The circadian clock within the heart: potential influence on myocardial gene expression, metabolism, and function

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Young, Martin E. The circadian clock within the heart: potential influence on myocardial gene expression, metabolism, and function. Am J Physiol Heart Circ Physiol 290: H1–H16, 2006; doi:10.1152/ajpheart.00582.2005.—It is becoming increasingly clear that the intrinsic properties of both the heart and vasculature exhibit dramatic oscillations over the course of the day. Diurnal variations in the responsiveness of the cardiovascular system to environmental stimuli are mediated by a complex interplay between extracellular (i.e., neurohumoral factors) and intracellular (i.e., circadian clock) influences. The intracellular circadian clock is composed of a series of transcriptional modulators that together allow the cell to perceive the time of day, thereby enabling preparation for an anticipated stimulus. These molecular timepieces have been characterized recently within both vascular smooth muscle cells and cardiomyocytes, giving rise to a multitude of hypotheses relating to the potential role(s) of the circadian clock as a modulator of physiological and pathophysiological cardiovascular events. For example, evidence strongly supports the hypothesis that the circadian clock within the heart modulates myocardial metabolism, which in turn facilitates anticipation of diurnal variations in workload, substrate availability, and/or the energy supply-to-demand ratio. The purpose of this review is therefore to summarize our current understanding of the molecular events governing diurnal variations in the intrinsic properties of the heart, with special emphasis on the intramyocardial circadian clock. Whether impairment of this molecular mechanism contributes toward cardiovascular disease associated with hypertension, diabetes mellitus, shift work, sleep apnea, and/or obesity will be discussed.

anticipation; cardiovascular; diurnal variations; zeitgeber

Since the initiation of life on Earth, one selective pressure that has been imposed continuously on terrestrial organisms is the time of day. Over the course of a normal 24 h, the environment of, and the demands placed on, an organism fluctuate dramatically. Despite environmental influences, biological processes must remain within specific physiological boundaries. Intuitively, homeostatic mechanisms would function more efficiently if the organism possessed a means for anticipation of daily routine changes in the environment. It is therefore not surprising that virtually every organism (with the exception of certain prokaryotes) has evolved specific mechanisms allowing for anticipation of environmental fluctuations over the course of the day. These mechanisms, collectively known as circadian clocks, allow individual cells to perceive the time of day (34). In doing so, circadian clocks confer the selective advantage of anticipation, conditioning the cell to changes in its environment before they occur.

Circadian clocks have been identified in every mammalian cell investigated to date, including key components of the cardiovascular system, such as cardiomyocytes and vascular smooth muscle cells (VSMCs) (26, 33, 34, 82, 90, 94). Given the universal appreciation for diurnal variations in both physiological and pathophysiological cardiovascular events, the recent exposure of a molecular machinery within the individual cells of the cardiovascular system that has the potential of modulating an array of cellular processes has sparked increasing interest. Historically, diurnal variations in blood pressure, heart rate, and cardiac output, as well fatal cardiovascular events, have been attributed primarily to diurnal variations in environmental stimuli, such as a sudden increase in sympathetic activity (89, 102, 105, 138). However, the ability of the cardiovascular system to respond in an appropriate and timely manner to neurohumoral stimuli is likely of equal importance. Intracellular circadian clocks provide a means by which the heart and vasculature can anticipate diurnal variations in stimuli, such as autonomic and sympathetic nervous activity, ensuring an optimal response. Attenuation of this molecular mechanism would therefore impair the ability of the heart and/or vasculature to respond appropriately to environmental stimuli, which in turn may contribute toward the development of cardiovascular disease.

The purpose of this review is to summarize our current knowledge regarding circadian clocks within the cardiovascular system (with special emphasis on the heart), the biological processes they influence, how they are regulated, and the factors that lead to their impairment. What should become apparent is that although circadian rhythmicity of cardiovas-
cular function is firmly established, our understanding of the role of the circadian clock as a mediator of this phenomenon remains in its infancy. Recent advances linking circadian clocks with cardiovascular function suggest it is timely to highlight the potential role(s) of this molecular mechanism within the heart.

