Analysis of Circadian Rhythm Gene Expression With Reference to Diurnal Pattern of Intraocular Pressure in Mice

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PURPOSE. To determine the expression of circadian rhythm clock genes in the iris-ciliary body complex of mice and their association with the diurnal pattern of intraocular pressure (IOP).

METHODS. Thirty wild-type C57BL/6 mice were acclimated to a 12-hour light-dark cycle. Intraocular pressure was measured with a rebound tonometer at six time points daily (circadian time [CT] 2, 6, 10, 14, 18, and 22 hours) for five consecutive days. On day 6, mice were euthanized at CT 2, 6, 10, 14, 18, and 22. Eyes were flash-frozen or fixed in 4% phosphate-buffered paraformaldehyde. Total RNA was extracted from the iris-ciliary body complex, and RNA expression of circadian rhythm genes *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2* was assessed by quantitative real-time PCR. Fixed eyes were paraffin embedded, and immunohistochemistry was performed to localize corresponding proteins (BMAL1, CLOCK, CRY1, CRY2, PER1, and PER2). Linear regression analysis was performed to correlate gene expression with IOP.

RESULTS. Intraocular pressure demonstrated a consistent circadian pattern. The clock genes *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2* showed a circadian pattern of expression in the iris-ciliary body complex of mice. *Bmal1*, *Clock*, *Cry1*, *Per1*, and *Per2* gene expression demonstrated statistically significant correlations with diurnal variations of IOP. BMAL1, CLOCK, CRY1, CRY2, PER1, and PER2 proteins were found to be expressed locally in the nonpigmented epithelium of the ciliary body.

CONCLUSIONS. Expression patterns of candidate circadian rhythm genes correlates with the diurnal pattern variation of IOP in mouse eyes, indicating a possible mechanism of IOP regulation through these genes.

Keywords: aqueous outflow, iris-ciliary body complex, clock genes, intraocular pressure, circadian rhythm

Elevated intraocular pressure (IOP) is a strong risk factor for primary open-angle glaucoma (POAG), and current standard of care focuses on the reduction in IOP as the primary modality to reduce optic nerve damage and prevent vision loss.¹ Characterization of molecular regulators of IOP would help identify novel molecules that can be used as therapeutic targets for the treatment of POAG.

Intraocular pressure is the result of a balance between secretion and outflow of aqueous humor, a colorless fluid in the anterior segment of the eye.² Three variables are of interest in this balance: the rate of aqueous humor formation, the resistance to outflow, and the episcleral venous pressure.³ The rate of formation and resistance to outflow are the primary targets for current drug therapy aimed at lowering IOP. The ciliary body is responsible for aqueous humor production, primarily via active secretion from its nonpigmented epithelium.⁴ Aqueous humor outflow is mediated through the trabecular meshwork (conventional outflow) and the uveoscleral region (uveoscleral outflow).⁵ The trabecular meshwork is responsible for approximately 75% of outflow resistance in humans and mice.^{6,7} Topical medications currently in use effectively lower IOP by decreasing ciliary body production of aqueous humor and/or increasing removal of aqueous humor

from the anterior segment of the eye through the trabecular meshwork and uveoscleral pathway.

Aqueous humor secretion is known to vary in a circadian manner, decreasing by up to 50% during sleep.⁸ Epinephrine may play a role in this change, but the mechanism behind decreased secretion remains poorly understood.⁹ Outflow facility also decreases slightly at night.^{10,11} However, the decrease in outflow does not appear to be significant enough to compensate for the decrease in aqueous secretion, and the cause of this decrease in outflow remains unknown.¹⁰ While the decrease in aqueous humor secretion and the slight decrease in aqueous humor outflow suggest a nocturnal normality or slight decrease in IOP, multiple studies have repeatedly demonstrated a sinusoidal pattern of IOP that peaks at night when animals are maintained on a 12-hour light-dark cycle.^{12–16} Interestingly, IOP is higher at night irrespective of diurnal or nocturnal habits of the animals, which suggests that IOP is regulated by light intensity and not by the level of activity.¹⁷ This circadian pattern of IOP is abolished in the absence of a distinct light-dark cycle.^{17,18} Previous studies to determine the cause of this pattern have demonstrated that impairments in the sympathetic nervous system abolish the nocturnal rise in IOP, implicating a role for adrenergic

stimulation in the circadian pattern.^{19,20} Additionally, exposure to short-wavelength light during the circadian dark phase eliminates the nocturnal IOP rise.²¹ Melatonin, which is synthesized by the ciliary epithelium in a circadian pattern with peak levels at night, has also been suggested to have an involvement in aqueous humor secretion from the nonpigmented epithelium.^{22–24} While the molecular mechanism for the diurnal variation in IOP is not yet known, these results suggest that the presence or absence of light may be a trigger for the molecular regulators of IOP. To examine this, we measured the diurnal variation in IOP of C57BL/6 wild-type mice and correlated this with clock gene expression in the iris-ciliary body complex.

