Disturbed Diurnal Rhythm Alters Gene Expression and Exacerbates Cardiovascular Disease With Rescue by Resynchronization

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Abstract—Day/night rhythms are recognized as important to normal cardiovascular physiology and timing of adverse cardiovascular events; however, their significance in disease has not been determined. We demonstrate that day/night rhythms play a critical role in compensatory remodeling of cardiovascular tissue, and disruption exacerabate disease pathophysiology. We use a murine model of pressure overload cardiac hypertrophy (transverse aortic constriction) in a rhythm-disruptive 20-hour versus 24-hour environment. Echocardiography reveals increased left ventricular end-systolic and -diastolic dimensions and reduced contractility in rhythm-disturbed transverse aortic constriction animals. Furthermore, cardiomyocytes and vascular smooth muscle cells exhibit reduced hypertrophy, despite increased pressure load. Microarray and real-time PCR demonstrate altered gene cycling in transverse aortic constriction myocardium and hypothalamic suprachiasmatic nucleus. With rhythm disturbance, there is a consequent altered cellular clock mechanism (per2 and bmal1), whereas key genes in hypertrophic pathways (ANF, BNP, ACE, and collagen) are downregulated paradoxical to the increased pressure. Phenotypic rescue, including reversal/attenuation of abnormal pathology and genes, only occurs when the external rhythm is allowed to correspond with the animals’ innate 24-hour internal rhythm. Our study establishes the importance of diurnal rhythm as a vital determinant in heart disease. Disrupted rhythms contribute to progression of organ dysfunction; restoration of normal diurnal schedules appears to be important for effective treatment of disease. (Hypertension. 2007;49:1104-1113.)

Key Words: cardiac hypertrophy ■ renin–angiotensin–aldosterone system pathway ■ remodeling ■ gene expression microarrays ■ circadian

Cardiovascular disease is a major and increasing cause of death worldwide. Epidemiological studies suggest an important role for day/night rhythms in the cyclic variation of heart rate and blood pressure,1,2 timing of endocrine hormone secretion,3,4 and susceptibility to adverse cardiovascular events (including myocardial infarction,5,6 stroke,7 angina,8 ventricular arhythmias,9 dissection/rupture of aortic aneurysm,10 and sudden cardiac death11). Shift workers and patients with sleep disorders are at increased risk of adverse cardiovascular events and poorer prognosis.12,13 However, there are no experimental data actually linking disturbed diurnal rhythms with cardiovascular pathophysiology and remodeling postinjury. Thus, relevance of diurnal rhythms is routinely ignored in clinical medicine; for example, diurnal rhythms are disturbed when multibedded rooms are used in intensive care units, and time of day is infrequently considered relevant for drug treatment or the efficacy of contemporary interventional procedures.

Daily behavioral and physiological rhythms in mammals are driven by the circadian pacemaker in the suprachiasmatic nucleus (SCN) of the hypothalamus,15,16 which orchestrates a hierarchy of molecular clocks in tissues throughout the organism, including heart and vasculature.17-20 This endogenous system is composed of oscillating levels of nuclear proteins and genes that interact via autoregulatory feedback loops. Core components include clock, period (per1 and per2), aryl hydrocarbon receptor nuclear translocator-like Arntl (Bmal1), rev/erbes, cryptochromes (cry1 and cry2), casein kinase 1 epsilon (ck1ε), dec1, and dec2, which cycle to maintain biological control in the normal 24-hour day/night environment.21-23 A major yet relatively unrecognized issue is how this biological control and its disruption affect disease processes. This study is the first to examine a role for...
disturbed rhythms in the pathophysiology of cardiovascular disease and provides a new target for therapy that could markedly benefit remodeling in vivo.

