Sustained Hemodynamic Stress Disrupts Normal Circadian Rhythms in Calcineurin-Dependent Signaling and Protein Phosphorylation in the Heart


Rationale: Despite overwhelming evidence of the importance of circadian rhythms in cardiovascular health and disease, little is known regarding the circadian regulation of intracellular signaling pathways controlling cardiac function and remodeling.

Objective: To assess circadian changes in processes dependent on the protein phosphatase calcineurin, relative to changes in phosphorylation of cardiac proteins, in normal, hypertrophic, and failing hearts.

Methods and Results: We found evidence of large circadian oscillations in calcineurin-dependent activities in the left ventricle of healthy C57BL/6 mice. Calcineurin-dependent transcript levels and nuclear occupancy of the NFAT (nuclear factor of activated T cells) regularly fluctuated as much as 20-fold over the course of a day, peaking in the morning when mice enter a period of rest. Phosphorylation of the protein phosphatase 1 inhibitor 1 (I-1), a direct calcineurin substrate, and phospholamban, an indirect target, oscillated directly out of phase with calcineurin-dependent signaling. Using a surgical model of cardiac pressure overload, we found that although calcineurin-dependent activities were markedly elevated, the circadian pattern of activation was maintained, whereas, oscillations in phospholamban and I-1 phosphorylation were lost. Changes in the expression of fetal gene markers of heart failure did not mirror the rhythm in calcineurin/NFAT activation, suggesting that these may not be direct transcriptional target genes. Cardiac function in mice subjected to pressure overload was significantly lower in the morning than in the evening when assessed by echocardiography.

Conclusions: Normal, opposing circadian oscillations in calcineurin-dependent activities and phosphorylation of proteins that regulate contractility are disrupted in heart failure. (Circ Res. 2011;108:437-445.)

Key Words: calcineurin ■ circadian rhythms ■ heart failure ■ RCAN1/MCIP1

Circadian rhythms are self-sustaining, 24-hour cycles in molecular, biochemical, and behavioral parameters that help an organism prepare for anticipated changes in physiological demand. Many important cardiovascular factors, including metabolism, heart rate, blood pressure, and hormone release, oscillate over a 24-hour period. In humans, the incidence of adverse cardiac events, such as myocardial infarction, ventricular tachycardia, and death from ischemic heart disease, vary according to the time of day. Despite overwhelming evidence of the importance of circadian rhythms in cardiovascular health and disease, little is known regarding the circadian regulation of intracellular signaling pathways in the heart.

The molecular basis of the circadian clock consists of cell-autonomous, positive and negative transcriptional and posttranscriptional feedback loops. The “master clock,” located in the suprachiasmatic nucleus within the hypothalamus, influences the phase of independent molecular clocks found in peripheral organs, including the heart. Many cells and tissues also display circadian fluctuations in cytoplasmic Ca²⁺ levels, although the source of these Ca²⁺ oscillations and their relationship to the transcriptional clock mechanism is not fully understood. Dysregulation of Ca²⁺ handling is a hallmark of heart disease. Several Ca²⁺-responsive signaling pathways, including the protein phosphatase calcineurin, have been causally linked to the progression of heart failure. Sustained activation of calcineurin...
is sufficient to drive pathological hypertrophic remodeling of the myocardium with subsequent heart failure and premature death.6 Calcineurin is activated by an elevation in intracellular Ca2+ and binding of a Ca2+/calmodulin complex. In a healthy heart, calcineurin is thought to be inactive and unresponsive to high-amplitude, high-frequency waves of Ca2+ that drive contraction. Calcineurin is activated in response to stress presumably when either diastolic resting Ca2+ or Ca2+ in subcellular domains exceed a required threshold.

Calcineurin has numerous substrates including the transcription factor NFAT (nuclear factor of activated T cells) through which calcineurin influences long-term changes in gene expression associated with pathological cardiac remodeling.6 When NFAT is dephosphorylated, it translocates to the nucleus where it binds to and activates calcineurin-responsive genes. Among target genes is the exon 4 isoform of the Rcan1.4 (regulator of calcineurin 1), previously called MCIPI1, DSCRI1, or calcipressin. RCAN1 proteins are potent inhibitors of calcineurin activity.7 Expression of the mouse Rcan1.4 gene is extremely responsive to changes in calci

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and provides expanded details for in situ hybridization, immunohistochemistry, chromatin immunoprecipitation, quantitative RT-PCR, and Western blot analysis.

