Adrenal and Glucocorticoid Effects on the Circadian Rhythm of Murine Intraocular Pressure

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Purpose. Intraocular pressure (IOP) fluctuates with a robust circadian rhythm, which is synchronized to organismal rhythmicity through the master circadian clock located in the suprachiasmatic nuclei. The mechanisms maintaining circadian synchrony between the suprachiasmatic nuclei and IOP rhythms are presently unknown. The purpose of this study was to evaluate the necessity and sufficiency of adrenal and glucocorticoid function for the entrainment of iris-ciliary body (iris-CB) and IOP circadian rhythms in mice.

Methods. Iris-CB complexes were dissected from C57Bl/6J mice that were kept in 12-hour light/dark cycles at 3-hour intervals, and their core clock gene (*Per1*, *Per2*, and *Bmal1*) and glucocorticoid receptor mRNA expression were quantified. Iris-CB complexes from *period2::luciferase* mice were dissected and cultured to measure in vitro rhythmicity. To determine the phase-shifting effect of glucocorticoids on the iris-CB, dexamethasone or vehicle was added to the cultured tissues at defined circadian phases. The diurnal IOP rhythms of adrenalectomized or sham-operated mice under the 12-hour light/dark cycles were also measured.

Results. In iris-CB complexes, glucocorticoid receptor mRNA expression remained stable throughout the day, whereas the mRNA of core clock genes showed a robust circadian rhythmicity. Dexamethasone significantly induced phase-delays when administered between circadian time 8 (CT8) to CT12 and phase-advance when given between CT16 to CT20. Adrenalectomy abolished circadian IOP rhythmicity, particularly diminishing nocturnal IOP elevation compared with sham-operated mice.

Conclusions. Glucocorticoids are sufficient for phase shifting the circadian clock in iris-CB. Intact adrenal function is required for manifest circadian rhythms of IOP in mice. Taken together, these data are consistent with the hypothesis that glucocorticoids mediate circadian entrainment of IOP to the master circadian oscillator.

Keywords: circadian, glucocorticoid, adrenalectomy

Elevated intraocular pressure (IOP) is a major risk factor of glaucoma. In mammals, IOP has long been known to have a strong circadian rhythm.[1,2] The rhythmicity of mammalian IOP is entrained by the light/dark cycle, showing peak during the subjective night and trough during the subjective daytime.[3,4] This diurnal IOP fluctuation is altered in patients with glaucoma,[5] and abnormal circadian rhythmicity of IOP has been correlated with the progression of visual field defects in glaucoma.[6] Understanding the mechanisms by which the circadian clock affects IOP may permit new therapeutic approaches to aid in the prevention of glaucoma.

The mechanisms by which the circadian clock regulates IOP are not fully understood. Maeda et al.[7] showed that mice lacking core circadian clock genes *Cry1* and *Cry2* lose their IOP rhythm, demonstrating that this rhythmicity is dependent on the core circadian clock mechanism. Dalvin et al.[8] showed strong correlations between the IOP diurnal curve and core clock gene expression in the iris-ciliary body (iris-CB) complex, where aqueous humor is produced and at least partly participates in the creation of the IOP rhythmicity in mammals.[9,10] We previously reported that entrainment of the IOP circadian rhythm to light/dark cycles uses a systemic signal rather than local cues from the retina or cornea[11] (which may be entrained directly by light/dark cycle via neuropsin signaling[12,13]). This result strongly suggests that the master circadian clock (which resides in the suprachiasmatic nuclei [SCN] of the hypothalamus) synchronizes IOP rhythmicity, as has been suggested in previous reports.[14,15] Several studies have tested candidates for mediating factors between the master clock and the IOP rhythm, including sympathetic nerves[16,17] and melatonin,[18] but none have yet demonstrated the definitive signaling pathways by which circadian synchrony is mediated between SCN and the eye.