**Methods**

**Animals**

Six-week-old male C57Bl/6 mice (20 g; Jackson) were entrained to a 12-hour light/12-hour dark cycle (LD 12:12) for 2 weeks. A total of 230 mice were used for these experiments: 122 with surgically acquired heart disease (cardiac hypertrophy) and 105 sham (SH); numerical differences take into account postsurgical mortality. Cardiac hypertrophy was induced mice anesthetized IP with ketamine (90 mg/kg) and xylazine (10 mg/kg) in saline solution. Animals were intubated (22G polyethylene IV catheter) and ventilated (0.3l/min O2, at a rate of 140 respirations per minute, using Harvard Apparatus model 687). A thoracotomy was performed in the second left intercostal space, aorta distal to the subclavian artery was cleared, and a silk suture (Ethicon 7-0) placed around a 27-gauge needle was used to constrict the arch. SH-operated animals underwent the same surgical procedure, except the ligature was not tightened.24 All of the animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. Activity was continuously recorded from animals in individual cages equipped with running wheels as required, using a Dataquest II data acquisition system coupled with ActiView Biological Rhythm Analysis Version 1.2 (Minimitter Co).

**RNA Preparation, Microarray Hybridization, and Analysis**

Animals were maintained after surgery under LD 12:12 for 4 weeks (lights on zeitgeber time=0; lights off zeitgeber time=12), euthanized, decapitated, and tissues (SCN, heart, and aorta) were collected every 4 hours starting at 1 hour before lights on, for 24-hour across the day/night cycle, for n=3 per time point (18 transverse aortic constriction [TAC] and 18 SH samples per tissue). Total RNA was prepared using Trizol reagent and was assessed for high quality by 1% agarose–formaldehyde gel electrophoresis and Agilent 2100 Bioanalyzer. Gene expression was performed using MOE430A murine arrays (representing 22,690 transcripts), in accordance with the manufacturer’s specifications (Affymetrix). Data were analyzed by COSOPT, an established wave-fitting algorithm used to analyze cosinar rhythms in microarray data, with multiple measures corrected (pMMC=β=0.1) to assess significance,25,26 along with RT-PCR for pertinent genes, as described previously (Affymetrix). Data were analyzed by COSOPT, an established wave-fitting algorithm used to analyze cosinar rhythms in microarray data, with multiple measures corrected (pMMC=β=0.1) to assess significance,25,26 along with RT-PCR for pertinent genes, as described previously (An expanded Supplemental Methods section is available in a data supplement available online at http://hyper.ahajournals.org). For rhythm disturbance studies, animals were maintained in LD 10:10 after surgery, and samples were collected for molecular analyses as described above, with n=3 mice per time (54 TAC and 54 SH total, see the data supplement).

**Histopathology, Echocardiography, and Hemodynamics**

In addition to the molecular studies listed above, mice were also dedicated for pathophysiologic analysis (33 TAC and 33 SH). For
Histopathology, tissues were fixed in 10% neutral-buffered formalin, processed, and stained with hematoxylin/eosin, Masson’s Trichrome, and/or Picrosirius Red staining. Tissues were collected from n=5 per phenotype. To measure cardiac function by echocardiography, mice were anesthetized with isoflurane gas, and transthoracic measurements were taken using Sequoia (Aquson) with a 13-MHz linear probe array. 2D M-mode images were acquired while the animal was in a semiconscious state using a high-resolution zoom with a sweep speed of 200 mm/s from the short axis view at the papillary muscle level. Fractional shortening percentage was calculated from the standard equation (left ventricular end-diastolic dimension−left ventricular end-systolic dimension)/left ventricular end-diastolic dimension×100%. For hemodynamics measurements, animals were anesthetized with isoflurane gas, and body temperature and heart rate were continuously monitored. The right common carotid artery was exposed and cannulated using a 1.4 French catheter (Millar), which was then fed to the proximal aorta and left ventricle. Data were acquired by an MP100 imaging system and analyzed using AcqKnowledge software (version 3.7.3; BIOPAC Systems, Inc). The number of animals used in each experiment is documented along with the data in Tables S1 and S2.

Results

Heart and Vessel Disease in Mice
To examine the role of diurnal rhythms in the pathophysiology of cardiovascular disease, a murine model of cardiac hypertrophy was created by TAC, under light anesthesia. The animals develop cardiac hypertrophy (Figure 1A and 1B), and there is an increased myocyte cross-sectional area and myocardial fibrosis (Figure 1C–1E). Gravimetrics are maintained in other peripheral organs.

