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Evidence Suggesting that the Cardiomyocyte Circadian Clock Modulates Responsiveness of the Heart to Hypertrophic Stimuli in Mice

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Abstract

Circadian dyssynchrony of an organism (at the whole body level) with its environment, either through light/dark cycle or genetic manipulation of clock genes, augments various cardiometabolic diseases. The cardiomyocyte circadian clock has recently been shown to influence multiple myocardial processes, ranging from transcriptional regulation and energy metabolism, to contractile function. We therefore reasoned that chronic dyssynchrony of the cardiomyocyte circadian clock with its environment would precipitate myocardial maladaptation to a circadian challenge (simulated shift work; SSW). To test this hypothesis, 2 and 20 month old wild-type and CCM (Cardiomyocyte Clock Mutant; a model with genetic temporal suspension of the cardiomyocyte circadian clock at the active-to-sleep phase transition) mice were subjected to chronic (16-wks) bi-weekly 12-hr phase shifts in the light/dark cycle (i.e., SSW). Assessment of adaptation/maladaptation at whole body homeostatic, gravimetric, humoral, histological, transcriptional, and cardiac contractile function levels revealed essentially identical responses between wild-type and CCM littermates. However, CCM hearts exhibit increased bi-ventricular weight, cardiomyocyte size, and molecular markers of hypertrophy (*anf*, *mcip1*) independent of aging and/or SSW. Similarly, a second genetic model of selective temporal suspension of the cardiomyocyte circadian clock (Cardiomyocyte-specific BMAL1 Knockout [CBK] mice) exhibits increased bi-ventricular weight and *mcip1* expression. Wild-type mice exhibit 5-fold greater cardiac hypertrophic growth (and 6-fold greater *anf* mRNA induction) when challenged with the hypertrophic agonist isoproterenol at the active-to-sleep phase transition, relative to isoproterenol administration at the sleep-to-active phase transition. This diurnal variation was absent in CCM

mice. Collectively, these data suggest that the cardiomyocyte circadian clock likely influences responsiveness of the heart to hypertrophic stimuli.

Keywords

Aging; Circadian Clocks; Hypertrophy; Shift Work

INTRODUCTION

Living organisms exhibit physiological time-of-day-dependent oscillations at multiple levels, both in anticipation of, and in response to, changes in the environment. Cell autonomous circadian clocks allow a cell/organ/organism to anticipate a forthcoming stimulus/stress, thereby facilitating a response of appropriate timing and intensity (Ederly 2000). At the core of the mammalian clock mechanism are two transcription factors, CLOCK and BMAL1 (Gekakis, et al. 1998; Hogenesch, et al. 1998; Takahashi, et al. 2008). Upon heterodimerization, CLOCK/BMAL1 activate a number of target genes, many of which will feedback in either a positive or negative manner, ultimately resulting in oscillations of target genes with a periodicity of approximately 24-hrs (Takahashi, et al. 2008). Target genes that are not integral clock components (i.e. do not feedback on CLOCK/BMAL1) are termed clock output genes (Ederly 2000; Takahashi, et al. 2008). Many of these output genes influence cellular processes, including metabolism, in a time-of-day-dependent manner (Marcheva, et al. 2009; Wijnen, et al. 2006).

Circadian clocks have been identified in nearly all mammalian cells investigated, including cardiovascular related cell types, such as cardiomyocytes, vascular smooth muscle cells, endothelial cells, and fibroblasts (Balsalobre, et al. 1998; Durgan, et al. 2005; McNamara, et al. 2001; Nonaka, et al. 2001; Takeda, et al. 2007). Although time-of-day-dependent rhythms in heart rate, blood pressure, and cardiac output have classically been attributed to rhythms in the neurohumoral milieu, recent studies have begun to highlight a role for circadian clocks as modulators of these cardiovascular parameters in both animal models and humans (Paschos, et al. 2010; Rudic, et al. 2009; Smolensky 1996; Young 2006). For example, genetic manipulation of circadian clock components, such as CLOCK and BMAL1, abolishes time-of-day-dependent oscillations in both blood pressure and heart rate (Curtis, et al. 2007). Similarly, variations within a tandem repeat of the human clock gene *Per3* is associated with alterations in heart rate (Viola, et al. 2008). More recently, several laboratories have initiated studies to elucidate the roles of cell-type specific circadian clocks on cardiovascular physiology. Genetic ablation of the circadian clock within endothelial or vascular smooth muscle cells results in marked attenuation of time-of-day-dependent rhythms in blood pressure and heart rate (Wang, et al. 2008). Through the use of a mouse model in which the cardiomyocyte circadian clock is temporally suspended at the beginning of the inactive/sleep phase (i.e., Cardiomyocyte-specific Clock Mutant [CCM] mice), we have shown that this molecular mechanism directly regulates myocardial gene expression, metabolism (e.g., triglyceride turnover), and contractile function (e.g., heart rate, cardiac output), in a time-of-day-dependent manner (Bray, et al. 2008; Durgan, et al. 2010b; Tsai, et al. 2010; Young 2009).

Given their role in a spectrum of cardiovascular functions, it is not surprising that disruption of circadian clocks has been implicated in the pathogenesis of various cardiovascular diseases (Rudic, et al. 2009; Young, et al. 2007). Circadian clocks are altered in numerous animal models of increased cardiovascular disease risk, including aging, diet-induced obesity, diabetes mellitus, hypertension- and pressure overload-induced hypertrophy, simulated shift work, and ischemia/reperfusion (i.e., myocardial infarction) (Durgan, et al.

2006; Kohsaka, et al. 2007; Kung, et al. 2007; Kunieda, et al. 2006; Mohri, et al. 2003; Young, et al. 2001; Young, et al. 2002). Importantly, circadian dyssynchrony of an organism (at the whole body level) with its environment, either through light/dark cycle or genetic manipulation, has been shown to augment the development of cardiovascular disease (Martino, et al. 2007; Martino, et al. 2008; Sole, et al. 2009). This includes shift work in humans (Knutsson, et al. 1986). What is less apparent is the relative role of cell-type specific circadian clocks in the pathogenesis of cardiovascular dysfunction. Initial studies designed to address this issue revealed that the cardiomyocyte circadian clock directly modulates myocardial ischemia/reperfusion tolerance in a time-of-day-dependent manner (Durgan, et al. 2010a). Whether this molecular mechanism influences responsiveness of the heart to additional adverse stresses is currently unknown.