Glucocorticoids (primarily corticosterone in rodents and cortisol in human) are rhythmically secreted by the adrenal glands in an SCN-dependent manner, with peak at the onset of activity.[19] Pezük et al.[20] have demonstrated that glucocorticoids can act as entraining signals and stabilize circadian coordination of peripheral clocks in several tissues, including the cornea. Glucocorticoid receptors (GR) are abundant throughout the

body, including the anterior segment of the eye,[21] but not in the SCN, which makes glucocorticoids a strong candidate for mediating entraining signals between SCN and peripheral clocks.[22] In the present study, we examined whether adrenal function and glucocorticoid signaling may be involved in the regulation of ciliary body and IOP circadian rhythmicity.

Methods

Animals

C57Bl/6J (Jackson Laboratory, Bar Harbor, ME, USA) and *Per2::LUC* knock-in mice in the C57Bl/6J background[23] were used in this study. Mice were 2 to 6 months old, and males and females were selected randomly except for the circadian IOP measurement study, which was performed with male mice only to limit any sex effects due to the estrous cycle. Mice used for quantitative PCR and IOP measurements were kept in a 12-hour light/dark cycle for at least 2 weeks before experiments began. Otherwise, mice were kept in a 14-hour/10-hour light/dark cycle with light supplied by fluorescent light tubes (~120 $\mu W/cm^2$ at cage level). All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all animal experiments were conducted following approved protocols by the institutional animal care and use committee at the University of Washington (approval number, PROTO201600738).

Quantitative Real-Time PCR

C57Bl/6J mice were euthanized at 3-hour intervals ($n = 4$ for each time point) from circadian time 0 (CT0) to CT21 with CO_2 aspiration after acclimatization to 12-hour light/dark cycles for at least 2 weeks. Iris-CB complexes were immediately extracted from the eye in cold Hank's balanced salt solution (HBSS; Life Technologies Corp., Grand Island, NY, USA) and directly placed into RNAlater (Qiagen, Germantown, MD, USA). Dissection procedures during the light and dark phase were conducted under room lights and dim red lights, respectively. RNA was purified from tissues using TRI Reagent (Sigma-Aldrich Corp., St. Louis, MO, USA). A total of 200 ng of RNA from each sample was used to generate cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA).

Quantitative reverse transcription PCR was performed by using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher, Waltham, MA, USA) and an Applied Biosystems 7500 Real Time PCR system. cDNA was amplified with specific primer pairs described below. The PCR products were assessed by melt curve to confirm the appropriate amplification of targets. Gene expression was determined by the standard curve method. To compare gene expression in different time points, expression was normalized by beta-actin mRNA expression in the same sample. The following PCR primers were used for each gene target: *Per1*, 5′-CCC AGC TTT ACC TGC AGA AG-3′ and 5′-ATG GTC GAA AGG AAG CCT CT-3′; *Per2*, 5′-CCA ACA CAG ACG ACA GCA TC-3′ and 5′-TCT CGC AGT AAA CAC AGC CT-3′; *Bmal1*, 5′-GAC ATT TCC TCA ACC ATC AGC G-3′ and 5′-GCA TTC TTG ATC CTT CCT TGG T-3′; *GR*, 5′-ATG GCG TGA GTA CCT CTG GA-3′ and 5′-TCC AGA CCC TTG GCA CCT AT-3′; and *beta-actin*, 5′-AGG TGA CAG CAT TGC TCC TG-3′ and 5′-GCT GCC TCA ACA CCT CAA C-3′. The conditions of the PCR were as follows: 95°C for 10 minutes for the initial denaturation, followed by 40 cycles of 15 seconds at 95°C for denaturation and 60 seconds for annealing/extension at 60°C.

Real-Time Bioluminescence Recording and Analysis of Circadian Rhythm

Per2::LUC mice were euthanized by CO_2 aspiration 3 to 5 hours before lights off in a 14-hour/10-hour light/dark cycle, and tissues were immediately dissected in a cold HBSS solution (Life Technologies). The Iris-CB complex with supporting tissues (i.e., a small rim of associated sclera) was cultured on cell culture inserts (PICM0RG50; Millipore, Burlington, MA, USA) in sealed dishes with Dulbecco's modified Eagle's medium containing a B-27 supplement (Life Technologies), 352.5 μg/ml sodium bicarbonate, 10 mM HEPES (Life Technologies), 25 U/ml penicillin, 25 μg/ml streptomycin (Life Technologies), and 0.1 mM luciferin potassium salt (Biosynth, Staad, Switzerland). All tissue cultures were incubated at 37°C, and their bioluminescence were recorded for 1 minute at 7.5-minute intervals by using a Lumicycle luminometer (Actimetrics, Wilmette, IL, USA). The overall recording period was at least 8 days. The obtained bioluminescence was detrended by using a polynomial fit line to eliminate a steady decline of background bioluminescence. The period of oscillation was decided by best-fit sine wave analysis in the Lumicycle Analysis software.