EXISTENCE OF CENTRAL AND PERIPHERAL CIRCADIAN CLOCKS

One of the first experiments suggesting the existence of an intrinsic mechanism allowing organisms to perceive the time of day also exemplifies the primary role of circadian clocks. In 1729, a French astronomer named Jean Jacques d’Ortous de Maran investigated whether plants that unfolded their leaves during the night did so in response to sunlight. He noted that plants kept in constant darkness still unfolded their leaves during daylight hours, despite never being exposed to the light, and concluded that the observed rhythm was not driven by the environment but was an intrinsic property of the organism (34). This rhythm provides a selective advantage by allowing the plant to anticipate when the sun will rise, thereby opening its leaves to increase photosynthetic potential at the correct time of day. By possessing an internal clock mechanism, cells/organisms are able to anticipate temporal changes in their environment, optimizing biological processes so that they occur at an advantageous time in the day. It has been suggested that primordial clocks may have allowed noncomplex cells to anticipate periods of high ionizing radiation from the sun (due to a lack of an ozone layer), so that DNA synthesis would occur only at night, thereby minimizing the occurrence of DNA mutations (99).

Circadian clocks can be defined as a set of proteins that generate self-sustained transcriptional positive and negative feedback loops with a free-running period of 24 h (32, 34, 49, 50). These circadian clocks are intrinsic to the cell, persisting when tissues and cells are isolated and cultured in vitro (4, 33, 82, 90, 152). To maintain their selective advantage, circadian clocks are reset (or entrained) by environmental cues (the most apparent being light); factors that influence the timing of this molecular mechanism are known as zeitgebers (or timekeepers) (34, 55). Given that the clock mechanism is transcriptionally based, initial output manifests at the level of altered gene expression. Those genes that are regulated directly by the circadian clock, but are not integral components of the clock mechanism, are termed clock-controlled, or output, genes. After posttranscriptional events, output from the clock ultimately modulates protein expression, metabolism, and/or function, depending on the cell or organ (39).

Through the use of sophisticated molecular and genetic approaches, considerable progress as been made within the last decade toward elucidation of the inner workings of eukaryotic circadian clocks. Initial studies in the fungus Neurospora and in the fruit fly Drosophila melanogaster led investigators to suggest that the circadian clock is composed of a single protein that inhibits its own synthesis, thereby creating an oscillator with a simple transcriptional loop (130). Although it is attractive, subsequent studies have dispelled this hypothesis, revealing a complex set of positive and negative feedback loops of which the molecular machinery still awaits full elucidation (32, 55). Mammalian orthologs of these circadian clock components are emerging (40, 132, 145, 162). Genetically manipulated mouse models have greatly accelerated characterization of the mammalian circadian clock, exposing an interplay among at least three negative loops and one positive loop that require a multitude of transcriptional, translational, and posttranslational events (39). One of the negative loops of the mammalian circadian clock that is understood to the greatest extent involves the period (PER, of which three isoforms have been identified, PER1, PER2, and PER3) and the cryptochrome (CRY, of which two isoforms are known, CRY1 and CRY2) proteins (85, 162). On heterodimerization, the basic helix-loop-helix/PER-arylhydrocarbon receptor nuclear translocator (ARNT)-SIM (bHLH/PAS) transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein 1 (BMAL1, also known as MOP3) recognize E-boxes located within the promoters of various target genes, including the per and cry genes (Fig. 1) (40, 57). Once translated, the PER and CRY proteins form heterodimers, translocate into the nucleus, and inhibit CLOCK/BMAL1 transactivation (potentially through interaction with the histone acetyltransferase p300, an essential coactivator for CLOCK/BMAL1-mediated transcription) (23, 36, 39, 73, 119). A key facet of the PER/CRY-mediated negative loop is a specific delay in the accumulation of the PER proteins by ~6 h, relative to their mRNA (35, 39, 49, 106). The PER proteins are targets for ubiquitin-mediated degradation, an event that is blocked by serine phosphorylation. Thus, once in the phosphorylated state [likely mediated by casein kinase 1ε (CK1ε)], PER proteins accumulate within the cytosol, allowing heterodimerization with the CRY proteins (67). Subsequent inhibition of CLOCK/BMAL1-mediated transcription decreases expression of the per and cry genes, ultimately relieving inhibition on CLOCK/BMAL1 (Fig. 1).