The purpose of the present study was to test the hypothesis that chronic dyssynchrony of the cardiomyocyte circadian clock with its environment would augment myocardial maladaptation to aging and/or a circadian challenge (simulated shift work [SSW]). Somewhat surprisingly, wild-type and CCM mice responded to aging and SSW in essentially identical manners. However, in-depth phenotypic analysis revealed that CCM hearts exhibit greater mass, cardiomyocyte size, and expression of hypertrophic markers (*anf*, *mcip1*), independent of aging and/or SSW. Similarly, a second genetic model of selective temporal suspension of the cardiomyocyte circadian clock (Cardiomyocyte-specific *BMAL1* Knockout [CBK] mice) exhibits a pro-hypertrophic phenotype. Given that CCM and CBK hearts are temporally suspended at the beginning of the inactive/sleep phase, we hypothesized that the normal heart exhibits greatest responsiveness to pro-hypertrophic stimuli at this time. Consistent with this hypothesis, we observed that wild-type mice treated with isoproterenol at the awake-to-sleep transition exhibit greater cardiac hypertrophy and *anf* mRNA induction, relative to mice treated with isoproterenol at the sleep-to-awake transition. This diurnal variation was absent in CCM mice. Collectively, these data suggest that the cardiomyocyte circadian clock likely influences responsiveness of the heart to pro-hypertrophic stimuli.

MATERIALS AND METHODS

Animals

Specific disruption of the cardiomyocyte circadian clock was achieved through the use of two distinct genetic models; CCM and CBK mice. CCM mice express the Clock Δ 19 mutant protein within cardiomyocytes, through use of the MHC α promoter (Durgan, et al. 2006). CCM (MHC α -Clock Δ 19^{+/-}) mice are compared to wild-type (MHC α -Clock Δ 19^{-/-}) littermates. *BMAL1* was ablated in CBK mice, through crossing *BMAL1*-floxed mice (Jackson Labs) with MHC α -CRE mice (kind gift from Michael Schneider). CBK (*BMAL1*^{flox/flox}/MHC α -CRE^{+/-}) mice are compared to wild-type (*BMAL1*^{flox/flox}/MHC α -CRE^{-/-}) littermates. Male CCM mice (on the FVB/N background), CBK mice (on the C57/Bl6J background), and appropriate littermate controls were housed at the Centers for Comparative Medicine at Baylor College of Medicine (aging, and simulated shift work studies) or at the University of Alabama at Birmingham (isoproterenol administration studies). Male wild-type C57/Bl6J mice were also housed at the Comparative Biology Core Facility at Pennington Biomedical Research Center (PBRC; circadian aging studies). All studies were reviewed and approved by the respective local IACUC committees prior to the conduct of these studies. All mice were housed under controlled conditions (23 $\pm$ 1°C; 12-h light/12-h dark cycle), and received standard laboratory chow and water *ad libitum*, with the exception of the C57/Bl6J circadian aging study and the simulated shift work study. For the latter, mice were subjected to a bi-weekly 12-hr phase shift in the light/dark cycle for a total of 16 weeks. Accordingly, on the 4th day of the week the light phase was extended to 24-hrs, while on the 7th day of the week the dark phase was extended to 24-hrs. For these studies,

measurements were made in the middle of the light phase (i.e., zeitgeber time 6; ZT6), 3 days following the last reversal of the light/dark cycle. For the C57/Bl6J aging study performed at PBMC, mice were transferred from 12-h light/12-h dark cycle to constant darkness (dim red lighting) for the five days immediately prior to euthanasia, to facilitate identification of the effects of aging on intrinsic circadian clocks. These studies conform to international ethical standards (Portaluppi, et al. 2010).

Isoproterenol-Induced Cardiac Hypertrophy

Cardiac hypertrophy was induced pharmacologically, through administration of the β -adrenergic agonist isoproterenol. Mice (6 months of age) were injected intraperitoneally with either isoproterenol (5mg/Kg/day) or saline (vehicle control) on a daily basis, for a 1 week period. Injections were made either at the dark-to-light phase transition (i.e., ZT0) or at the light-to-dark phase transition (i.e., ZT12). Hearts were isolated exactly 24-hrs following the last injection, and stored at -80°C prior to subsequent biochemical analysis.

Non-invasive Mouse Monitoring

Body weight was monitored in mice at one-week intervals. Twenty-four hour patterns of body temperature (telemetry), energy expenditure (indirect calorimetry), and physical activity (beam breaks) were measured using a Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments Inc., Columbus, OH). For body temperature measurements, mice were allowed to recover for 2 weeks following intra-abdominal implantation of radiotelemetric probes, prior to data acquisition (using the Mini-Mitter system).

Humoral Factor Analysis

Non-fasted (i.e., fed state) plasma glucose, triglyceride, insulin, leptin, adiponectin, and corticosterone concentrations were measured using commercially available kits (Stanbio, Boerne, TX; Millipore, St. Charles, MO; MP Biomedicals, LLC, Orangeburg, NY).

Histological Analysis

Following perfusion with ice cold cardioplegic solution, hearts underwent Z-fix aqueous buffered zinc formalin perfusion fixation at constant pressure (approximately 16mmHg for 10 minutes). 5 μm longitudinal four-chamber view sections were subsequently obtained, mounted on slides, deparaffinized in xylene and rehydrated in 100%, 95%, 85%, 70% solutions of ethanol. For enzyme antigen retrieval, sections were treated with Proteinase K, 20 $\mu\text{g}/\text{ml}$ in 10mM Tris/HCl for 30 minutes at 37°C . After blocking with 5% goat serum in 1% bovine serum/PBS, sections were stained with laminin rabbit polyclonal antibody (Abcam #11575) at 4°C overnight, followed with goat anti-rabbit AlexaFluor 488 secondary antibody (Molecular Probes #11034), to demonstrate the basement membrane. Images of tissue in cross-sectional orientation were acquired (40x objective), total field size was measured, and myocytes were counted within the field to determine the average myocyte cross-sectional area. At least 100 myocytes were counted per heart. Picosirius Red staining of collagen fibers was used for semi-quantitative assessment of outer left ventricular wall fibrosis, using ImagePro software.