Drug Treatment

Dexamethasone (DEX; Sigma-Aldrich Corp.), a synthetic glucocorticoid analogue, was dissolved in HBSS (10 μM). After 3 to 4 days of bioluminescence recording, dishes were gently taken out from LumiCycle, and 12 μl of the DEX solution (final concentration, 100 nM; $n = 38$) or 12 μl vehicle (HBSS; $n = 39$) was applied to the culture dishes. Cultures were resealed and placed in the same position as before the treatments. The culture dishes were kept in the luminometer without washing out the drugs. All the procedures were conducted under dim red light.

Construction of Phase Response Curve

The period of the baseline-subtracted data (τ) was determined by a best-fit sine wave analysis within the LumiCycle Analysis software package. The phase of the rhythm before a drug treatment was determined by the peaks in luminescence 3 days before the drug application. The time of estimated peaks following the treatments was calculated by adding the period in hours to the time of the last peak before the treatments. The phase of the rhythm after the drug applications was similarly determined by the time of three or more peaks of luminescence after the drug applications. Phase shifts were obtained by subtracting the time of observed peaks following the treatments from the time of expected peaks and were adjusted for CT (one circadian hour = τ/24). Positive and negative values of phase shift indicate phase advance and phase delay, respectively.

Adrenalectomy (ADX)

Bilateral ADX was performed on C57BL/6J mice ($n = 6$) in a dorsal approach under general anesthesia. Mice were fed with 0.9% saline solution ad libitum instead of water postoperatively due to resulting mineralocorticoid deficiency. Mice in the control group ($n = 7$) underwent sham surgery by using the identical procedure as ADX but without removing the adrenal glands. The IOP experiments on these mice were conducted at least 2 weeks after surgery. All surgical procedures were conducted in 11-week-old mice.

Monitoring of Wheel-Running Behavior

To determine whether ADX affects the systemic circadian rhythmicity, sham-operated and adrenalectomized mice were