### Animal Procedures

Male C57BL/6 mice were housed and fed under standard laboratory conditions with a strict 12:12 hour light:dark cycle with lights turning on at 6:00 AM, circadian time 0 (CT0), and off at 6:00 PM (CT12). For pressure-overload experiments, mice were subjected to thoracic aortic constriction (TAC) or severe (s)TAC for 3 weeks as described previously.18 Mice were shifted to constant darkness at the end of the normal light cycle for 24 hours before harvesting. Hearts were removed and the ventricles flash frozen within 30 seconds of the beginning of the day (CT1 to CT3) and lowest at the end of the day (CT11 to CT13) (Figure 1A). In comparison, there were no significant changes in either the level of the exon 1 isoform of Rcan1 (RCAN1.1) or tubulin. A similar circadian pattern in Rcan1.4 protein levels was found in the hearts of 129/Sv and C3H/He inbred lines demonstrating that the oscillations were not strain dependent (data not shown). These findings are consistent with genome-wide microarray analysis identifying Rcan1 as having a circadian pattern of mRNA expression in the mouse heart.18 We found a 20-fold oscillation in Rcan1.4 mRNA levels with a peak at CT23 to CT1 and a trough at CT11 (Figure 1B) including Ser67,12 The in vivo consequence of phosphorylation at these various sites remains controversial, however, calcineurin can dephosphorylate both I-1Ser67 and I-1Thr35,12,13 In vitro and in vivo studies suggest that calcineurin activity can promote dephosphorylation of PLB via regulation of I-1,14,15

Given the need for the heart to adapt to daily changes in cardiac demand and the potentially antagonistic roles of PKA and calcineurin, we asked whether calcineurin activity and/or PLB phosphorylation change over the course of 24 hours in a healthy heart. We found circadian oscillations in both these parameters that were directly out of phase with each other. We then tested what happens to these rhythms when both β-adrenergic activity and calcineurin activity increase in the pressure stressed myocardium.

### Results

#### Changes in Rcan1.4 Protein and mRNA Levels Display Circadian Rhythmlicity

Biochemical assays of calcineurin activity are limited to measuring the potential activity of the entire cellular pool of calcineurin, rather than the fraction of the pool that was active in vivo. We therefore used multiple indirect methods to assess calcineurin activity. Initially, we quantified changes in both protein and mRNA levels of the Rcan1.4 gene, a direct target of calcineurin/NFAT. Male C57BL/6 mice were entrained to a 12:12 light:dark cycle then shifted to constant darkness at circadian time 12 (CT12) the day before samples were harvested for analysis. Rcan1.4 protein levels were highest in the heart at the beginning of the day (CT1 to CT3) and lowest at the end of the day (CT11 to CT13) (Figure 1A). In comparison, there were no significant changes in either the level of the exon 1 isoform of RCAN1 (RCAN1.1) or tubulin. A similar circadian pattern in Rcan1.4 protein levels was found in the hearts of 129/Sv and C3H/He inbred lines demonstrating that the oscillations were not strain dependent (data not shown). These findings are consistent with genome-wide microarray analysis identifying Rcan1 as having a circadian pattern of mRNA expression in the mouse heart.18
directly preceding the peak and trough in RCAN1.4 protein levels. In contrast, there were no significant circadian changes in the transcript levels of either Rcan1.1 or I-1 (Figure 1C and 1D). Thus, circadian regulation of Rcan1 expression is unique to the Rcan1.4 isoform and controlled at the level of transcript abundance. Transcription of the circadian clock gene Period 2 (Per2) oscillated with 24-hour periodicity (Figure 1E) verifying the presence of a functional clock in these samples.

Calcineurin-Dependent Signaling Is Most Active in a Mouse Heart as the Animal Enters a Period of Decreased Physical Activity

Immunohistochemical analysis for NFATc1 in the left ventricle revealed nuclear staining at 6:00 AM (CT0) (Figure 2A) but not at 6:00 PM (CT12) (Figure 2B). Although only a modest number of nuclei stained positive for NFATc1 even at the peak of activity, these positive nuclei were always

Figure 2. More NFATc1 is located in the nucleus and bound to chromatin at CT0 than at CT12. Left ventricular free-wall harvested at 6:00 AM (CT0) (A, C, and D) and 6 PM (CT12) (B) were stained with a FITC-labeled NFATc1 antibody (yellow/green) and propidium iodide (PI) (red). Cardiac myocytes have a high level of autofluorescence because of the abundance of mitochondrial flavins and flavoproteins, which emit in a broad band overlapping the FITC-NFATc1 signal. In image C, the gain on the green channel has been turned down to obtain a clear outline of the nuclear PI signal. In D, the intensity of the green overlay has been restored, so that the autofluorescence of the sarcomere now obscures the myocyte-localized NFATc1-positive nuclei marked with arrows. A nonmyocyte nucleus is marked with an asterisk (*) and is not obscured. NFATc1 occupancy of the RCAN1.4 promoter was determined by chromatin immunoprecipitation from ventricular lysates using either preimmune IgG or NFATc1 antibodies (E) (n=4 each time point). In situ hybridization was carried out using an Rcan1.4-specific probe on the left ventricular wall harvested at 6:00 AM (CT12). Antisense probe (F) and sense probe (G) are shown. White bars denote 20 μm (A, B, F, and G) or 10 μm (C and D).
embedded within sarcomere-positive cells and never observed in nonmyocyte nuclei (Figure 2C and 2D).