RNA Isolation and Real-Time RT-PCR

RNA was extracted from hearts using standard procedures (Chomczynski, et al. 1987). Candidate gene expression analysis was performed by quantitative RT-PCR using previously described methods (Gibson, et al. 1996; Heid, et al. 1996). Specific assays were designed for each gene from mouse sequences available in GenBank. Primer and probe

sequences have been reported previously (Bray, et al. 2008; Young, et al. 2001). RT-PCR data are represented as fold change from an appropriate reference group.

In Vivo Assessment of Cardiovascular Function

Myocardial contractile function was assessed *in vivo* in mice under anesthesia (isoflurane) through MRI (using a Bruker BioSpin MRI PharmaScan 70/16; 7.0 Tesla). Blood pressure was assessed through tail cuff measurements (using a Facility - IITC Inc./Life Science Non-Invasive Blood Pressure system).

Western Blotting

Qualitative analysis of protein expression was performed using standard Western blotting techniques. Briefly, powdered tissue (~20mg) was homogenized in tissue protein extraction reagent containing 2mM PMSF, 2mM EDTA pH 8.0, 0.25% NP-40 alternative with protease and phosphatase inhibitor cocktails. Lysates (10ug) were separated on a 7.5% bis-acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to a PVDF membrane and probed with Bmal-1 (1:2000 – Millipore), Cry-2 (1:2000 – Millipore) or Calsequestrin (1:4000 – Abcam) diluted in 1% Casein/PBS for 1.5 hours at room temperature. Membranes were incubated with goat anti-rabbit horse radish peroxidase-conjugated secondary antibody (1:10,000–1:40,000 – Santa Cruz). Bands were visualized with Immunstar Western C detection kit (Biorad) on x-ray film, scanned and quantified using Scion Image version 4.0.3.2 and normalized to Calsequestrin.

Statistical Analysis

For circadian aging studies, Cosinor analysis (Nelson, et al. 1979; Xu, et al. 2009) was performed using the Nonlinear Regression function of PASW 18 software with the following equation where the initial values and default parameter ranges were set manually:

$$f(t) = \text{Mesor} + A \cdot \cos[(2\pi t/T) + \text{Acrophase}]$$

Mesor (acronym for midline estimating statistic of rhythm) = the mean of the oscillation

A = the amplitude

T = the period (24 h)

Acrophase = the timing of the cosine maximum

t = a timepoint

R² is the resulting statistic that measures the percent variance accounted for by a 24 h rhythm (or rhythm of any period τ). When the cosinor analyses were performed based on the assumption that the free-running period is fixed at 24 h, the R² values for the expression levels of the three genes were as follows:

$$\begin{aligned} per2 \text{ (Young): } R^2 &= 0.737; per2 \text{ (Aged): } R^2 = 0.795 \\ bmal1 \text{ (Young): } R^2 &= 0.968; bmal1 \text{ (Aged): } R^2 = 0.968 \\ dbp \text{ (Young): } R^2 &= 0.899; dbp \text{ (Aged): } R^2 = 0.887 \end{aligned}$$

Individual cosinor parameters (mesor, amplitude, and acrophase) for each age group were compared for each gene with *t*-tests with significance ascribed at $p < 0.05$.

Two- and three- way analysis of variance (ANOVA) was performed to investigate the main effects of group (e.g., genotype, age, intervention) and time, using Stata version 10.0 (Stata Corp., San Antonio, TX). The null hypothesis of no model effects was rejected at $p < 0.05$. For analyses in which the full model was statistically significant (i.e., $p < 0.05$), Bonferroni *post-hoc* analyses were performed to examine all possible pair-wise comparisons. A full model including second-order interactions was conducted for each experiment. Statistically significant differences were determined using Type III sums of squares. Main effects of groups are reported in the top left hand corner of the figures, as well as in supplemental tables. Significant pair-wise comparisons are indicated directly within tables and figures, using appropriate symbols (as defined in legends).

RESULTS

Aging Influences Myocardial Circadian Clock Gene Expression in a Time-of-Day-Dependent Manner

We have previously investigated the rate of resynchronization of the circadian clock within the rat heart following reversal of the light/dark cycle; full resynchronization takes between 5 and 8 days (Durgan, et al. 2006). Although previously published studies have shown that the circadian clock in extra-cardiac tissues is altered with aging (Kunieda, et al. 2006), whether aging influences the circadian clock within the heart is unknown. To address this issue, circadian oscillations in myocardial circadian clock components were investigated in young (6 months) versus aged (24 months) wild-type mice. Cosinor analysis did not reveal any significant differences in amplitude, acrophase, or mesor between young versus aged hearts, for the circadian clock (*bmal1*, *per2*) and clock output (*dbp*) genes investigated, although a trend for a slight (≤ 1 hour) phase advance is noteworthy in aged hearts (e.g., $p = 0.066$ for a 0.68 hour phase advance in *bmal1*; Supplemental Table 1). Analysis of variance revealed significant time main effects for all genes investigated, independent of age (Supplemental Table 2). Subsequent post hoc pair-wise comparisons identified significant time-of-day-dependent age effects in expression of the genes investigated (Figure 1).

Adaptation of Wild-type and CCM Littermates to Aging and Simulated Shift Work

In order to investigate whether genetic disruption of the cardiomyocyte circadian clock predisposes towards development of myocardial contractile dysfunction in response to aging and/or manipulation of the light/dark cycle, 2 month old (young) and 18 month old (aged) wild-type and CCM mice were randomized into two major groups; control and SSW (simulated shift work). Control mice were housed in a normal 12-hr/12-hr light/dark cycle. SSW mice were subjected to a bi-weekly 12-hr phase shift in the light/dark cycle for a total of 16-wks (as described in the Materials and Methods section). Mice were therefore 6 months (young) and 22 months (aged) old at the end of the study. For 6 out of the 8 groups investigated, no mice died before the end of the study protocol. However, 1 mouse (out of 11) died in the aged wild-type SSW group, while 3 mice (out of 10) died in the aged CCM SSW group; in all four cases, the cause of death was euthanasia following development of overt infections.