MATERIALS AND METHODS

Animals

Thirty wild-type C57BL/6 mice (retired breeders; approximately 8 months old) were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice were housed at room temperature with food and water readily available. Average body weight of mice was 27.8 ± 2.8 g at the beginning of the experiment and 27.5 ± 3.0 g at the end of the experiment ($P = 0.7$). All mice were acclimated to a 12-hour light–dark cycle for 7 days before IOP measurements were initiated. All animal studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Mayo Institutional Animal Care and Use Committee (IACUC A42713).

IOP Measurements

Mice were gently restrained using a decapiCone (Braintree Scientific, Inc., Braintree, MA, USA), and IOP of right and left eyes was measured using rebound tonometry with an Icare TonoLab (Colonial Medical Supply, Franconia, NH, USA) as previously described.²⁵ Intraocular pressure was measured every 4 hours at circadian time (CT) 2, 6, 10, 14, 18, and 22 hours for five consecutive days. Intraocular pressure measurements during the light phase (CT 2, 6, and 10 hours) were taken under room lighting conditions while IOP measurements during the dark phase (CT 14, 18, and 22 hours) were taken under indirect red light, which has previously been shown not to interfere with the circadian cycle in humans.^{26–28} For each time point, four IOP measurements were recorded for both left and right eyes, and the average of the eight IOP measurements (four from each eye) was reported as that animal's IOP.

On day 6 after IOP measurements, mice were euthanized at CT 2, 6, 10, 14, 18, and 22 hours ($n = 5$ mice per time point) using a CO₂ chamber, in accordance with IACUC regulations. At each time point, seven eyes were flash frozen in liquid nitrogen and stored at -80°C . The remaining three eyes (from three separate animals) were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer.

Gene Expression

Whole, flash-frozen eyes were transitioned from -80°C to -20°C by soaking in RNAlater-ICE (Life Technologies, Carlsbad, CA, USA) for a minimum of 16 hours. The iris-ciliary body complex was dissected out, and total RNA was extracted using the RNeasy-Micro Kit (Life Technologies).

Following RNA extraction, 500 ng total RNA from each mouse was used to generate cDNA from iris-ciliary body RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Throughout the process, tissue

products from each individual eye were handled and stored separately.

Quantitative real-time PCR was performed using TaqMan probes (Applied Biosystems, Inc., Carlsbad, CA, USA) for *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2*. Polymerase chain reaction for all genes was performed in triplicate for five different cDNA samples per time point (CT 2, 6, 10, 14, 18, and 22 hours). Each cDNA sample originated from a different mouse. All gene expression data were normalized to 18s rRNA.

Immunohistochemistry

Paraformaldehyde-fixed eyes were embedded whole into paraffin and sectioned onto slides. Slides were deparaffinized and subjected to 10 minutes of antigen retrieval in a 1:1 mixture of 1 mM EDTA and 0.1 M sodium citrate at 95°C . After antigen retrieval, slides were incubated with blocking buffer (1% glycine and 1% ovalbumin in phosphate-buffered saline [PBS] with Tween 20) for 45 minutes at room temperature followed by 2-hour incubation with primary antibody at room temperature. Representative sets of six slides (CT 2, 6, 10, 14, 18, and 22 hours) were incubated with one of six primary antibodies: BMAL1 (1:100; Abcam, Cambridge, MA, USA), CLOCK (1:100; Abcam), CRY1 (1:25; Thermo Fisher Scientific, Rockford, IL, USA), CRY2 (1:100; Novus Biologicals, LLC, Littleton, CO, USA), PER1 (1:100; Abcam), and PER2 (1:100; Novus Biologicals, LLC).

Slides were washed with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:100; Life Technologies) for 1 hour at room temperature. Following secondary antibody incubation, slides were washed in PBS and mounted with Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Images were captured using a Zeiss LSM 510 confocal microscope and Zen 2009 digital image software (Carl Zeiss, Inc., Thornwood, NY, USA).