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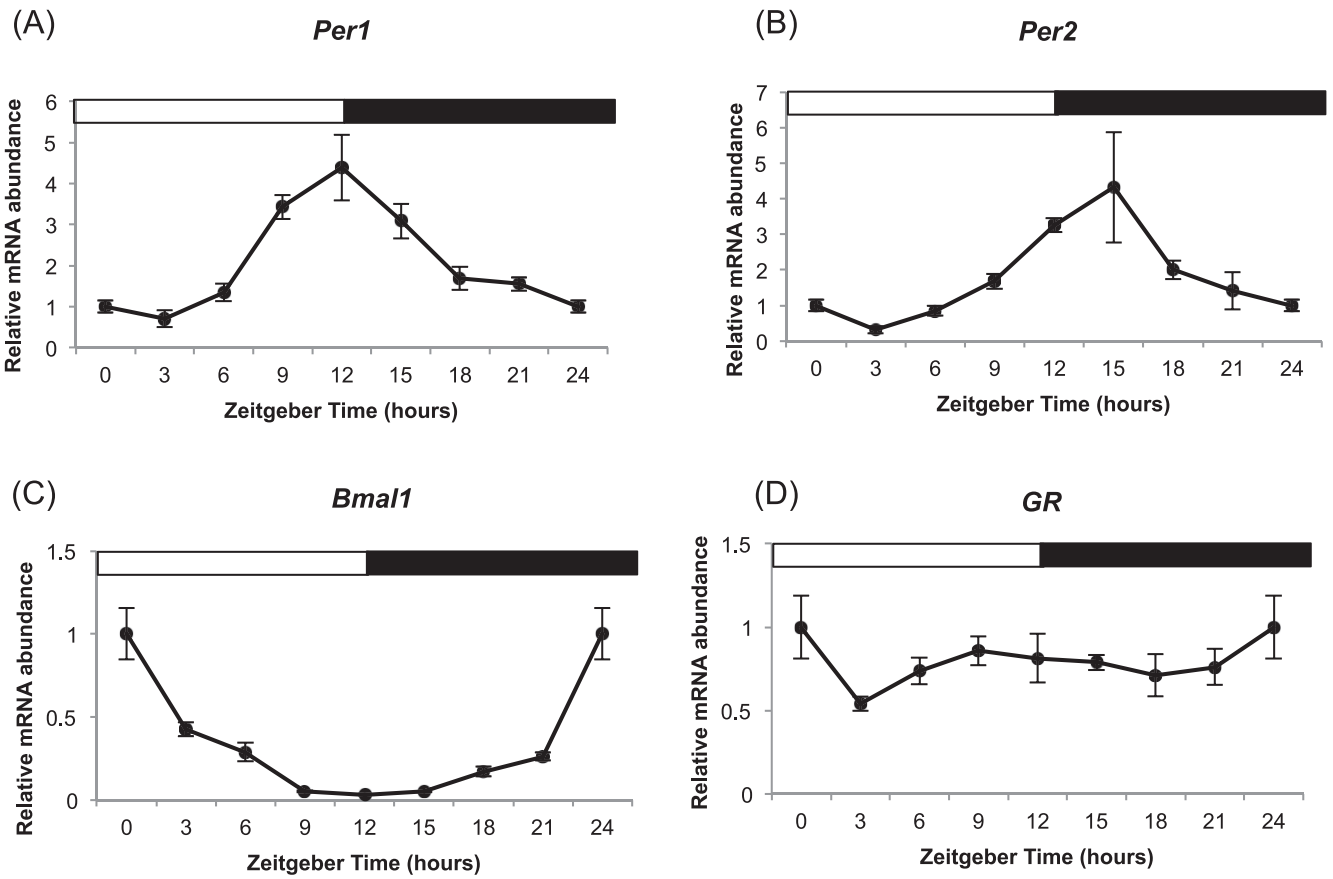

Figure 1. The diurnal mRNA expression of *Per1*, *Per2*, *Bmal1*, and *GR* in the mouse iris-CB complex at 3-hour intervals. (**A**) *Per1*, (**B**) *Per2*, and (**C**) *Bmal1* mRNA expression in the iris-CB complex showed robust circadian rhythmicity ($P < 0.001$; 1-way ANOVA). However, the level of *GR* (**D**) mRNA did not show a significant fluctuation. $n = 4$ for each time point. *Bars* represent means ± 1 SEM. The values at ZT0 were double plotted as the values at ZT24. The data at each time points were normalized to the value at ZT0.

kept in individual cages equipped with running wheels connected to a data collection device under 12-hour light/dark conditions. Locomotor activities and light/dark cycles were recorded by the Clock Lab data collection system (Actimetrics).

IOP Measurement

For IOP measurements, only male C57Bl/6J mice were used to avoid any effect from the estrous cycle. The IOPs of adrenalectomized or sham-operated mice were obtained with a rebound tonometer (Icare TonoLab; Icare Finland Oy, Espmoo, Finland) as previously described.[11] Unanesthetized mice were gently held using a decapiCone (Braintree Scientific, Inc., Braintree, MA, USA) and a molded plastic restrainer with velcro fasteners (catalog no. MR01C; Colonial Medical Supply, Windham, NH, USA). The IOP diurnal curve was obtained by measuring IOP at Zeitgeber time 2 (ZT2), 6, 10, 14, 18, and 22. All mice were kept in a 12-hour light/dark cycle more than 2 weeks before the IOP measurements began. ZT0 and ZT12 were determined by time of lights-on and lights-off, respectively. IOP measurements during the light phase were conducted under room lighting conditions, and the measurements during the dark phase were performed under dim red light conditions. At each time point, eight IOP measurements (four measurements in each eye) were obtained and the average of the values was considered as IOP of the mouse at that time point and used for further statistical analysis as described below.