The rate of re-entrainment of body temperature, energy expenditure, and physical activity rhythms following a 12-hr phase shift in the light/dark cycle were identical in wild-type versus CCM littermates (Figure 2A), consistent with the cardiomyocyte-restricted nature of the CCM model. Body weight during the 16-wk study was significantly higher in aged versus young animals, independent of genotype or light/dark cycle manipulation (Figure 2B). Significant main effects for age were also observed for body weight, lung weight, kidney weight, epididymal fat pad weight and bi-ventricular weight (all were higher in the aged mice compared to young mice, independent of SSW and genotype; Supplemental Table

3). A main effect of SSW was observed only for epididymal fat pad weight (higher in the SSW compared to control group, independent of age and genotype; Supplemental Table 3). Main effects of genotype were observed for epididymal fat pad weight, bi-ventricular weight, and bi-ventricular weight-to-body weight ratio in CCM mice independent of age and SSW (Supplemental Table 3). Interestingly, *post hoc* analyses (Table 1) demonstrated that bi-ventricular weight-to-body weight ratio is significantly higher in CCM hearts relative to wild-type hearts for 3 out of 4 possible pair-wise comparisons.

We next investigated whether age, SSW, and/or genotype altered distinct humoral factors known to influence whole body metabolic homeostasis and/or heart function (Supplemental Table 3 [main effects] and Table 2 [individual group data and *post hoc* pair-wise comparisons]). Of the humoral factors investigated, main effects of age were observed only for plasma glucose (higher in the aged versus young animals, independent of SSW and genotype; Supplemental Table 3). SSW had main effects on plasma triglyceride and corticosterone levels (both higher in the SSW group compared to the control group, independent of age and genotype; Supplemental Table 3). In contrast, none of the humoral factors investigated were influenced by genotype (Supplemental Table 3 and Table 2).

To characterize further wild-type versus CCM hearts following aging and/or SSW, histological and gene expression analyses were next performed. Consistent with bi-ventricular weight, three-way analysis of variance revealed main effects of age and genotype for myocyte cross sectional area (i.e., higher in aged and in CCM mice; Supplemental Table 3 and Figure 3A). However, only a main effect of age was observed for fibrosis; no significant main effects were detected in fibrosis for genotype or SSW (Supplemental Table 3 and Figure 3B). Gene expression analysis included measurements of established molecular markers of cardiac hypertrophy (wherein *anf*, *mcip1*, *mhcβ* are generally induced, while *serca2a* and *mhca* are generally repressed, in hypertrophied rodent hearts (Depre, et al. 1998)). Significant main effects for age were observed for *anf*, *mhcβ*, and *mhca* independent of SSW and genotype (Supplemental Table 3 and Figure 4). Main effects of SSW were also observed for *serca2a*, *mhcβ*, and *mhca* (increased with SSW, independent of age and genotype; Supplemental Table 3). Consistent with increased myocyte cross sectional area in CCM hearts (Figure 3A), three-way analysis of variance revealed main effects of genotype on *anf*, *mcip1*, *serca2a*, and *mhca* independent of age and SSW (Supplemental Table 3 and Figure 4). Importantly, Figure 4 shows similar transcriptional response patterns of wild-type and CCM hearts to aging and SSW.

MRI analysis was performed next, to investigate whether distinct adaptation of CCM hearts at gravimetric, histological, and transcriptional levels resulted in differences in myocardial contractile function. Somewhat surprisingly, only septal wall thickness was significantly different between CCM and wild-type groups, independent of age and SSW (Supplemental Table 3 and Table 3). Neither aging nor SSW significantly influenced any measures of myocardial contractile function, as assessed by MRI (Supplemental Table 3 and Table 3). Blood pressure measurements revealed no significant differences in this parameter between the investigated groups (Supplemental Table 3 and Table 3).

Selective Genetic Ablation of BMAL1 in Cardiomyocytes Results in a Pro-Hypertrophic Phenotype

Data presented thus far are consistent with the hypothesis that temporal suspension of the cardiomyocyte circadian clock at the beginning of the light phase (i.e., ZT0) results in a hypertrophic phenotype. These data are based on expression of the CLOCKΔ19 mutant protein in the cardiomyocytes of mice on the FVB/N background (i.e., CCM mice; see above). To gain further insight, we investigated a second novel mouse model of temporal suspension of the cardiomyocyte circadian clock through selective genetic ablation of

BMAL1 from cardiomyocytes (i.e., CBK mice) on the C57/B16J background. As anticipated, hearts from CBK mice show decreased expression of *bmal1* mRNA (Figure 5Ai) and BMAL1 protein (Figure 5Aii), versus wild-type hearts; residual expression is likely from non-cardiomyocyte cells present in intact hearts. Similar to CCM hearts (Figure 5Bi), CBK hearts (Figure 5Bii) exhibit attenuated *dbp* mRNA diurnal variations (Supplemental Table 4). Importantly, CBK hearts exhibit a significant increase in bi-ventricular weight-to-body weight ratio relative to wild-type littermates (Figure 5Ci), as well as increased expression of the hypertrophic marker *mcip1* (Figure 5Cii).