Two additional negative loops of the mammalian circadian clock involve the bHLH transcription factor differentially expressed in chondrocytes (DEC, of which two isoforms have been identified, DEC1 and DEC2) and the nuclear receptor reverse transcriptase c-ERBαα (REV-ERBαα) (58, 101). Both the dec1/2 and rev-erbαα genes are under direct transcriptional control by the CLOCK/BMAL1 heterodimer (Fig. 1). After translation of the corresponding proteins and translocation into the nucleus, both DEC1/2 and REV-ERBαα attenuate CLOCK/BMAL1-mediated transcription: DEC1/2 appear to associate with the CLOCK/BMAL1 heterodimer, impairing transactivation capacity, whereas REV-ERBαα specifically represses bmal1 transcription (potentially through recruitment of the N-CoR/histone deacetylase 3 corepressor) (112, 153). In contrast, the CLOCK/BMAL1- and/or PER2-mediated induction of bmal1 expression constitutes the positive loop in the mammalian circadian clock (Fig. 1) (41, 119).

Although by definition the circadian clock involves an interplay of transcriptional positive and negative feedback loops, multiple posttranscriptional events are essential for complete clock function, including protein synthesis/degradation, reversible phosphorylation, and nucleocytoplasmic translocations (35, 55, 150). Each of these events represents a potential site of regulation, enabling “fine-tuning” of this molecular clock by extracellular factors (i.e., zeitgebers). CLOCK, BMAL1, and CRY, as well as the PER proteins, have all been shown to undergo phosphorylation in a circadian-like fashion; in addition to its influence on protein stability, increasing evidence
suggests that phosphorylation status is closely associated with nucleocytoplasmic trafficking of specific circadian clock components (e.g., PER and CLOCK proteins) (71, 111, 144). Multiple protein kinases likely play significant roles in the mammalian clock mechanism, including CK1ε, MAPK, GSK-3β, and PKG (type II), thereby providing avenues for modulation of the circadian clock by an array of extracellular stimuli (79, 111, 134, 144).

Mammalian circadian clocks can be divided into two major classes, depending on the cell type within which they are found: the central and peripheral circadian clocks (18, 55, 106). The central circadian clock (often termed the master clock) is located within the suprachiasmatic nucleus (SCN; located in the hypothalamus) of the brain, whereas peripheral clocks are those clocks found within all non-SCN cells of the organism, including other regions of the central nervous system. Zeitgebers are factors that reset or entrain central and/or peripheral circadian clocks. The central clock is reset by light (via electrical signals transmitted along the retinohypothalamic tract), whereas peripheral circadian clocks are influenced by multiple neurohumoral factors (as well as diurnal variations in temperature) (7, 14, 25, 55). One of the strongest entrainers of peripheral circadian clocks appears to be feeding (25, 124). It is believed that the central clock entrains peripheral clocks via modulation of neurohumoral stimuli, either directly (i.e., interaction between the SCN and specific peripheral tissues) and/or indirectly (e.g., through alterations in feeding behavior).

The CLOCK/BMAL1 heterodimer binds to E-boxes in the promoters of various genes that are not believed to be an integral component of the clock mechanism. These clock-controlled genes include lactate dehydrogenase A (ldha), vasopressin, weel, prokineticin2, and the rich in proline and acidic amino acid residues (PAR) transcription factors d-element binding protein (dbp), hepatic leukemia factor (hlf), and thyrotrrophic embryonic factor (tef) (21, 37, 38, 60, 81, 107, 108). The latter family of transcription factors, which in turn have the potential to modulate expression of a host of target genes, are antagonized by another bHLH transcription factor, E4BP4; e4bp4 is an additional example of a clock-controlled gene, of which the expression is likely induced by REV-ERBAα (84, 139). Consistent with their reciprocal function, oscillations in PAR transcription factors and E4BP4 have been reported to be antiphase to one another, in various tissues, including the heart (see CIRCADIAN CLOCKS WITHIN THE CARDIOVASCULAR SYSTEM).