Microarrays Demonstrate Conserved Global Gene Rhythms in Heart/Vessel Disease
Microarrays of gene cycling in SCN and cardiac tissues have been reported recently for healthy animals but, surprisingly, never for disease. We anticipated that, because circadian rhythms are essential to normal physiology, then altered cycling contributes to disease; remarkably, we found the converse result. Animals were euthanized 4 weeks post-surgery, tissues were collected across the diurnal cycle, RNA was purified, and gene expression was analyzed. At a conservative cutoff value (P<0.05 on every array), we found 8350 genes expressed in TAC heart (n=18), 8776 in SH (n=18), and 8017 common between data sets (91% to 96%). The slight difference is likely because of conservative cutoff values and because a few genes are downregulated at some times. Rhythms are identified by COSOPT and illustrated using radar diagrams. When plotted by peak phase of expression, genes in normal hearts exhibit a biphasic pattern that peaks once during the day and once at night. Remarkably, genes in hypertrophied hearts exhibit the same pattern, peaking at identical times as control tissues (Figure 2A and 2B and data supplement).

We next determined whether the pattern was cardiac specific or applied to vasculature as well. The same approach identified 9578 genes in TAC aorta, 9300 in SH, and 8667 (90% to 93%) common. Although global phase and gene composition differed from the heart and revealed a broader distribution with 3 peaks, the overall phase angle was again conserved. Profiles showed remarkable superimposition, even among individual genes (Figure 3). This first demonstration that global gene rhythms in disease can remain unchanged suggests that rhythmicity is an important contributing mechanism to compensatory hypertrophy.

Disturbing Central and Peripheral Clocks Exacerbates Heart Disease In Vivo
Because conservation of rhythmicity is important, as indicated by the results above, we anticipate that disturbing rhythms adversely affects disease. According to current paradigms, the master SCN clock is set daily by light; thus, to achieve rhythm disturbance, we altered the light cycle. Mice were randomized into either a 20-hour (LD 10:10) rhythm disruptive or 24-hour (LD 12:12) normal environment 24 hours after surgery. Wheel running activity, a documented entrainment to LD, and activity suppression in light (Figure 4A through 4C).

We found that rhythm disturbance adversely affected cardiac structure and function. We first examined cardiac pathology as a global measure of matrix remodeling that does not vary across the diurnal cycle. Twenty-four–hour TAC mice exhibited myocardial fibrosis, hypertrophy, and perivascular remodeling (primarily the smooth muscle layer in arterioles), as anticipated, in response to increased pressure. However, 20-hour mice exhibited markedly altered pathology. Abnormal thinning as opposed to hyperplasia of vessel walls quantified by digital scanning (*P<0.01) and reduced myocyte cross sectional area (*P<0.0001; Figure 4D and 4E). Cardiomyocytes and
vascular muscle cells in 20-hour TAC mice showed significantly less hypertrophy than 24-hour cohorts, in spite of the increase in blood pressure.

Echocardiography revealed differences in left ventricular dimensions in 20-hour versus 24-hour TAC mice and decreased fractional shortening indicative of reduced ventricular contractile strength (Figure 4F through 4H and Table S1). Hemodynamics revealed increased systemic blood pressure in 20-hour versus 24-hour littermates (Figure 4I and Table S2). Differences were not related to time of day, because circadian cabinets were synchronized so that all of the animals were examined zeitgeber time 10 to 14, and 24-hour versus 20-hour SH mice exhibited similar functional parameters. Moreover, structural-functional changes are reflected in tissue pathology, and these do not vary with time of day and are shown in Figure 5. Thus, taken together, findings of abnormal pathology, reduced contractility, and increased blood pressure demonstrate that diurnal rhythm disturbance adversely impacts on cardiac disease phenotype in vivo.

Rhythm Disturbance Alters Gene Expression Profiles in Heart and Brain

We used microarrays and RT-PCR to show that cyclic expression of key genes important in cardiac hypertrophy are altered with rhythm disturbance. This includes atrial natriuretic factor (ANF) and brain natriuretic factor (BNP), which are normally upregulated and compensatory in hypertrophy; angiotensin converting enzyme (ACE), which modulates blood pressure; and collagens involved in fibrosis (Figure 6A). Disturbing rhythms reduces gene expression, converse to what would be expected with increased pressure (Figure 6B).