Statistical Analysis

To analyze circadian variations of mRNA expression, 1-way ANOVA was performed. For exploring the differences between DEX and vehicle groups in the phase response curve experiments, 2-way ANOVA was performed. To compare the patterns of the diurnal IOP rhythms of sham-operated and ADX mice, 2-way repeated measures ANOVA was performed. In case of the significant results of an interaction effect between investigated factors in the 2-way ANOVA, 1-way ANOVA was performed separately for each group. Differences at each time points in phase-shift and IOP experiments were analyzed by Student's *t*-test with Bonferroni corrections for multiple comparisons. All statistical analyses were performed with SPSS version 23.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to indicate statistical significance.

Results

Clock Gene and Glucocorticoid mRNA Are Expressed in the Iris-CB Complex

Robust diurnal expression of *Per1*, *Per2*, and *Bmal1* in the iris-CB complex was observed by quantitative PCR (Figs. 1A, 1B, 1C; $P < 0.001$). An analysis of mRNA levels at 3-hour intervals showed that the *Per1* mRNA expression in the iris-CB reached circadian peak at ZT12 and circadian trough at ZT3. *Per2* mRNA expression in the iris-CB reached peak at ZT18 and

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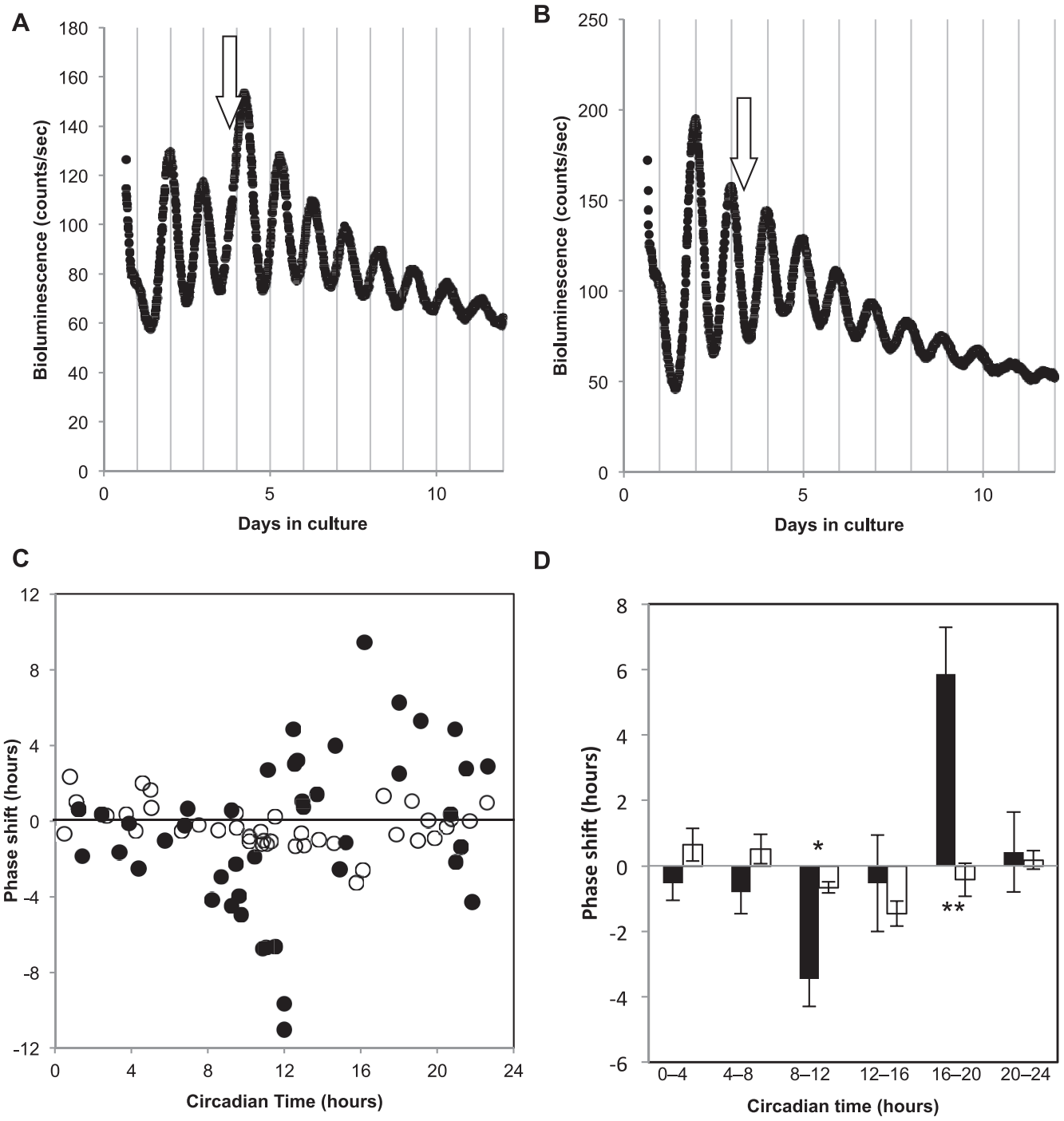