NFAT binding to the Rcan1.4 promoter was assessed by chromatin immunoprecipitation studies. A six-fold increase in NFAT occupancy of the Rcan1.4 promoter was detected at CT0 compared with at CT12 (Figure 2E), verifying that the circadian expression pattern of Rcan1.4 was driven by changes in NFAT nuclear translocation. An in situ hybridization specific for Rcan1.4 indicated that transcription was elevated uniformly across the wall of the myocardium (Figure 2F and 2G). Peak Rcan1.4 transcript levels were blunted in the hearts of mice with cardiomyocyte-specific expression of a transgene encoding RCAN1 to inhibit calcineurin (Figure 3A) or treated with the calcineurin inhibitor cyclosporine (Figure 3B). Taken as a whole, these results suggest that activation of the calcineurin/NFAT signaling pathway occurs throughout the left ventricular myocardium and is greatest when the animal is entering its rest phase and cardiac demand decreases.

Phosphorylation of I-1 and PLB Oscillates Out of Phase With Calcineurin Activity

Immunoblot analyses were conducted to assess phosphorylation of the calcineurin substrate I-1 at Thr35 and Ser67. Unfortunately, we were not able to detect phosphorylation of Thr35 in either heart extracts or forskolin-treated cells transfected with an I-1 expression construct (data not shown). There were, however, pronounced circadian changes in Ser67 phosphorylation. Phospho–I-1Ser67 was lowest in the morning (CT1 to CT8), increased notably at CT11 as the animals became active, and peaked at CT14 directly opposed to circadian changes in calcineurin activity (Figure 3C and 3D). In contrast, total I-1 protein (Figure 3C) and transcript levels (Figure 1D) did not change.

Cardiac contractility and β-adrenergic drive are both higher at night in the hearts of nocturnal rodents.19,20 Antibodies specific for phospho-PLB^Ser16^ showed a peak at CT14, coincident with the peak in I-1^Ser67^ phosphorylation (Figure 3C). The change in phosphorylation was even more pronounced when protein extracts were run such that PLB was maintained as a pentameric complex. Phospho-PLB^Ser16^ and phospho-PLB^Thr17^ were both elevated at CT14 compared with CT2 (Figure 4A, 4B, 4D, and 4E). This was evident in the slower electrophoretic migration of total PLB complexes from CT14 lysates (Figure 4C). Thus, the overall phosphorylation state of PLB was elevated during the period when calcineurin-dependent activities were lowest.

Although we were not able to detect phosphorylation of I-1 at Thr35 we predict that phosphorylation of this site by PKA should parallel PLB^Ser16^ phosphorylation. The kinase responsible for I-1^Ser67^ phosphorylation in the heart has not yet been identified definitively. To test whether I-1^Ser67^ is phosphorylated...
in response to β-adrenergic stimulation, we injected wild type mice with 200ng of isoproterenol. Phospho-I-1Ser67 was maximal 5 minutes after injection and dissipated within an hour (Figure 4F). Phospho-PLBSer16 followed a similar time course (Figure 4G), suggesting that in response to β-adrenergic stimulation, the kinetics of I-1Ser67 phosphorylation and dephosphorylation may be similar to that of PLBSer16.

It is likely that the flux through many kinase/phosphatase signaling pathways display circadian rhythmicity in the heart. Glycogen synthase kinase (GSK)β can antagonize calcineurin activity by rephosphorylating NFAT.21 We found a mild increase in GSK3β phosphorylation indicating inactivation of GSK3β at CT14 (Figure 3C and 3E), consistent with insulin activation of Akt kinase when the mouse begins to feed. Therefore, circadian changes in GSK3β activity are not responsible for the observed changes in NFAT localization. We also assessed changes for I-fos and I-fos3 transcript levels which did not change significantly (data not shown), and thus, are unlikely to account for the magnitude of the changes observed in Rcan1.4 mRNA and NFAT nuclear localization. Taken as a whole, we document an inverse correlation between calcineurin-dependent activities and the phosphorylation of proteins that either inhibit PP1 or promote contractility.

