Fig. 5B). The lack of an increase in ongoing axon degeneration in telmisartan-treated mice lends further support for a neuroprotective effect of telmisartan treatment.

The observed effect of telmisartan seems to be directly neuroprotective, rather than due to IOP reduction, because IOP responses to bead injection were similar in vehicle-treated and telmisartan-treated mice, with the average IOP over the experimental period of 19.9 mm Hg for vehicle and 20.8 mm Hg for telmisartan-treated mice, above uninjected control values of 16.5 mm Hg and 15.9 mm Hg, respectively (Fig. 2). Our previous study indicated that telmisartan and two other ARBs, irbesartan and losartan, were able to cross the blood/retinal barrier, which would allow for direct neuroprotective interaction of the drugs with RGCs.[17] Previous studies support neuroprotective effects of ARBs,[29] including telmisartan.[30,31] Specifically, in the retina, losartan was shown to protect against RGC death in retinal explants.[32]

Previously, we found that telmisartan lowered IOP in normal C57BL/6J mice over a 7-day treatment period.[17] In the present study, comparing IOPs in eyes that were not injected with microbeads, we found that telmisartan lowered IOP (Fig. 2B), consistent with our previous finding and extending the effect to 12 weeks of treatment. The reduction in IOP seen in this study was modest (5.8% averaged over the experimental time course), also similar to our previous study. Lack of progressive reduction of IOP would argue against long-term remodeling of the extracellular matrix of

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the trabecular meshwork as a possible mechanism of IOP lowering by telmisartan, because this would likely lead to enhanced lowering of IOP over the longer duration treatment in this study, which we did not observe. From an experimental point of view, reproducing the IOP-lowering effect in uninjected eyes confirm that the mice consumed enough of the telmisartan solid food to receive a physiologically effective dose. Although we did not investigate the mechanisms of telmisartan-induced IOP lowering, it must act either by decreasing aqueous humor outflow resistance, reducing aqueous humor production, or lowering episcleral venous pressure, or a combination of these mechanisms. However, in microbead-injected eyes, we saw no evidence of an IOP-lowering effect of telmisartan (Fig. 2C). Although our data do not rule out that bead-induced IOP elevation in telmisartan-treated mice could have been higher in the absence of telmisartan, the similarity of IOP responses in telmisartan- and vehicle-treated groups (Fig. 2C) argues against this possibility. A reasonable explanation for the apparent lack of an IOP-lowering effect of telmisartan on microbead-induced IOP elevation is that the physical blockage of the outflow pathway due to accumulation of microbeads in the corneoscleral angle has a much greater effect on IOP than does telmisartan.

The IOP responses of individual mice to bead injection were quite heterogenous both in magnitude and temporal pattern (Supplementary Fig.). Obvious sources of variability include variations in bead injection, variable responses to bead injections and variability in IOP readings. Heterogeneity is not unexpected in the microbead model, as such variability is apparent in other studies and may be intrinsic to this method that perturbs the equilibrium achieved by normal aqueous humor dynamics.[21,33] Despite this variability, averaging IOP across mice results similar responses to microbead injection for the vehicle-treated and telmisartan-treated mice (Fig. 2A), which included elevation to a peak between days 14 and 28, followed by a somewhat rapid decline to a lower elevation from day 35 to the end of the experiment on day 77.

To validate our results, total axon number was determined by two methods, each with their advantages and disadvantages: manually and using an automated counting program, AxonJ. Although manual counting is the standard by which automated methods are evaluated, it requires subjective identification of axons, and is very time-consuming. Calculation of the total axon number with the manual method (normal and degenerating axons included) assumes similar density throughout the nerve, which is somewhat problematic, especially for glaucoma, in which sectorial and focal loss of RGC axons is often a feature, although not observed in this study. To reduce subjective bias, we use a counting mask consisting of an array of boxes that is the same for all nerves, and the counter is blinded to treatment condition. AxonJ does not require subjective identification of axons and rapidly counts all axons in the nerve, except those with darkly stained axoplasm. Counting all axons avoids the equal density assumption but could still suffer from focal regions with severe degeneration where debris and activated glial cells may be erroneously counted as axons. Although well-validated in the original report,[28] in our hands, AxonJ undercounted compared to manual counts, with undercounting increasing with increasing axon number (Fig. 4A). Delineating outlines of axons in regions of high density is challenging, and, consistent with this, there was a strong correlation between undercounting and axon density (Fig. 4B), a relationship confirmed by inspection of counting masks generated by AxonJ showing multiple axons sometimes grouped as one, particularly in regions of high axon density. Although exclusion of axons with darkly stained axoplasm would contribute to undercounting by AxonJ compared to our manual counting that includes all axons, this could only account for a small proportion since we found that only ~2 to 3% of total axons were degenerating, whereas the average undercounting was ~20%. To compensate for undercounting, AxonJ counts were adjusted for axon density, which accounted for 71% of the variation in the AxonJ/manual count ratio ($r^2 = 0.70667$, Fig. 4B). Density-adjusted AxonJ counts yielded similar results to manual counting, indicating a protective effect of telmisartan against axon loss due to elevated IOP. Obtaining the same result using two different methods of identifying axons adds further validation to our conclusion that telmisartan protected against RGC axon degeneration.

Although our data are consistent with a direct neuroprotective effect, telmisartan also lowers systemic BP as shown in our previous study, and results in loss of body weight as shown in the current study (Fig. 1B). The relationship between BP and glaucoma is complex.[34] Although retrospective studies suggest a protective effect of lowering BP, possibly by lowering IOP, detrimental effects are also possible due to vascular dysregulation or decreased ocular perfusion pressure. Interestingly, ARBs, including telmisartan, have been shown to reduce body weight, as found in this study (Fig. 1B) and adipose tissue[35,36] and, furthermore, reduction of body weight and adipose tissue is associated with lowering of IOP.[37,38] However, the similarity of IOP responses to bead injection argues against IOP-mediated effects of weight loss or BP lowering as contributing to the observed neuroprotection.

In summary, our study using a mouse glaucoma model suggests that telmisartan provides neuroprotection against RGC degeneration in response to elevation of IOP. Telmisartan is a well-tolerated drug in common clinical use to treat systemic hypertension and should be further investigated as a potential treatment for glaucoma, especially in patients predisposed to both hypertension and glaucoma.

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