Figure 2. DEX phase shifts PER2::LUC bioluminescence of iris-CB complex ex vivo. Representative data of DEX (**A**) and vehicle (**B**) treatments are illustrated. *White arrows* indicate the time of the treatments. (**C**) Individual phase-shift results. *Black* and *white dots* indicate DEX and vehicle treatments, respectively, for individual experiments. (**D**) Aggregate phase-shift results. For statistical analysis, phase-shift results were divided into 4-hour interval bins. *Black* and *white bars* indicate DEX and vehicle treatments, respectively. $n = 4$ to 12 for each bin. $^{*}P < 0.05$, $^{**}P < 0.01$; Student's *t*-test with Bonferroni corrections. *Error bars* indicate standard deviations.

trough at ZT3. Thus, the diurnal rhythm of *Per1* mRNA preceded that of *Per2* mRNA. In addition, *Bmal1* mRNA expression showed an antiphasic pattern to those of *Per1* and *Per2*, which reached diurnal peak at ZT0 and the circadian trough at ZT12. This clock gene expression pattern was consistent with a previous report.[8] Conversely, the mRNA expression of GR was detected in iris-CB but did not show a circadian rhythmic pattern (Fig. 1C; $P = 0.279$).

DEX Phase Shifts the PER2::LUC Bioluminescence Rhythms of Iris-CB in a Phase-Dependent Manner

Robust circadian rhythms of iris-CB complex PER2::LUC bioluminescence were observed from all explants. The average circadian period in vitro before treatments was 23.7 ± 0.8 hours (mean ± SD; $n = 82$). Pilot experiments measuring phase shifts to DEX at CT12 suggested robust phase shifting

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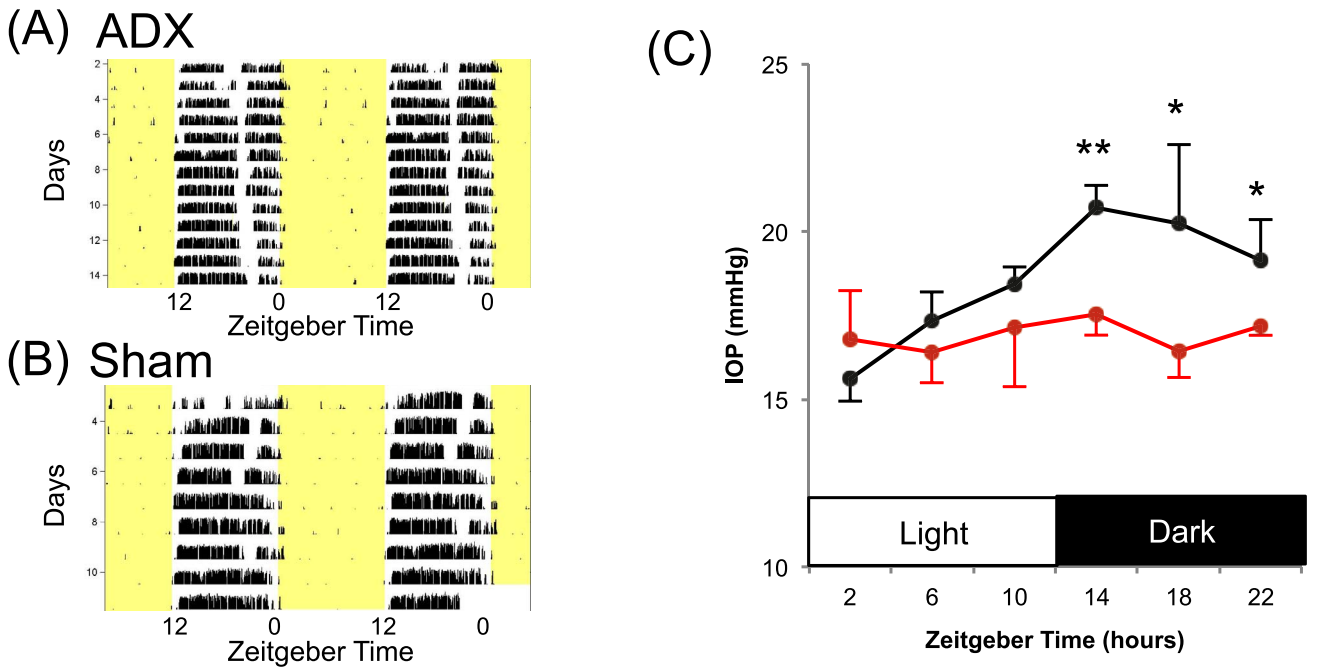