**Calcineurin Activity Is Elevated But Maintains Rhythmicity in Hypertrophic and Failing Hearts**

To determine whether cardiac hypertrophy or failure alters the temporal relationship between changes in calcineurin activity and protein phosphorylation, mice were subjected to either TAC surgery to induce stable compensated hypertrophy or severe sTAC to induce decompensated heart failure.16 Three weeks after surgery hearts from TAC mice showed a 29% increase in heart weight to body weight ratio compared with sham-operated controls (Figure 5A). Cardiac function was preserved in these mice as assessed by echocardiography (data not shown) and lung weight (Figure 5B). In sTAC mice, there was a 78% increase in heart weight and a doubling of lung weight compared with sham controls indicating that these mice had progressed to heart failure. Cardiac function was significantly reduced after sTAC (Figure 5C). Remarkably, the decline in function was more pronounced when echocardiography was performed in the morning between 5:00 and 7:00 AM than if function was assessed in the evening between 5:00 and 7:00 PM at the end of the light period, regardless of whether the initial echo was performed in the morning or evening. The time of day differences in function in the sTAC mice were primarily systolic (Figure 5D), whereas the decline in diastolic function was similar regardless of the time of day (Figure 5E). In the control sham mice, there was also a trend toward improved function if the mice were echoed in the evening, but the difference did not reach statistical significance.

In both TAC and sTAC hearts, Rcan1.4 mRNA levels were elevated at all times of the day compared with controls, however, circadian rhythmicity was maintained in both the hypertrophic and failing hearts (Figure 6A), suggesting that although calcineurin activity increased greatly, it retained a circadian pattern of activation. Maximal Rcan1.4 expression was only slightly higher in failing hearts compared with hypertrophic hearts. However, trough expression at CT12 was much higher in the sTAC hearts than in the TAC hearts. Our results suggest that although calcineurin activity remains circadian, there is a progressive elevation in trough activity and a dampening of the fold change between peak and trough activity with increasing stress.

Rcan1.1 transcript levels remained constant throughout (Figure 6B). Per2 expression retained rhythmicity (Figure 6C) indicating maintenance of a cardiac-specific circadian clock, although Per2 oscillation was dampened slightly in sTAC hearts, consistent with previous reports.22 Expression of the fetal genes β-myosin heavy chain (βMHC) and atrial natriuretic factor (ANF) was elevated in TAC and sTAC hearts compared with sham controls indicative of cardiac stress and failure (Figure 6D and 6E). Expression of the high-affinity glucose transporter (Glut4) was depressed in TAC and sTAC hearts compared with sham controls consistent with a failure phenotype (Figure 6F).22,23 Importantly, there were no pronounced circadian oscillations in ANF, βMHC, and Glut4 expression in control hearts suggesting that daily activation of calcineurin in healthy hearts is not sufficient to alter expression of these genes associated with heart failure. Whether the circadian changes in βMHC and ANF expression observed in the TAC and sTAC hearts are linked to changes in calcineurin activity or other signaling pathways is not yet known.

**The Circadian Pattern of PLB and I-1 Phosphorylation Is Disrupted**

Western blot analysis of RCAN1.4 protein in control, TAC, and sTAC hearts at CT2 and CT14 reflect the changes seen in

**Figure 5. Changes in cardiac parameters were assessed in control mice (c) and mice subjected to TAC or sTAC. Analysis included: heart weight/body weight ratios (A) and lung weight/body weight ratios (B), as well as echocardiographic analysis to quantify percent fractional shortening (%FS) (C), left ventricular end diastolic dimension (LVEDD) (D), and left ventricular end diastolic dimension (LVEDD) (E) in sham and sTAC mice (n=21 each in A and B; n=10 each in C, D, and E).**
Rcan1.4 mRNA (Figure 7A and 7B). Within each experimental group, RCAN1.4 protein was more abundant at CT2 than at CT14. Within a given time point, RCAN1.4 protein was consistently higher in the TAC and sTAC hearts than in control hearts.

The normal circadian pattern of I-1 and PLB phosphorylation was lost in the TAC and sTAC hearts. Phosphorylation of PLB was elevated throughout the day in both TAC and sTAC hearts (Figure 7C and 7D; Online Figure I). This is most easily seen by comparing the delay in migration of the PLB pentamer in the TAC and sTAC samples to the migration of PLB in the control sham samples at CT2. Importantly, phosphorylation of I-1Ser67 could not be detected in either TAC or sTAC hearts at any time of day (Figure 7E and 7F), demonstrating that there is a loss of phosphorylation in addition to a loss of circadian changes. This was not attributable to loss of total I-1 protein levels because these were similar in control, TAC, and sTAC hearts.

