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Molecular Clock Mechanisms and Circadian Rhythms Intrinsic to the Heart

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Circadian rhythms are the external expression of an internal clock mechanism that measures daily time.[1] Periodic environmental cues entrain or set the circadian clocks. The daily light-dark cycle represents the most dominant and potent entraining stimulus in mammals. An entrained clock coordinates physiological events to the 24-hour day. Normally, cardiovascular or hemodynamic parameters, such as heart rate and blood pressure, exhibit variations consistent with circadian rhythm. Additionally, several types of acute pathological cardiac events exhibit circadian or at least diurnal rhythm patterns. Specifically, the incidences of acute myocardial infarction, myocardial ischemia, out-of-hospital cardiac arrest, ventricular tachycardia, postmyocardial infarction, and sudden death in heart failure all vary according to the time of day.[2–5] Also, diurnal rhythms can influence degree and form of cardiac hypertrophy and remodeling.[6,7] For instance, the degree of nocturnal blood pressure elevation in patients with systemic hypertension correlates with the severity and concentricity of left ventricular hypertrophy.[8,9] Investigators postulate that these circadian or diurnal variations depend on centrally mediated autonomic or neurohumoral activation. However, peak incidence for some acute events, such as sudden death, does not temporally correspond to the circadian sympathetic activation. Thus, alternative inputs or mechanisms for these rhythm patterns have been postulated. Regardless of the input, the intrinsic clock mechanism must respond and regulate some of the circadian rhythms within the heart itself.

The intrinsic response elements for the putative external circadian inputs had not until recently been identified or characterized in the heart.[10,11] Circadian rhythms are controlled by a transcriptional feedback system fluctuating as a function of the light-dark cycle. Molecular control of a circadian clock mechanism has been described in detail in the fruit fly.[12] Similarities between the core clock mechanisms in fruit flies and mice occur with both exhibiting interlocking positive and negative transcriptional and translational feedback loops.[1,10] Molecular clock mechanisms have been identified in the suprachiasmatic nuclei comprising the master circadian clock mechanism in the mammalian brain. This master clock presumably sets the phase for intrinsic molecular clocks identified in peripheral tissues including heart.[1,10,11]

The negative-feedback loop of the molecular clock mechanism involves dynamic regulation of three *Period* genes, designated *Per 1–3* in rats and mice and two cryptochrome genes (*cry 1–2*).[10,11] Two key transcription factors forming a heterodimer, CLOCK and BMAL1, regulate the rhythmic transcription for the mammalian *Per* and *Cry* genes (see review by Reppert and Weaver[1]). After PER and CRY translation, these proteins form a variety of multimeric complexes that are translocated into the nucleus. The CRY proteins act as negative regulators by directly interacting with CLOCK and/or BMAL1 and inhibiting transcriptional activation by the BMAL1-CLOCK heterodimer. Concurrently, PER2 enhances *bmal1* transcription, which is the phase opposite to *Per/cry*, and initiates the positive-feedback loop. The BMAL1-CLOCK heterodimer binds to *cis*-acting elements in the promoter region for multiple target genes including *Per*, *cry*, and *bmal1*. A delay of ≈6 hours between peak gene and peak protein expression contributes to the phasic nature of the positive- and negative-feedback loops.[13]

The circadian rhythm for the genes involved in the intrinsic molecular clock has recently been confirmed and characterized in the rat heart.[11] Posttranscriptional regulation of the specific transcription factors, such as CLOCK:BMAL1, still requires detailing, including confirmation that changes in protein expression follows the phasic changes in gene expression. The circadian rhythm of the molecular clock presumably enables the heart to adapt to various physiological stimuli, which change during the day. Thus, the response of the targets for the transcriptional factors involved in regulation of the clock requires determination. Furthermore, characterization of the relationship between intrinsic circadian rhythms and adaptation of the heart to chronic stress might elucidate disease process mechanisms.[11]

A study in this issue of *Circulation Research*[14] follows previous work[11] by the same investigative group in defining intrinsic circadian rhythms in heart. Previously, Young et al[11] demonstrated that the rhythm of major genes involved in the clock mechanism was not disturbed in a rat model of myocardial hypertrophy, induced by aortic banding. However, rhythm changes were blunted for various clock output or target genes including PAR (rich in proline and amino acid residues) transcription factors *dbp* (albumin D-element binding protein), *hlf* (hepatic leukocyte factor), and *tef* (thyrotrophic embryonic factor). These data led to the hypothesis that the heart with pressure overload–induced hypertrophy loses

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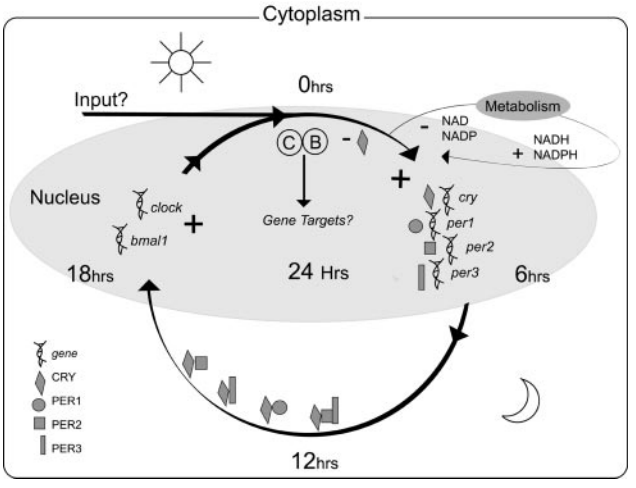
its ability to anticipate environmental changes and adapt to daily stresses.