Diurnal Variation in Responsiveness of the Heart to the Hypertrophic Stimulus Isoproterenol

Results presented thus far suggest that temporal suspension of the cardiomyocyte circadian clock at ZT0 results in a pro-hypertrophic phenotype. These observations led to the hypothesis that the cardiomyocyte circadian clock increases responsiveness of the heart to pro-hypertrophic stimuli at the awake-to-sleep phase transition (i.e., ZT0). To address this hypothesis, wild-type and CCM mice were treated with the pro-hypertrophic agonist isoproterenol at one of two distinct times of the day; the awake-to-sleep phase transition (ZT0) or the sleep-to-awake phase transition (ZT12). Following seven consecutive days of isoproterenol treatment, three-way analysis of variance revealed main effects of time, treatment and genotype for bi-ventricular weight-to-body weight ratio (i.e., highest at ZT0 following treatment with isoproterenol, in CCM mice; Supplemental Table 5 and Figure 6A). The effect of isoproterenol treatment on bi-ventricular weight-to-body weight ratio was approximately 5-fold greater for wild-type mice treated with isoproterenol at ZT0 (21% increase; $p < 0.05$) versus ZT12 (4% increase; NS) (Figure 6A). In contrast, this time-of-day-dependence in the isoproterenol response was not observed in CCM hearts (9% increase at ZT0 [NS] versus 5% increase at ZT12 [NS]) (Figure 6A). Similarly, gene expression analysis revealed main effects of treatment and genotype for *anf* mRNA (increased with isoproterenol and in CCM hearts; Supplemental Table 5 and Figure 6B). An approximate 6-fold greater induction of *anf* mRNA was observed in wild-type hearts treated with isoproterenol at ZT0 (604% increase; $p < 0.05$) versus ZT12 (101% increase; NS), a diurnal variation that was again absent in CCM hearts (Figure 6B).

Diurnal Variation in Cryptochrome 2 in Wild-type, but not CCM and CBK, Hearts

We next decided to investigate the possible mechanism by which the circadian clock influences time-of-day-dependent oscillations in the hypertrophic response of the heart to the β -adrenergic agonist, isoproterenol. Possible mediators include the cryptochromes (CRY), integral clock proteins, of which CRY2 is the dominant isoform in the heart (Young 2006). CRY proteins have recently been shown to directly bind to GS α in the liver, thereby reducing adrenergic responsiveness (Zhang, et al. 2010). We report that CRY2 protein oscillates in a diurnal manner in wild-type hearts, with higher expression at ZT12 (Figures 7A and 7B). These time-of-day-dependent oscillations are absent in CCM and CBK hearts, wherein CRY2 protein expression is essentially suspended at ZT0 (Figures 7A and 7B).

DISCUSSION

The purpose of the present study was to investigate whether chronic circadian dyssynchrony of the heart with its environment (through genetic temporal suspension of the cardiomyocyte circadian clock) would augment maladaptation to a circadian challenge (i.e., aging and/or simulated shift work). Counter to this hypothesis, CCM mice exhibit essentially identical responsiveness to aging and SSW relative to wild-type littermates, at whole body homeostatic, gravimetric, humoral, histological, transcriptional, and cardiac contractile function levels. However, CCM hearts appear to have a pro-hypertrophic phenotype, as

observed through gravimetric, histological, transcriptional, and imaging analyses. Similarly, a second genetic model of selective disruption of the cardiomyocyte circadian clock (namely CBK mice) exhibits a pro-hypertrophic phenotype. To investigate further the potential role of the cardiomyocyte circadian clock in modulating responsiveness of the heart to hypertrophic stimuli, wild-type and CCM mice were treated with isoproterenol in a time-of-day-dependent manner. We report that isoproterenol induced cardiac hypertrophy and *anf* expression to the greatest extent when administered to wild-type mice at the awake-to-sleep transition (relative to administration at the sleep-to-wake transition). This diurnal variation in isoproterenol responsiveness is absent in CCM mice. Collectively, these data suggest that the cardiomyocyte circadian clock likely influences responsiveness of the heart to hypertrophic stimuli.

The primary function of cell autonomous circadian clocks is to confer anticipation of extracellular/environmental stimuli, thereby promoting synchrony of the cell/organ/organism with its environment (Edery 2000). As such, impairment or loss of this mechanism would likely promote maladaptive responses. With regards to the cardiovascular system, the recent exposure of circadian clocks as critical regulators of numerous aspects of cardiovascular function over the course of the day has led to speculation that impairment of these molecular timekeepers may precipitate cardiovascular disease (Young, et al. 2007). Evidence in support of this hypothesis is several fold: 1) circadian clocks are altered/impaired in many animal models of increased cardiovascular disease risk (e.g., DIO, diabetes, hypertension, shift-work, etc.) (Durgan, et al. 2006; Kohsaka, et al. 2007; Kung, et al. 2007; Kunieda, et al. 2006; Mohri, et al. 2003; Young, et al. 2001; Young, et al. 2002); 2) common environmental factors that are implicated as significant contributors towards cardiovascular disease in Western society (i.e., quality and quantity of calories ingested, physical activity, and sleep) are also known to be strong entrainment factors regulating circadian clocks (Burioka, et al. 2008; Damiola, et al. 2000; Kohsaka, et al. 2007; Zambon, et al. 2003); 3) genetic polymorphisms in human circadian clock genes have been shown to be associated with cardiovascular disease development, such as hypertension (Woon, et al. 2007); and 4) multiple animal models of environment- and genetic- induced perturbations in molecular clocks generally exhibit increased susceptibility towards cardiovascular disease development (Sole, et al. 2009). Several examples exist for the latter. This includes increased mortality in cardiomyopathic hamsters following light/dark cycle manipulation (Penev, et al. 1998). Similarly, Martino *et al* have reported that light/dark cycle manipulation augments myocardial dysfunction in mice subjected to pressure overload, an effect that could be reversed following re-entrainment to a normal light/dark cycle (Martino, et al. 2007). More recently, the same group has shown that housing heterozygous *tau* hamsters (that possess an altered circadian clock, with a periodicity of 22 hours) in a normal 24-hr light/dark cycle results in profound cardiorenal disease, which is absent when these rodents remain synchronized with their environment (by housing in a 22-hr light/dark cycle) (Martino, et al. 2008). Targeted genetic ablation of circadian clock components has also been shown to influence susceptibility of mice to adverse cardiovascular stresses, such as vascular injury and high salt intake (Anea, et al. 2009; Doi, et al. 2010).