Statistical Analysis

Intraocular pressure and gene expression data were calculated as mean $\pm$ standard deviation. Peak and trough values for IOP and gene expression were evaluated by a two-tailed *t*-test. Intraocular pressure and gene expression data were correlated using linear regression analysis. *P* values ≤ 0.05 were considered statistically significant.

RESULTS

Intraocular Pressure

Intraocular pressure demonstrated a significant circadian rhythm ($P < 0.01$ for peak versus trough IOPs). Intraocular pressure of right and left eyes together was lowest at CT 2 (15.2 ± 0.2 mm Hg, $n = 30$), the first measured time point after lights on and highest at CT 14 (17.8 ± 0.2 mm Hg, $n = 30$), the first measured time point after lights off (Fig. 1). No significant difference between IOP of right eyes (16.6 ± 1.0 mm Hg) and left eyes (16.5 ± 0.9 mm Hg) was noted for individual CTs and for all time points combined ($P > 0.4$).

Gene Expression

Bmal1, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2* each demonstrated a significant 24-hour pattern of expression (Fig. 2). *Bmal1* and *Clock* trough expression occurred at CT 10, with peak expression between CT 22 and CT 2. *Cry1* gene expression peaked at CT 14 and troughed at CT 6. Finally, *Cry2*, *Per1*, and *Per2* gene expression peaked at CT 10 and troughed between CT 22 and CT 2.

FIGURE 1. Circadian pattern of IOP in C57BL/6 mice maintained on a 12-hour light-dark cycle. Measurements were taken by using a rebound tonometer. Intraocular pressure was highest at CT 14 (17.8 ± 0.2 mm Hg, $n = 30$) and lowest at CT 2 (15.2 ± 0.2 mm Hg, $n = 30$). Data are from the average of eight IOP measurements (four from each eye) and are expressed as mean $\pm$ SD.

Correlation of Gene Expression With IOP

Gene expression correlated with IOP (Fig. 3). *Bmal1* and *Clock* gene expressions were inversely related to IOP, while *Cry1*, *Cry2*, *Per1*, and *Per2* gene expression followed similar trends with IOP. *Cry1* peak gene expression occurred

concurrently with peak IOP, while gene expression of *Bmal1*, *Clock*, *Cry2*, *Per1*, and *Per2* preceded IOP by one CT time point. *Bmal1*, *Clock*, *Cry1*, *Per1*, and *Per2* gene expression had significant correlations with IOP ($P \leq 0.01$ for all genes). *Cry2* gene expression trended toward but did not meet statistical significance when correlated with IOP ($P < 0.1$).

FIGURE 2. Circadian pattern of clock gene expression in the iris-ciliary body complex of C57BL/6 mice. *Bmal1* and *Clock* were expressed antiphase to *Cry1*, *Cry2*, *Per1*, and *Per2*. All data were normalized to 18s rRNA. Data are expressed as mean $\pm$ SD.

FIGURE 3. Correlation of IOP with clock gene expression. All correlations were statistically significant ($P \leq 0.01$) except *Cry2* ($P = 0.07$). Gene expression changes occurred one time point prior to IOP change for *Bmal1*, *Clock*, *Cry2*, *Per1*, and *Per2*, but gene expression changed concurrently with IOP for *Cry1*.

Immunohistochemistry

BMAL1, CLOCK, CRY1, CRY2, PER1, and PER2 proteins showed localization to the cytoplasm of the nonpigmented epithelium of the ciliary body (Fig. 4). Time-variable expression was observed throughout the circadian cycle, which appeared to be related to gene expression. CLOCK protein expression peaked at CT 10 and troughed at CT 18, an approximate 8-hour delay from peak and trough gene expression (Fig. 4A). Similarly, BMAL1 peaked at CT 10 (Fig. 4B); CRY2 and PER1 peaked at CT 18 (Figs. 4C, 4D, respectively); and PER2 peaked between CT 14 and 18 (Fig. 4E); all peaking 4 to 8 hours after gene expression induction. CRY1 appeared to peak at CT 22 but had weak staining throughout the circadian cycle (Fig. 4F).