Discussion

These studies provide several important new insights into the dynamics and consequences of calcineurin signaling in the
heart. First, we present evidence that there is a circadian rhythm in calcineurin activity in a normal, healthy mouse heart. This assertion is supported by 5 different assessments of calcineurin activity: Rcan1.4 protein levels, Rcan1.4 mRNA levels, NFATc1 nuclear translocation, NFATc1 occupancy of the Rcan1.4 promoter, and phosphorylation levels of the calcineurin substrate I-1 Ser67. Second, although activation of calcineurin has been viewed primarily as a stress response driving pathological hypertrophic remodeling, these daily increases in calcineurin activity are not associated with transcriptional changes indicative of pathological remodeling. Third, calcineurin activity oscillates out of phase with phosphorylation of proteins that promote cardiac contractility. Fourth, cardiac function in failing hearts shows diurnal variation. Finally, in failing hearts, calcineurin activity is elevated above normal peak activity throughout the day. Although a pronounced circadian oscillation in calcineurin-dependent activities persists, the amplitude of circadian variation declines correlating to a decline in cardiac function. Circadian rhythmicity in the phosphorylation of the regulatory proteins I-1 and PLB is lost in hypertrophic and failing hearts. Based on our findings we propose that circadian oscillations provide temporal separation of kinase and phosphatase activities that help to regulate changes in cardiac function in response to physiological demand and that this temporal relationship is disrupted when the heart is placed under sustained hemodynamic load.

In nocturnal animals, the light to dark transition at CT12 is anticipated by a rapid increase in physical activity, blood pressure, and β-adrenergic drive, all factors shown to increase calcineurin activity in cardiomyocytes. We therefore expected Rcan1.4 expression to increase in parallel with these physiological parameters at dusk. Instead, there was a gradual increase in Rcan1.4 expression throughout the course of the night suggesting a progressive activation of calcineurin that peaked at dawn when β-adrenergic drive is minimal in a mouse. Figure 8A depicts a model of how calcineurin activity could crosstalk with cardiac function via dephosphorylation of I-1, thus promoting reversal of phosphorylation-driven increases in cardiac contractility as discussed in the introduction. The findings we present here document that in a normal heart, circadian control provides temporal separation of these opposing processes as illustrated in Figure 8B.

The peak in NFAT activity and Rcan1.4 transcription at dawn (CT0) implies that calcineurin activity and cytoplasmic Ca²⁺ levels are also maximal at this time of day. Conversely, Ca²⁺ levels are likely lowest at dusk (CT12) when these indicators of calcineurin activity are lowest. Oscillating out of phase with calcineurin-dependent activities is phosphorylation of I-1 and PLB, increased in hypertrophic and failing hearts. Induced expression of calcineurin activity is greatly increased but maintains circadian rhythmicity in both hypertrophic and failing hearts. In contrast, PLB phosphorylation becomes erratic and elevated throughout the day, whereas I-1 phosphorylation can no longer be detected during the second half of the night. We postulate that the peak in calcineurin activity at the dark to light transition (CT0) helps maintain PLB and other key proteins in an unphosphorylated state as the mouse transitions to a time of lower cardiac demand. Conversely, a trough in calcineurin activity around dusk (CT12) would allow maximal β-adrenergic responses when the animal enters its waking hours. Consistent with this, fractional shortening of sTAC mice was higher in the evening than in the morning (Figure 5C) potentially reflecting circadian differences in the adrenergic response of the unanesthetized mice to handling. Likewise, a recent study demonstrated circadian differences in the response of isolated adult cardiomyocytes to β-adrenergic stimulation.

Although we used changes in Rcan1.4 transcript levels primarily as an indication of changes in calcineurin activity in this study, the RCAN1 protein itself likely contributes to shaping the dynamics of calcineurin-dependent signaling. It is interesting to note that cardiac damage from ischemia-reperfusion (I/R) is greater in mice lacking Rcan1 than in
wild-type hearts. Furthermore, I/R damage in wild type mice has been shown to be greater when the procedure is performed at CT12 than when I/R is performed at CT0. This time of greater susceptibility corresponds to the time when we find RCAN1.4 protein levels in the heart are lowest.

An important shortcoming of the present study is our inability to monitor changes in I-1Thr67 phosphorylation and the as yet incomplete understanding of the cumulative effect of changes in I-1 phosphorylation at other sites including Ser67. Taking these limitations into account, the model in Figure 8B suggests that daily oscillations in Ca^{2+} handling and calcineurin activity form interdependent positive and negative-feedback loops typical of circadian rhythms that result in a separation between times of day when kinase activities predominate and times of day when phosphatase activities predominate. Clearly, this simple model does not take into account many additional factors that can influence calcineurin activity, PP1 activity, and cardiac contractility. For instance, the density of β-adrenergic receptors, adenylyl cyclase activity, and phosphodiesterase activity have all been shown to undergo circadian cycling both in neurons and the heart.