Although a number of studies have been performed investigating whether disruption/impairment of circadian clocks in a ubiquitous fashion influences the etiology of cardiovascular disease, few studies have similarly reported a role for distinct cell-type specific clocks. Through the use of CCM mice, we recently revealed that the cardiomyocyte circadian clock directly influences myocardial ischemia/reperfusion tolerance, in a time-of-day-dependent manner (Durgan, et al. 2010a). However, the role of this clock in the etiology of cardiovascular disease in response to other clinically-relevant stressors remains unknown. To address whether the cardiomyocyte circadian clock plays a significant role in the development of cardiovascular disease in response to aging and/or light/dark cycle

manipulations (i.e., simulated shift work), we utilized a genetic mouse model wherein the heart is dyssynchronized with the rest of the organism, through temporal suspension of the cardiomyocyte circadian clock (i.e., CCM mice) (Young 2009). Contrary to expectation, wild-type and CCM littermates respond to aging and SSW in essentially identical manners (at whole body homeostatic, gravimetric, humoral, histological, transcriptional, and cardiac contractile function levels; Tables 1–3 and Figures 2–4). These observations suggest that extra-cardiac factors likely play a dominant role in the adaptation/maladaptation of the myocardium to these distinct stresses (i.e., aging and light/dark cycle manipulation). Such factors will likely include paracrine and endocrine factors, many of which are themselves under direct control of non-cardiomyocyte circadian clocks. For this reason, ubiquitous disruption of circadian clocks likely augments cardiovascular disease progression to a greater extent than selective temporal suspension of the cardiomyocyte circadian clock.

One potential limitation of this aspect of the present study is the fact that neither aging nor simulated shift work precipitated cardiac dysfunction (in wild-type or CCM mice). It is conceivable that further aging and/or extending the light/dark cycle manipulation regime for greater than 16 weeks, might induce contractile dysfunction. However, approximately 20% of the aged mice died within 16 weeks of the SSW regime, thereby limiting justification for prolongation. Importantly, previous studies have shown that manipulation of the light/dark cycle for this duration is a significant stress, particularly for aged mice (Davidson, et al. 2006).

During extensive characterization of wild-type and CCM littermates in the present study, we revealed a pro-hypertrophic phenotype in CCM mice, independent of age and SSW. More specifically, CCM mice exhibit a greater bi-ventricular weight-to-body weight ratio (Table 1), increased cardiomyocyte cross sectional area (Figure 3), increased septal wall thickness (Table 3), and a transcriptional profile indicative of cardiac hypertrophy (i.e., induction of *anf* and *mcip1*, as well as a repression of *mhca* and *serca2a*; Figure 4). Previous studies have shown that the genetic manipulation performed to disrupt the circadian clock can dramatically influence the resulting phenotype. For example, ubiquitous expression of the CLOCK Δ 19 mutant protein has been reported to result in a pro-obesity phenotype, while knockout of BMAL1 in a ubiquitous manner results in a lean phenotype (Bunger, et al. 2005; Turek, et al. 2005). Genetic background of the mouse model also significantly influences the phenotype observed; CLOCK Δ 19 mutant mice on the C57/Bl6J background are obesity-prone, while the same mice on either the ICR or CBA background are obesity-resistant (Kennaway, et al. 2007; Oishi, et al. 2006; Turek, et al. 2005). To ensure the pro-hypertrophic phenotype observed in CCM mice was neither model nor background specific, we investigated a novel, second mouse model of temporal suspension of the cardiomyocyte circadian clock on a distinct genetic background (i.e., CBK mice on C57/Bl6J background). Similar to CCM hearts (Young 2009), clock output from CBK hearts is essentially suspended at ZT0 (Figures 5Bi and 5Bii). Importantly, genetic disruption of the cardiomyocyte circadian clock in CBK mice was associated with a pro-hypertrophic phenotype (Figures 5Ci and 5Cii).

Given that CCM and CBK hearts both appear to be temporally suspended at the awake-to-sleep phase transition, we hypothesized that wild-type hearts may be more responsive to pro-hypertrophic stimuli at this time of the day. To test this hypothesis, mice were treated with the hypertrophic agonist isoproterenol at either the awake-to-sleep phase transition or the sleep-to-awake phase transition. Consistent with the hypothesis, wild-type mice exhibited greatest hypertrophic growth and *anf* induction when treated with isoproterenol at the awake-to-sleep phase transition (Figure 6). In contrast, this diurnal variation in responsiveness to isoproterenol was absent in CCM mice (Figure 6). Collectively, these data

support the hypothesis that the cardiomyocyte circadian clock modulates responsiveness of the heart to pro-hypertrophic stimuli in a time-of-day-dependent manner.

An important question relates to the identity of potential mechanisms by which the cardiomyocyte circadian clock modulates responsiveness of the myocardium to hypertrophic stimuli. Similar to our data regarding hypertrophic responsiveness, Collins and Rodrigo reported that the inotropic response of adult cardiomyocytes to isoproterenol exhibits a time-of-day-dependent variation (Collins, et al.). Isoproterenol is a β -adrenergic agonist (Lefkowitz, et al. 2000). Zhang *et al* recently revealed a mechanistic link between β -adrenergic signaling and the circadian clock in hepatocytes (Zhang, et al. 2010). Through a series of elegant studies, the investigators reported that cryptochromes (an integral clock component) can bind directly to Gs α , resulting in subsequent decreases in G-protein coupled receptor signaling (Zhang, et al. 2010). Consistent with such a model, we report that CRY2 protein levels exhibit a diurnal variation in wild type, but not CCM or CBK hearts, with approximate 1.5-fold greater levels at ZT12 (the time of day at which isoproterenol-induced hypertrophy was lowest; Figure 7A–B). These data are consistent with the hypothesis that the cardiomyocyte circadian clock influences myocardial β -adrenergic responsiveness, through time-of-day-dependent attenuation of Gs α by cryptochromes. However, it is important to note that additional potential molecular links may exist. For example, we have recently shown that the phosphorylation status of the growth signaling kinases Akt and GSK3 β oscillates in wild-type hearts, in a cardiomyocyte circadian clock dependent manner (Durgan, et al. 2010a); both Akt and GSK3 β are known modulators of cardiac hypertrophic growth (Heineke, et al. 2006). Whether CRY2, Akt and/or GSK3 β serve as molecular links between the cardiomyocyte circadian clock and hypertrophic growth requires full elucidation.