Disturbing diurnal rhythms also affects the clock genes, consistent with the notion that altering LD affects circadian mechanisms. Per2 and bmal profiles changed in SCN (Figure 7A and 7B), and heart, although they remained in relative phase with each other (Figure 7C). In heart, rhythmic expression remained robust (peak/trough ratio) but with horizontal compression of the waveform likely reflecting different phase
relationships between circadian rhythm and zeitgeber (Figure 7A through 7C). Mechanistically, we suggest that altered gene cycling in SCN leads to altered gene cycling downstream in the heart and that this ultimately affects cardiac disease phenotype (Figure 8).

**Rescue of Abnormal Pathophysiology and Gene Cycling After Rhythm Restoration**

Lastly, we show that returning to 24-hour LD rescues animals from the adverse effects of rhythm disturbance. TAC animals in 20-hour LD for 8 weeks, then returned to 24-hour LD for
a further 8 weeks, demonstrate a return to consolidated activity (Figure 4C). There is attenuation/reversal of myocardial pathology, including perivascular fibrosis and myocyte hypertrophy (Figures 4D, 4E, 5A, and 5B). Cyclic expression of genes important to hypertrophy is restored, including that for ANF, BNP, ACE, and collagen, similar to 24-hour TAC cohorts (Figures 6A, 6B, 8A). Finally, and importantly, per2 and bmal gene profiles in SCN and the heart are also restored, consistent with the notion that clock disturbance can be rescued (Figures 7 and 8A). With appropriate circadian manipulation, there is restoration of molecular gene rhythms, with beneficial cardiac remodeling in vivo (Figure 8B).

**Discussion**

In this study, we demonstrate for the first time a role for diurnal rhythms in remodeling myocardium in cardiovascular disease using a model of pressure overload–induced cardiac hypertrophy in mice. We initially describe the effect of compensated heart and vessel disease on cognate cardiovascular clocks. We further demonstrate conservation of global gene rhythms in remodeling myocardium, the first ever demonstration of global gene cycling in heart disease using microarrays and bioinformatics analyses. Although hypertrophy is viewed as disease, initially it is also an adaptive response allowing the organism to survive with cardiac stress.
(eg, hypertension), and maintenance of normal biological rhythmicity is key to this adaptive response.

Next, we show that disturbing rhythms has a devastating effect on the disease phenotype. Surprisingly, this is also the first demonstration of the remarkably adverse consequences of rhythm disturbance on a disease process outside of the brain. For these experiments, rhythms are disturbed by altering light:dark cycles. We chose this approach because it is consistent with current paradigms of a hierarchical system in which SCN sets the pace and drives oscillations in peripheral tissues in vivo. Also, this approach obviates pleiotropic gene effects, as could happen with mutant or knockout models. Using this model, we show that, with rhythm disturbance, cellular hypertrophy is profoundly decreased, and cardiac compensation is reduced. These observations support our hypothesis that rhythmicity is integral to compensatory hypertrophy and normal remodeling. This has particular relevance to humans, indeed, people with heart disease are often subjected to altered LD, such as in shiftwork, jetlag, sleep disorders, or even in intensive care units.

In terms of physiological mechanism, many of the myocardial changes may be ascribed to increased blood pressure. This is relevant to translational medicine, because human hypertension is associated with sleep disturbances, particularly sleep apnea. At a molecular level, altered gene expression provides a second mechanism that likely also contributes to myocardial change. With rhythm disturbance, clock genes per2 and bmal and cardiac remodeling genes BNP, ANF, ACE, and collagens exhibit gene expression that is paradoxically altered opposite to what one would expect from an increase in blood pressure alone; it is inappropriate to the pressure load.