Sustained hemodynamic stress elicits increases in both calcineurin activity and β-adrenergic drive. The model in Figure 8A suggests that these are opposing processes. Our data indicates that in hypertrophy and failure the normal temporal separation of calcineurin activity and phosphorylation of contractile proteins is disrupted. PLB phosphorylation was elevated throughout the day even during the sedentary period of the animal. In contrast, I-1Thr67 phosphorylation was completely lost, consistent with increased calcineurin activity. A corresponding loss of I-1Thr35 phosphorylation would release PP1 inhibition and result in uncoupling of circadian changes in calcineurin activity from regulation of PLB phosphorylation (Figure 8C). Initially, release of I-1 inhibition would be compensatory, helping to reverse hyperphosphorylation of regulatory proteins; however, sustained loss of a circadian pattern of PP1 inhibition could ultimately contribute to declining cardiac function. Consistent with our observations, patients with heart failure demonstrate an increase in PP1 activity coincident with a decrease in I-1 phosphorylation.

The present studies provide a deeper understanding of the dynamics of calcineurin regulation in the heart and draw attention to the need to control for normal, underlying circadian changes in the activity of intracellular signaling pathways. In a human heart, it is likely that calcineurin activity cycles with a reverse phase compared with a mouse heart, although as yet, this is not known. In humans, there is disproportionate ischemic activity, arrhythmic activity, and acute cardiovascular events in the first few hours after waking. Interestingly, this would correspond to the time of day when calcineurin activity would be lowest, allowing maximal β-adrenergic responsiveness in a normal heart. Elevation of the trough in calcineurin activity in heart failure would compromise the ability to respond appropriately. There is a growing appreciation for the potential therapeutic benefit of timing drug delivery to correlate with maximal biological need. Our findings highlight the importance of remaining mindful of inherent circadian oscillations in the cardiovascular system during both the study and treatment of heart disease.

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Disclosures
None.

References


Novelty and Significance

What Is Known?

• Circadian rhythms are important for maintaining cardiovascular health.

• Activation of the protein phosphatase calcineurin is known to promote cardiac hypertrophy and heart failure.

What New Information Does This Article Contribute?

• In healthy hearts, there is an apparent circadian rhythm in calcineurin activity that oscillates out of phase with phosphorylation of proteins, such as phospholamban (PLB) and inhibitor 1 (I-1), that regulate cardiac contractility.

• In heart failure, calcineurin activity increases but continues to cycle, whereas cycling of PLB and I-1 phosphorylation is lost.

Despite overwhelming evidence that circadian rhythms are important in cardiovascular health and disease, little is known regarding circadian regulation of intracellular signaling pathways that control cardiac function and remodeling. Activation of calcineurin is known to promote pathological cardiac remodeling. Here, we present evidence that there is a circadian rhythm in calcineurin activity in normal, healthy hearts that is not associated with transcriptional changes indicative of pathophysiological remodeling. Calcineurin activity oscillates out of phase with phosphorylation of proteins, such as PLB and I-1 that promote cardiac contractility. In heart failure, calcineurin activity increases but continues to cycle, whereas cycling of PLB and I-1 phosphorylation is lost. We propose a model in which daily oscillations in Ca^{2+} handling and calcineurin activity form interdependent positive and negative-feedback loops typical of circadian rhythms that result in separation between times of day when kinase activities predominate and times of day when phosphatase activities predominate. These studies provide a deeper understanding of the dynamics of calcineurin regulation in the heart and draw attention to the importance of normal, underlying circadian changes in regulating the activity of intracellular signaling pathways.
Sustained Hemodynamic Stress Disrupts Normal Circadian Rhythms in Calcineurin-Dependent Signaling and Protein Phosphorylation in the Heart