The findings of the present study have significant clinical implications. Non-dipping hypertensive patients (whose blood pressure drops <10% during sleep, and therefore exhibit inappropriate stress on the heart during the sleep phase) exhibit increased left ventricular hypertrophy and are at increased risk of cardiovascular and renal disease (compared to dipping hypertensive subjects) (Bianchi, et al. 1994; Hermida, et al. 2005; Palatini, et al. 1992). Similarly, obstructive sleep apnea (OSA) is associated with increased sympathetic stimulation on the heart during the sleep phase, adverse cardiac remodeling and increased risk of heart failure (Bradley, et al. 2009; Kario 2009). Recently, Sole and Martino hypothesized that the sleep phase corresponds to a time of increased myocardial renewal and growth, and as such pressure overload during this phase may accelerate the development of pathological hypertrophy (Sole, et al. 2009). Our findings are consistent with this hypothesis, and also suggest an important mediatory role for the cardiomyocyte circadian clock.

In conclusion, we report that genetic, temporal suspension of the cardiomyocyte circadian clock has little impact on adaptation of the mouse to aging and/or simulated shift work. However, these studies highlight a novel potential role for the cardiomyocyte circadian clock in modulating responsiveness of the heart to pro-hypertrophic stimuli in a time-of-day-dependent manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Circadian rhythms in expression of clock genes (*bmal1* (A) and *per2* (B)), as well as a clock output gene (*dbp*; C) in young (6 month old) versus aged (24 month old) mouse hearts. Values are expressed as mean $\pm$ SEM (n=3). * denotes $p<0.05$ for young versus aged mice at the same zeitgeber time.

Figure 2.

Re-synchronization of body temperature (i), energy expenditure (ii), and physical activity (iii) diurnal variations in wild-type versus CCM mice following a reversal of the light/dark cycle (LDR; panel A). Changes in body weight between young/aged, wild-type/CCM, and control/SSM mice during the 16 week protocol (panel B). Values are expressed as mean $\pm$ SEM (n=3–16).

Figure 3. Cardiomyocyte size (A) and fibrosis (B) assessment. Images (i) are representative of between 3 and 6 independent observations per group. Semi-quantitative values are expressed as mean $\pm$ SEM (n=3–6). # denotes $p < 0.05$ for young versus aged hearts within the same light/dark cycle and genotype groups. \$ denotes $p < 0.05$ for control versus SSW hearts within the same age and genotype groups. * denotes $p < 0.05$ for wild-type versus CCM hearts within the same age and light/dark cycle groups.

Figure 4.

Gene expression markers of cardiac hypertrophy in young versus old wild-type and CCM mice subjected to simulated shift work (SSW). Values are expressed as mean $\pm$ SEM ($n=3-7$). # denotes $p < 0.05$ for young versus aged hearts within the same light/dark cycle and genotype groups. \$ denotes $p < 0.05$ for control versus SSW hearts within the same age and genotype groups. * denotes $p < 0.05$ for wild-type versus CCM hearts within the same age and light/dark cycle groups.

Figure 5.

Phenotypic characterization of CBK, versus wild-type littermate, hearts. Gene (A) and protein (B) expression of BMAL1. Diurnal variations in *dbp* mRNA in CCM (C) and CBK (D) hearts. Biventricular weight-to-body weight ratio (E) and *mcip1* mRNA (F). Values are expressed as mean $\pm$ SEM (n=3–19). # denotes p<0.05 for ZT0 versus ZT12 hearts within the same genotype group. * denotes p<0.05 for wild-type versus CCM/CBK hearts within the same ZT group.

A)

B)

Figure 6.

Gravimetric (A) and gene expression (B) markers of cardiac hypertrophy in wild-type and CCM mice administered with isoproterenol in a time-of-day-dependent manner. Values are expressed as mean $\pm$ SEM (n=3–9). # denotes $p < 0.05$ for ZT0 versus ZT12 hearts within the same treatment and genotype groups. \$ denotes $p < 0.05$ for vehicle versus isoproterenol treated hearts within the same ZT and genotype groups. * denotes $p < 0.05$ for wild-type versus CCM hearts within the same ZT and treatment groups.

Figure 7.

Diurnal variations in CRY2 protein in CCM (A) and CBK (B) hearts. Values are expressed as mean $\pm$ SEM (n=5–6). # denotes $p<0.05$ for ZT0 versus ZT12 hearts within the same genotype group. * denotes $p<0.05$ for wild-type versus CCM/CBK hearts within the same ZT group.

Table 1

Mean values for gravimetric measures in post hoc comparison groups.

Parameter	Group [(Young/Aged)-(Control/SSW)-(Wild-type/CCM)]									
	Y-C-W	Y-S-W	A-C-W	A-S-W	Y-C-C	Y-S-C	A-C-C	A-S-C		
BW (g)	32.4 ± 1.6	31.0 ± 2.2	38.1 ± 2.5	36.6 ± 1.6 [#]	27.8 ± 1.7	32.4 ± 1.6 ^{\$}	34.0 ± 2.6 [#]	35.8 ± 1.6		
LW (mg)	156 ± 8	147 ± 11	197 ± 23 [#]	241 ± 57	170 ± 6	150 ± 10	197 ± 11 [#]	166 ± 19		
KW (mg)	226 ± 15	241 ± 22	269 ± 11	278 ± 11	231 ± 8	220 ± 16	269 ± 23	243 ± 11		
EFW (g)	0.94 ± 0.12	1.01 ± 0.11	1.35 ± 0.15	1.53 ± 0.18 [#]	0.49 ± 0.15 [*]	1.00 ± 0.15 ^{\$}	1.06 ± 0.19 [#]	1.41 ± 0.17		
BVW (mg)	173 ± 11	145 ± 6	183 ± 16	193 ± 14 [#]	198 ± 9	198 ± 19 [*]	215 ± 19	212 ± 11		
BVW/BW (mg/g)	5.36 ± 0.31	4.69 ± 0.17	4.78 ± 0.11	5.36 ± 0.55	7.27 ± 0.55 [*]	6.08 ± 0.40 [*]	6.37 ± 0.59 [*]	5.93 ± 0.32		

Definitions include: Y, young (6 month old) mice; A, aged (22 month old) mice; C, control light/dark cycle; S, simulated shift work (i.e., repetitive reversal of the light/dark cycle for 4 months); W, wild-type mice; C, cardiomyocyte-specific circadian clock mutant (CCM) mice; SSW, simulated shift work, light/dark cycle manipulation; BW, body weight; LW, lung weight; KW, kidney weight; EFW, epididymal fat weight; BVW, bi-ventricular weight; NS, not significant. Values are expressed as mean ± SEM (n=3-7).