Importantly, we also show rescue of the adverse affects on cardiovascular health by resynchronization of rhythms. When rhythm disturbance was rescued, there was reversal/attenuation of abnormal pathophysiology. The normal disease phenotype was restored, including appropriate compensatory cellular hypertrophic responses. It was striking that in 20-hour TAC hearts, myocytes in heart and vessel wall exhibited less hypertrophy and more fibrosis than in 24-hour TAC.

Figure 6. Rhythm disturbance alters hypertrophic gene expression. (A) ANF, BNP, ACE, and Col3a1 gene expression. Twent-four–hour samples plotted on primary y axis (top graphs); green, 24-hour SCN; black, 24-hour SH heart; red, 24-hour TAC heart; blue, 20-hour to 24-hour rescue TAC hearts. Twenty-hour samples plotted on secondary y axis (bottom dotted graphs); red, 20-hour TAC heart; black, 20-hour sham. (B) Mean expression profile/cycle, significant reduction at peak time of expression in 20-hour versus 24-hour TAC. *P<0.05.
hearts in spite of the increase in pressure load. Restoration of the normal 24-hour diurnal rhythm led to regression of fibrosis and further myocyte hypertrophy. With return to a normal synchronous 24-hour environment, gene profiles were also reestablished in cellular clock and hypertrophic pathways. Thus, appropriate tissue compensation occurred, but only when the external rhythm was allowed to correspond with the animals’ innate internal rhythm, that is, return to a 24-hour day. This further underscores the notion that normal diurnal rhythms are crucially important in remodeling processes in cardiovascular disease.

Finally, there are several key areas elucidated by this study that obviously merit further investigation. For example, the affects of altering L:D cycling of cardiac proteins is not known, although they would undoubtedly prove interesting. Indeed, the phosphorylation status of glycogen synthase kinase-3β is important to both the core circadian clock mechanism and development of cardiac hypertrophy and may contribute to the adverse remodeling that occurs. Also, key proteins in the renin–angiotensin–aldosterone system cycle and perturbations of renin–angiotensin–aldosterone system rhythms in heart disease have never been investigated but could be highly relevant to humans.

We have almost no understanding of relationships between a disease, such as heart or blood vessel disease, and the relevant peripheral and central clocks. Save for sleep apnea, the issue of sleep or biological rhythms is rarely considered as a contributing pathophysiologic factor. Here we show that altering rhythms can have major adverse effects on cardiovascular health and probably other disease processes as well. This should open discussion among basic researchers, clinicians, and, ultimately, industry, the military, and the general public, where the importance of work schedules and sleep have been part of contemporary discourse.

**Perspectives**

Here, we present a novel mechanism contributing to cardiovascular disease, disturbed diurnal rhythms; there are important clinical implications from this study that demand further consideration and evaluation. Clinicians mostly ignore diurnal rhythms; however, as this study shows, there are profound adverse cardiovascular consequences with rhythm disturbance. Moreover, daily rhythms, such as sleep, are viewed mainly from a neuroscience perspective, which should be re-evaluated in light of our results in the cardiovascular system. There are also additional implications for specific demographic groups. For example, rhythms are disturbed in shiftworkers and patients with sleep disorders, and these are associated with an increased risk of adverse cardiovascular events and poorer prognosis. Effects of aging also warrant investigation. The elderly exhibit sleep disorders, and recent studies have shown that senescence impairs cellular circadian gene expression; one would be expected to iteratively compound the other. Patients in intensive care units, particularly in multibedded rooms, are usually exposed to abnormal light:dark cycles at a time when organ repair is critical. Finally, contemporary drug testing does not routinely consider time of day. Drug studies are conducted in the day, for convenience of both scientists and subjects. However, as this study showed, gene expression exhibits a unique phase for each tissue, and this could have obvious impact on timing of therapy. For example, the rapidly emerging field of chrono-
therapeutics relies on delivering therapeutics at an optimal time in coordination with daily physiology; further benefits could arise by coordinating to heart- or vessel-specific diurnal phase. Appreciating relationships between central and peripheral clocks and normal versus abnormal organ physiology will have significant impact relevant not only to cardiovascular health but also to the general treatment and prognosis of human disease.

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Disclosures
None.

References


26. Martino et al Disturbed Diurnal Rhythm Exacerbates Disease 1113


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