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Supplemental Methods

Surgical Models of Pressure-Overload Hypertrophy and Failure

Wild type, inbred male C57BL6 mice (6-8 weeks old) were subjected to pressure overload by thoracic aortic constriction (TAC). In this procedure a constriction was placed in the transverse aorta between the innominate and left common carotid arteries. Preanesthesia was achieved using ketamine (100mg/kg IP) plus xylazine (5 mg/kg IP). A warming pad was used to maintain body temperature with in a range of 34°C to 37°C. Respiratory rate, body temperature, and heart rate were monitored continuously during the procedure. The aorta was accessed via a left lateral thoracotomy and suture material (5−0 silk) used to ligate the transverse aorta along with an overlying 27G needle. After ligation, the 27G needle was removed leaving a discrete region of stenosis in the aorta. The chest was closed and the animals observed during recovery from anesthesia. At 3 weeks, when the hypertrophic response reaches steady state, integrity of aortic banding was confirmed by inspection of the surgical constriction and by visualization of marked differences in caliber of the right and left carotid arteries. TAC constriction to a 27G stenosis induced moderate hypertrophy (∼40% increase in heart mass) without clinical signs of heart failure or malignant ventricular arrhythmia. Severe, decompensated hypertrophy was induced by constricting the thoracic aorta to a 28G diameter (sTAC).

Mini-osmotic pump implants

Animals were anesthetized with Avertin (0.02 ml/gram) and the hair shaved at the site of insertion. A 1 cm incision was made between the scapulae and extended with gentle blunt dissection into a pocket for the pump. The mini-osmotic pump (Alzet, Palo Alto, CA) was inserted under the skin and the incision closed using a wound clip.

Quantitative RT-PCR

Total RNA was isolated using Tripure reagent (Roche). Random hexamers were used to reverse-transcribe reactions using superscript II (Invitrogen). Real-time PCR was performed using SYBR green (Applied biosystems). Data for each transcript were normalized to both GAPDH and 18S rRNA as internal controls using the 2−ΔΔCt method. Primers used are listed in supplementary table 1.

Western Blot Analysis

Tissue was homogenized in a buffer containing 10 mmol/L Tris-HCl, pH 7.5, 4% glycerol, 0.1% Triton-X, 0.5 mmol/L sodium bisulfite, 4mM sodium orthovanadate, 100 mmol/L sodium fluoride, and protease inhibitors using a Dounce homogenizer then solubilized by adding an equal volume of 2X SDS loading buffer. For resolving PLB pentamers, samples were run directly on SDS-PAGE without boiling. Total I-1 antibody was a generous gift from Dr. Angus Nairn (Yale University). Phospho-I-1Ser67, and MCIP1/RCAN1 antibodies have been described previously. Antibodies used included: PLB and NFATc1 (Affinity Bioreagents) tubulin (Sigma), pPLB-Ser16 (Upstate), pPLB-Thr17 (Barilla, Leeds, UK), ERK1/2 and pGSK3 (Ser9) (Cell Signaling Technology).

Quantification of Western Blot Analysis

Images for all westerns were obtained using the BIO-RAD Molecular Imager Gel Doc XR system and quantified using QuantityOne 1-D Analysis software (BIO-RAD). Values were then averaged for each time point and presented as ± sd. Statistical significance was determined (StatView) with Student’s unpaired, 2-tailed t test.

RNA In Situ Hybridization: 35S-labeled sense and antisense probes specific for exon 4 of the mouse Rcan1 gene were generated using the Maxiscript kit (Ambion). Radioisotopic in-situ hybridization was performed as previously described.
**Immunohistochemistry**: Monoclonal antibody against NFATc1 (clone 7a6; Affinity BioReagents) (diluted 1:300) was used for immunolabeling of paraformaldehyde-fixed paraffin-sections following Vector Laboratories’ mouse-on-mouse detection system.\(^6\)