Figure 3. Effect of ADX on the mouse IOP rhythmicity. Representative actogram of wheel-running behavior of ADX (**A**) and sham-operated mice (**B**). *Black area* indicates time of day when the mouse was running in the wheel, and *yellow area* indicates times of day when lights were on. (**C**) The circadian IOP rhythmicity of ADX mice (*red line*) was abolished, whereas sham-operated mice (*black line*) showed a robust rhythmicity. $^{*}P < 0.05$, $^{**}P < 0.01$; Student's *t*-test with Bonferroni corrections. $n = 6$ for ADX group and $n = 7$ for sham group. *Error bars* indicate standard deviations.

with 100-nM concentration. Tissue toxicity in culture was observed at higher concentrations. Representative measurements of the circadian PER2::LUC activity of an iris-CB complex treated with 100 nM DEX and vehicle are illustrated in Figures 2A and 2B, respectively. As shown in Figures 2C and 2D, compared with the vehicle treatments, a significant shift delay by DEX treatments was observed at CT8 to 12 (–3.5 ± 2.9 hour; $P = 0.042$), whereas a significant shift advance by DEX treatments was observed at CT16 to 20 (5.9 ± 2.9 hour; $P = 0.006$) by using an analysis of 4-hour bins.

Adrenalectomized Mice Lose Diurnal IOP Rhythmicity

The ADX and sham-operated mice were behaviorally entrained by the external 12-hour light/dark cycle, as demonstrated by wheel-running behavior (Figs. 3A, 3B). A 2-way repeated measures ANOVA for IOP fluctuation revealed a significant interaction effect between ADX operation and time ($P = 0.001$). Therefore, the IOP fluctuation of each group was analyzed separately by 1-way repeated measures ANOVA. This analysis showed that the diurnal fluctuation of the IOP in ADX mice was dampened, whereas sham-operated mice showed that a robust IOP rhythmicity ($P = 0.007$), which showed peak at ZT 14, early in the dark phase, and trough at ZT 2, early in the light phase. The levels of IOP were significantly higher in sham-operated mice than ADX mice in the dark phase at ZT14, ZT18, and ZT22 ($P < 0.001$, $P = 0.036$, $P = 0.018$, respectively) but not in the light phase.

Discussion

The present studies reveal that the glucocorticoid DEX is able to substantially reset the circadian clock of the iris-CB complex ex vivo, and the removal of the adrenal glands results in the loss of the diurnal IOP rhythm in vivo while not affecting behavioral rhythmicity. These results are consistent with the hypothesis that glucocorticoids rhythmically secreted from adrenal glands act as an entraining signal for iris-CB and IOP rhythmicity.