The present study follows with analyses of diurnal variations in myocardial metabolic flux and contractile function and how these variations relate to circadian expression of metabolic genes. Contractile performance, carbohydrate oxidation, and oxygen consumption in isolated working rat hearts were greatest in the middle of the night, with little variation in fatty acid oxidation. The expression for all the metabolic genes investigated, which represented a wide variety of genes involved in regulation of carbohydrate and fatty acid metabolism, exhibited circadian rhythm. The study has clearly linked diurnal expression of certain genes related to regulation of carbohydrate metabolism to diurnal variation in carbohydrate oxidation. These genes include those regulating glucose transport, incorporation into glycogen, and pyruvate oxidation. A presumption of the present study is that an appropriate time passed between peak gene expression and time for measurement of peak metabolic flux and contractile function. The data indicate that metabolic flux was measured only 3 hours after peak gene expression. According to studies defining circadian phases of gene and protein expression as noted earlier, then flux and contractile function were likely measured during their ascending limbs.[1] This represents a limitation in the study design, in that metabolic flux and function might exhibit even more extreme changes than reported by these investigators.

Regulation of the clock genes by the redox state of nicotinamide adenine dinucleotide cofactors (NAD and NADP) has been demonstrated in a human neuroblastoma cell culture system.[15] The reduced forms of these cofactors, NADH and NADPH, strongly enhance DNA binding activity of the CLOCK:BMAL1 and CLOCK:NPAS2 heterodimers (*NPAS2* gene encodes a functional analogue of CLOCK). In contrast, the oxidized form of these redox factors inhibits DNA binding by these heterodimers.[15] The regulation by NADH in vitro implies that oscillations in metabolic flux participate in feedback loops with the clock genes. In particular, glycolytic flux results in cytosolic NADH production and depends on shuttle mechanisms for NADH transport into the mitochondria. Therefore, the variations in carbohydrate flux demonstrated in this study in intact heart could represent an interacting feedback mechanism for the clock. If so, then abnormalities in the metabolic flux and NADH generation could disrupt or reset the clock. Reentrainment of the clock would produce a cascade of cellular events, assuming a wide range exists in yet undefined target genes for the clock mechanism.

The present study in *Circulation Research*[14] further demonstrates that pressure-overload hypertrophy impairs the circadian rhythms of the many metabolic genes. Unfortunately, the investigators did not search for circadian rhythms in metabolic flux and contractile function in the hypertrophied hearts. Lack of confirming functional data represents a true limitation in the study, as gene expression does not always coordinate with protein expression in the hypertrophied or remodeled heart. For instance, shifts in myosin isoform gene profile in remodeled human failure are not accompanied by comparable shifts in protein expression.[16] Nevertheless, the



The molecular components of a 24-hour circadian clock are illustrated in the schematic. Approximate Zeitgeber times are noted. The CLOCK:BMAL1 (circles labeled C and B) protein heterodimer, functioning as a transcription factor, activates transcription of *cry* and *Per 1–3* (denoted by geometric symbols with double helix). The protein products of these genes (geometric symbols) form various multimeric complexes and positively regulate transcription for *Bmal1* and *Clock*. CRY negatively inhibits DNA binding of CLOCK:BMAL1 forming a negative-feedback loop. The nicotinamide adenine dinucleotide cofactors (NAD and NADP) in the reduced state (NADH and NADPH) positively effect CLOCK:BMAL1 binding, and in the oxidized state negatively effect binding of this heterodimer. Circadian clock phases are adapted from Reppert and Weaver.[1] Future research areas include identification of inputs and targets for components of clock.

impairments in circadian rhythm represent an important and novel finding. Previously, these investigators demonstrated that pressure-overload hypertrophy did not alter circadian rhythm of the clock mechanism genes. However, the present results suggest that circadian variations in expression for the putative target genes of the clock are attenuated or even abolished. The authors speculate that this attenuation limits the responsiveness of the hypertrophied heart to stress and can lead to energy starvation and failure.

Thus, this study raises several mechanistic issues, which must be addressed and are illustrated in the Figure. As an example, the input signals for the intrinsic circadian clock mechanism require identification. Several inputs have been considered and might operate in specific cells or systems. Glucocorticoid hormones represent one group of candidate regulators of clock oscillations in peripheral tissues. Dexamethasone, a glucocorticoid analogue, induces gene expression for *cry1* and *Per 1–3* and can cause phase shift in the circadian rhythm of these genes in heart, as well as in kidney and liver of mice in vivo.[17] Yet, intrinsic oscillations of these genes are identical in livers of wild-type mice and mice with a hepatocyte-specific glucocorticoid receptor inactivation.[17] Therefore, glucocorticoids cannot be the only signals setting the phase of peripheral clocks. These dexamethasone experiments illustrate the complexities involved in designing experiments, which will elucidate clock mechanisms and regulations.

The function of proteins under putative regulation by the peripheral clocks must be studied to determine the impor-

tance of posttranscriptional and posttranslational processes on the clock mechanisms. Additionally, future mechanistic studies should address defining whether abnormalities in metabolic flux cause or result from disruptions in the clock mechanisms.

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