**Chromatin Immunoprecipitation From Heart Tissue** was performed as described previously.\(^7\) Frozen tissue was pulverized in liquid N\(_2\) then transferred to a 15 ml polypropylene tube and resuspended in five volumes of 1X PBS. Crosslinking was initiated by the addition of formaldehyde to a final concentration of 1%. Samples were incubated on a shaker at room temperature for 15 minutes. The crosslinking reaction was stopped by the addition of 0.125 mol/L glycine. Samples were then centrifuged (5000 rpm X 2 min at room temperature). Pellets were washed with 5ml of PBS and centrifuged again. Pellets were re-suspended in 250 ml of PBS containing protease inhibitor cocktail and PMSF (5 mmol/L) and homogenized using a hand-held homogenizer. Contents were transferred to an eppendorf tube and microfuged at 5000 rpm for 2 min at 4°C. 500 ml of cell lysis buffer (5 mmol/L PIPES pH 8.0, 85 mmol/L KC1, 0.5% NP40) supplement with 20 ml of protease inhibitor cocktail and 5 mmol/L PMSF was added to the pellet. Following 15 minutes of incubation on ice samples were centrifuged again at the above settings. Pelleted nuclei were lysed in 500 ml of nuclear lysis buffer (50 mmol/L TRIS-Cl pH 8.0, 10 mmol/L EDTA, 1% SDS) containing the usual protease inhibitor cocktail and PMSF. Samples were sonicated to shear chromatin to an average length of 500 bp (40% output, 15 sec continuous pulse for 5 times). Sonicated samples were then centrifuged at 15000 rpm for 10 min at 4°C. The supernatants were collected and 50 ml of the chromatin preparations were used for each immunoprecipitation (IP). The IP was performed in a final volume of 500 ml containing 10 mg salmon sperm DNA in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mmol/L EDTA, 16.7 mmol/L TRIS-Cl pH 8.0, 167 mmol/L NaCl). Anti-NFATc1 antibody (2 mg, clone 7a6, Affinity Bioreagents) was added and incubated gently rocking at 4°C for an hour. Normal mouse serum was used as negative control IgG. Protein A/G PLUS agarose (20 ml) was added and incubated over night at 4°C. Pellets were washed three times with IP buffer prior to isolation for real-time PCR. The primers used to assess NFATc1 binding to the Rcan1.4 promoter amplified a 65 bp fragment between –282 and –217 in a region with the highest conservation between human, mouse and frog. Primers used for the Rcan1.4 promoter were: forward 5’- TGGGAACTATGCCGCAAGAG-3’ and reverse 5’- GGTGGAAAAGGCGCTAAGGT-3’. Q-PCR was performed using SYBR Green kit (Qiagen) on an i-Cycler (Bio-Rad) and analyzed by the 2-ΔΔC(T) method.\(^8\) Data were normalized to the input DNA and plotted as fold values relative to the signal corresponding to a negative control primer specific to a non coding genomic DNA between Gapdh and Cnap1 genes. Chromatin immunoprecipitations were performed three times in triplicate. Error bars represent the standard error of the mean. Similar results were obtained with either the 7A6 anti-NFATc1 antibody from Affinity BioReagents or the K-18 antibody from Santa Cruz.
**Supplemental Table I:** Primer pairs used for quantitative RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pair</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rcan1.4</em></td>
<td>5’-CCC GTG AAA AAG CAG AAT GC –3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCC TTG TCA TAT GTT CTG AAG AGG G-3’</td>
</tr>
<tr>
<td><em>Rcan1.1</em></td>
<td>5’-GAC CCG CGC GTG TTC- 3’</td>
</tr>
<tr>
<td></td>
<td>5’-TGT CAT ATG TTC TGA AGA GGG ATT C-3’</td>
</tr>
<tr>
<td><em>Per2</em></td>
<td>5’-ATG CTC GCC ATC CAC AAG A-3’</td>
</tr>
<tr>
<td></td>
<td>5’-GCG GAA TCG AAT GGG AGA AT-3’</td>
</tr>
<tr>
<td><em>b-MHC</em></td>
<td>5’-GCATTCTCCTGCTGTTTCCTT-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TGGATTCTCAAACGTGTCTAGTGA</td>
</tr>
<tr>
<td><em>ANF</em></td>
<td>5’-GTA CAG TGC GGT GTC CAA CA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCT CCT CCA GGT GGT CTA GCA-3’</td>
</tr>
<tr>
<td><em>I-1</em></td>
<td>5’-AAG ACC GGA TCC CCA ACT C-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCT CCT CCA GGT GGT CTA GCA-3’</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>5’-CAA GGT CAT CCA TGA CAA CTT TG-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCT CCT CCA GGT GGT CTA GCA-3’</td>
</tr>
<tr>
<td><em>18S rRNA</em></td>
<td>5’-ACC GCA GCT AGG AAT AAT GGA-3’</td>
</tr>
<tr>
<td></td>
<td>5’-TCT CCT CCA GGT GGT CTA GCA-3’</td>
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</tbody>
</table>
Online Figure I: Enlarged images of the western blots from Figure 7C and 7D showing changes in the migration of PLB pentamers from sham operated control, TAB (A), and sTAB (B) heart protein lysates isolated at the times indicated. Each monomer contains two potential phosphorylation sites, one at serine 16 and another at threonine 17, resulting in a total of ten potential sites for phosphorylation in the pentamer complex. The arrows to the left indicate the location of differentially phosphorylated complexes migrating to distinct locations in the SDS-PAGE gel. Five separate complexes can be seen in the TAC samples, whereas six could be resolved in the sTAC samples. The horizontal dashed line is drawn at mid-level migration of the Sham CT2 pentamer. Regardless of time of day, the mean distribution of all TAC and sTAC samples are above this line suggesting an increase in total phosphorylation. Because of the rapid-rate of dephosphorylation of PLB, all hearts were snap frozen precisely 30 seconds after cervical-dislocation.
Supplemental References