In this study, the phase response analysis demonstrated that DEX treatments to cultured iris-CB caused a significant phase delay between CT8 and CT12 and a significant phase advance between CT16 and CT20. This biphasic phase response curve is consistent with nonparametric mechanisms of clock entrainment.[24] Plausible mechanisms for how glucocorticoids can shift the clock have been described. Glucocorticoids bind and activate GRs to bind to glucocorticoid response elements in DNA, which are located in the promoter regions of clock genes, such as *Per1* and *Per2*, and modulate gene transcription.[25] Additionally, circadian clock components such as CLOCK and CRY proteins can interact with the GRs and inhibit their activation.[26,27] A recent study showed that activation of GRs induces a rapid increase of *Per2* mRNA expression via the glucocorticoid response element and E-box in the *Per2* promoter region and results in phase shift.[28] Circulating glucocorticoids in mammals show a strong circadian rhythm that is dependent on the suprachiasmatic nucleus and the adrenal glands.[29] In the present study, *GR* mRNA expression in iris-CB was demonstrated. Taken together, these results suggest a model whereby the phase of the circadian rhythm of the iris-CB clock is set by the SCN via circulating glucocorticoid levels.

Consistent with this model, adrenalectomized mice showed a significant dampening of the circadian IOP rhythmicity compared with sham-operated mice. These results suggest that glucocorticoids, which are rhythmically released from adrenal glands at the onset of the activity (i.e., at CT12 of mice)[19] play a critical role in the regulation of the IOP rhythmicity. Weinreb et al.[30] demonstrated that oral DEX administration causes an acute IOP elevation in humans. In addition, intravenous

injection of DEX rapidly increased the IOP of dogs.[31] In rodents, IOP has been shown to peak during the dark phase,[3,4] which corresponds with the endogenous glucocorticoid peak. Given that the IOP of ADX mice was significantly lower than that of sham-operated mice in the dark phase, glucocorticoid may induce the nocturnal increase of the murine IOP rhythm.

Similar to our findings, a previous study showed that mice lacking core clock genes *Cry1* and *Cry2* also lost their circadian rhythmicity of IOP. However, unlike adrenalectomized wild-type animals, these mice do not show free-running behavioral circadian rhythms because CRY proteins are part of the core circadian transcriptional feedback loop in the master pacemaker of the suprachiasmatic nucleus.[32] In the present study, ADX mice maintained their behavioral rhythms, but their IOP clock was disturbed. This finding suggests that adrenal hormones mediate signals from the circadian master clock to the peripheral IOP circadian clock but do not affect the SCN, which is consistent with previous studies.[22]

The present study has several limitations. First, the ADX leads to the loss of not only glucocorticoid but also other adrenal cortex hormones such as aldosterone. These hormones are secreted in a circadian manner and might contribute to the circadian entrainment of IOP as well.[33] In future studies, the supplementation of adrenalectomized mice with timed pulse corticosteroid treatment with a diurnal measurement of IOP may allow us to determine if corticosteroids are sufficient to drive circadian rhythmicity in vivo as they appear to be in vitro. Second, in positing that circadian effects of glucocorticoids are mediated through the iris-CB, we assume that the primary effects are mediated through circadian changes in aqueous production. Aqueous humor outflow components (i.e., trabecular meshwork and uveoscleral outflow) are also known to be involved in the IOP circadian rhythm.[9,10] Unfortunately, it is not possible for us at present to culture sufficient quantities of trabecular meshwork cells to allow for bioluminescent circadian analysis, although recent advances in magnetic bead culture enrichment methods may make this feasible in the near future.[34] Third, the finding of arrhythmicity of IOP in adrenalectomized animals could be due to internal desynchrony of oscillators within the eye or due to loss of amplitude of the aggregate circadian rhythm. The present experiments cannot distinguish these possibilities.

In conclusion, we have demonstrated that glucocorticoids are sufficient to reset the iris-CB circadian clock and that adrenal gland function is necessary for manifest diurnal rhythmicity of IOP. These results suggest that circulating glucocorticoids may constitute a systematic timing cue from the SCN to the IOP clock as they do for other peripheral clocks. GRs within the eye might, therefore, be a therapeutic target for controlling the diurnal IOP fluctuation for glaucoma patients.

Acknowledgments

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