[#] denotes p<0.05 for young versus aged hearts within the same light/dark cycle and genotype groups.

^{\$} denotes p<0.05 for control versus SSW hearts within the same age and genotype groups.

^{*} denotes p<0.05 for wild-type versus CCM hearts within the same age and light/dark cycle groups.

Table 2

Mean values for humoral factors in post hoc comparison groups.

Parameter	Group [(Young/Aged)-(Control/SSW)-(Wild-type/CCM)]							
	Y-C-W	Y-S-W	A-C-W	A-S-W	Y-C-C	Y-S-C	A-C-C	A-S-C
Glucose (mg/dl)	401 ± 29	238 ± 38 [§]	370 ± 36	366 ± 31 [#]	327 ± 32	243 ± 42	335 ± 47	313 ± 35
Triglyceride (mg/dl)	99 ± 18	100 ± 24	93 ± 23	237 ± 47 [§]	90 ± 31	217 ± 71	155 ± 24	175 ± 22
Insulin (ng/ml)	0.65 ± 0.10	0.38 ± 0.16	0.75 ± 0.26	1.72 ± 0.73	0.52 ± 0.11	0.74 ± 0.25	0.54 ± 0.11	0.49 ± 0.13
Leptin (ng/ml)	7.9 ± 1.3	9.8 ± 3.1	18.5 ± 9.2	13.9 ± 3.4	5.3 ± 1.0	20.4 ± 6.7	8.8 ± 1.2	11.3 ± 3.4
Adiponectin (µg/ml)	5.0 ± 0.6	4.5 ± 0.5	5.5 ± 0.8	4.4 ± 0.3	4.6 ± 0.6	5.0 ± 0.6	4.7 ± 0.6	4.3 ± 0.7
Corticosterone (ng/ml)	282 ± 16	312 ± 22	249 ± 23	327 ± 22 [§]	246 ± 24	344 ± 35	257 ± 18	280 ± 37

Definitions include: Y, young (6 month old) mice; A, aged (22 month old) mice; C, control light/dark cycle; S, simulated shift work (i.e., repetitive reversal of the light/dark cycle for 4 months); W, wild-type mice; C, cardiomyocyte-specific circadian clock mutant (CCM) mice; SSW, simulated shift work, light/dark cycle manipulation; NS, not significant. Values are expressed as mean ± SEM (n=3–7).

[#] denotes p<0.05 for young versus aged hearts within the same light/dark cycle and genotype groups.

[§] denotes p<0.05 for control versus SSW hearts within the same age and genotype groups.

Table 3

Mean values for *in vivo* cardiovascular function measures in post hoc comparison groups.

Parameter	Group [(Young/Aged)-(Control/SSW)-(Wild-type/CCM)]									
	Y-C-W	Y-S-W	A-C-W	A-S-W	Y-C-C	Y-S-C	A-C-C	A-S-C		
LVEF	0.62 ± 0.03	0.60 ± 0.05	0.68 ± 0.04	0.69 ± 0.05	0.65 ± 0.07	0.69 ± 0.05	0.56 ± 0.10	0.66 ± 0.03		
RVEF	0.53 ± 0.02	0.51 ± 0.04	0.55 ± 0.05	0.55 ± 0.05	0.49 ± 0.07	0.61 ± 0.04	0.51 ± 0.08	0.65 ± 0.03		
LVFS	0.39 ± 0.04	0.33 ± 0.04	0.40 ± 0.03	0.43 ± 0.05	0.45 ± 0.05	0.48 ± 0.08	0.40 ± 0.05	0.41 ± 0.03		
RVFS	0.40 ± 0.07	0.42 ± 0.07	0.48 ± 0.12	0.35 ± 0.10	0.32 ± 0.14	0.26 ± 0.06	0.32 ± 0.07	0.44 ± 0.06		
LVEDD (mm)	4.54 ± 0.05	4.31 ± 0.33	4.65 ± 0.18	4.62 ± 0.15	4.73 ± 0.32	4.66 ± 0.33	5.02 ± 0.20	4.74 ± 0.09		
RVEDD (mm)	1.83 ± 0.11	2.04 ± 0.18	1.74 ± 0.18	1.82 ± 0.14	2.02 ± 0.11	1.94 ± 0.26	1.96 ± 0.16	1.82 ± 0.18		
SWT (mm)	1.25 ± 0.07	1.13 ± 0.09	1.30 ± 0.10	1.20 ± 0.09	1.73 ± 0.16*	1.54 ± 0.26	1.56 ± 0.16	1.71 ± 0.19*		
OWT (mm)	1.02 ± 0.17	1.16 ± 0.11	1.12 ± 0.23	1.38 ± 0.22	1.07 ± 0.12	1.11 ± 0.08	1.32 ± 0.06	1.15 ± 0.25		
BP (mmHg)	129 ± 4	132 ± 4	133 ± 5	121 ± 6	129 ± 3	131 ± 6	139 ± 5	135 ± 10		

Definitions include: Y, young (6 month old) mice; A, aged (22 month old) mice; C, control light/dark cycle; S, simulated shift work (i.e., repetitive reversal of the light/dark cycle for 4 months); W, wild-type mice; C, cardiomyocyte-specific circadian clock mutant (CCM) mice; SSW, simulated shift work, light/dark cycle manipulation; LVEF, left ventricular ejection fraction; RVEF, right ventricular ejection fraction; LVFS, left ventricular fractional shortening; RVFS, right ventricular fractional shortening; LVEDD, left ventricular end diastolic diameter; RVEDD, right ventricular end diastolic diameter; SWT, septal wall thickness; OWT, outer wall thickness; BP, peak systolic blood pressure; NS, not significant. Values are expressed as mean ± SEM (n=3-6).

* denotes p<0.05 for wild-type versus CCM hearts within the same age and light/dark cycle groups.