DISCUSSION

In diurnal and nocturnal animals, IOP is elevated at night but lowers during the day. While studies have shown that aqueous secretion and aqueous outflow decrease at night, the combination of these two effects suggests a decrease in IOP, not an increase. In this study, a consistent circadian pattern of IOP was observed in wild-type C57BL/6 mice that confirmed other patterns previously described in the literature.^{12–17} Intraocular pressure demonstrated a sinusoidal variation, with a trough early in the light phase and a peak early in the dark phase. This remains in contradiction to the expected decrease in IOP due to the known decrease in aqueous humor secretion at night without a significant compensatory decrease in

outflow facility. However, this idiosyncrasy might be explained by the timing of aqueous flow measurements, which have been taken during the midnocturnal period (2 AM–4 AM) or as an average over a longer period during the night in previous studies.^{10,11} It could be that this decrease in aqueous humor secretion is actually a compensatory response following an increase in IOP that occurred earlier in the night. These paradoxical findings suggest an alternative regulatory mechanism may be involved in IOP regulation during the day and night. The current study suggests that expression of circadian rhythm clock genes and proteins in the iris-ciliary body complex strongly correlate with a diurnal pattern variation of IOP. These results suggest that clock genes may be molecular candidates involved in regulating the diurnal pattern of IOP.

Circadian rhythm genes or clock genes are endogenous oscillators of circadian rhythm processes in mammals.^{29–31} *Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2* are well established clock genes whose protein products (BMAL1, CLOCK, CRY1, CRY2, PER1, and PER2) interact with one another in an autoregulatory feedback cycle that varies over the course of 24 hours.^{31–34} During the day, BMAL1 and CLOCK proteins form a complex that induces transcription of the cryptochrome (CRY) and period (PER) genes. At night, CRY and PER proteins form a heterologous dimer that translocates to the nucleus of the cell and represses CLOCK-BMAL1-mediated transcription.³⁵ While the master circadian rhythm clock is located in the suprachiasmatic nucleus of the brain, local circadian clocks have been identified in the immune system, liver, heart, adipose tissue, and retina.^{29,36–41} In the retina, these clocks function

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independently of the suprachiasmatic nucleus.³⁹ Moreover, previous *in vivo* work with *Cry1*^(-/-) and *Cry2*^(-/-) mice demonstrated that these clock genes were essential for maintaining the diurnal variation in IOP.⁴² Taking this into consideration, we hypothesized that the 24-hour diurnal pattern of IOP may be regulated by differential expression of clock genes in the iris-ciliary body complex.

The current study has shown that six core clock genes (*Bmal1*, *Clock*, *Cry1*, *Cry2*, *Per1*, and *Per2*) are expressed in the iris-ciliary body complex of mice, with protein products localized to the nonpigmented epithelium of the ciliary body. *Bmal1* and *Clock* gene expression show an inverse relationship with IOP while *Cry1*, *Cry2*, *Per1*, and *Per2* have a direct correlation with IOP. Furthermore, *Bmal1*, *Clock*, *Cry2*, *Per1*, and *Per2* expression changes preceded changes in IOP. From these results, it is tempting to speculate that clock genes may act in diurnal IOP regulation. Maeda et al.³⁸ showed that diurnal IOP response was eliminated in *Cry1*^(-/-) and *Cry2*^(-/-) knockout mice, indicating an importance of these genes in circadian control of IOP. This is consistent with our results that show gene and protein expression of *Cry1* and *Cry2* preceded or correlated with elevated IOP during the diurnal cycle. How gene expression changes in the clock genes may regulate IOP is unknown. While speculative, they may have a role in increasing aqueous humor secretion gradually throughout the light period, resulting in a subsequent rise in IOP early in the dark cycle. A change in clock gene expression during the dark period may then trigger a decrease in aqueous secretion, resulting in a gradual fall in IOP until the light period returns and the cycle repeats. While our results suggest a role of the clock genes in the iris-ciliary body complex, it is also possible that similar gene expression events are occurring in the outflow pathways. Future studies are warranted to determine whether there is a clock gene-mediated interplay between aqueous humor secretion and outflow facility.

This study also showed an apparent time-variable expression of corresponding clock proteins locally in the nonpigmented epithelium of the ciliary body. Moreover, protein expression appeared to relate to gene expression, with a 4- to 8-hour delay from changes in gene expression to changes in protein expression. This suggests that the variation in gene and protein expression can affect changes in activity in these cells throughout the circadian cycle and this further supports the hypothesis that these genes play a role in aqueous humor secretion. Although more quantitative methods such as western blotting would be required to confirm this circadian pattern of protein expression, our results support a hypothesis that clock genes may be involved in regulation of diurnal IOP.

Taken together, this study shows a correlation of local clock gene expression in the iris-ciliary body complex with circadian variation of IOP. These results suggest that clock genes expressed locally in the iris-ciliary body complex may function as molecular regulators of IOP. Future studies will be aimed at determining whether there is a causative relationship between clock gene expression in the ciliary body and IOP. If causation can be proven, clock genes could become molecular targets for novel glaucoma treatments.

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