CIRCADIAN CLOCKS WITHIN THE CARDIOVASCULAR SYSTEM

Oscillations in circadian clock gene expression have been reported by numerous investigators, for various cardiovascular components. Gene expression measurements of hearts and blood vessels (e.g., aorta) isolated from rodents at specific times of the day have revealed dramatic rhythmicity in the expression of genes encoding for both core circadian clock components and output genes (23, 33, 48, 69, 80, 82, 86, 92–95, 101, 109, 110, 122, 125, 154, 159, 160). Figure 2 provides precise quantitative 24-h expression profiles for circadian clock genes in the intact hearts of rats kept in a normal 12:12-h light-dark cycle [lights on at zeitgeber time (ZT) 0]. Oscillations in bmal1, clock, and neuronal pas2 (napas2) (a redox-sensitive clock homologue, also known as mop2) mRNA molecules are essentially identical to one another, in terms of both peak (i.e., zenith) level of expression and phase (Fig. 2A) (104). However, trough (i.e., nadir) expression levels of bmal1 and napas2 are significantly lower than those of clock, suggesting that the former transcription factors likely become limiting at the light-to-dark phase transition. The heart expresses all
known isofoms of the _cry_ and _per_ genes; _cry_2 is the predom-
ninant cryptochrome in the heart, whereas _per_1 and _per_2 are
epressed to greater extents than _per_3 (Fig. 2B). Peak levels of
_cry_2, _per_1, and _per_2 expression are observed at the light-to-
dark phase transition in the rat heart, with similar absolute
levels of expression relative to one another. However, unlike
_cry_2 expression, _per_1 and _per_2 expression decreases dramat-
ically at the dark-to-light phase transition, consistent with the
known clock mechanism, wherein PER expression becomes
limiting (relative to the CRY proteins). It is also worthy to note
that the antiphase nature of _nat_ and _per_2 expression becomes
consistent with the latter antagoniz-
ing (as well as the activity of the other
PAR transcription factor family members) at the dark-to-light
phase transition.

Taken together, the data presented in Fig. 2 are consistent
with the operation of a fully functional circadian clock within
the heart. However, one could hypothesize that oscillations in
circadian clock components observed in intact organs in vivo
are the result of diurnal variations in one or more neurohumoral
factors that directly influence expression of these genes. By
definition, the circadian clock is intrinsic to the cell. Therefore,
to conclusively demonstrate that the components of the car-
diovascular system possess an intrinsic circadian clock mech-
anism, one must expose the cell autonomous nature through the
use of cultured organs or cells. The latter has been performed
for cultured vessels, VSMCs, and cardiomyocytes. Davidson et
al. (26) have recently cultured both arteries and veins isolated
from transgenic mice overexpressing the luciferase reporter
gene under control of the _per_1 promoter. They report that
oscillations in _per_1-driven bioluminescence persist in cultured
vessels, for between 3 and 12 days. McNamara et al. (82)
reported that exposure of human VSMCs to media containing
50% serum for 2 h (serum shock, a strategy utilized in numer-
ous studies for the reestablishment of circadian clock gene
oscillations in cultured cells) induced rhythmic expression of
_bmal1_. Furthermore, this rhythm was phase advanced by dex-
methasone and phase delayed by retinoic acid (82). Similarly,
Nonaka et al. (94) have shown that angiotensin II induces
significant oscillations in _bmal1, per_2, and _dbp_ in VSMCs,
with periodicitics close to 24 h. Together, these observations
expose an intrinsic circadian clock mechanism within VSMCs,
of which the timing is influenced by humoral factors such as
corticosterone, retinoic acid, and angiotensin II.

We (33) have recently examined the circadian clock within
the cardiomyocyte. Through the use of adult rat cardiomy-
cocytes, we (33) found that the presence of 2.5% FCS within
the culture medium is sufficient to maintain oscillations in circa-
dian clock ( _bmal1, rev-erbaa_, and _per_2) and output ( _dbp_)
geenes; in serum-free media, these oscillations are either se-
verely attenuated ( _bmal1 and dbp_) or completely abolished
(_rev-erbaa_ and _per_2). Studies by Welch et al. (148) and
Nagoshi et al. (91) provide a plausible explanation for the

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Fig. 2. Quantitative circadian expression levels
for clock (A), _bmal1_ (A), neuronal PER-aryl-
hydrocarbon receptor nuclear translocator-
SIM 2 ( _npas2_), cry1 (B), cry2 (B), _per_1
(B), _per_2 (B), _per_3 (B), _rev-erbaa_ (C), __dec1
(C), __dbp_ (D), __hlf_ (D), __tef_ (D), and __e4bp4__ (D)
in intact rat hearts. Circadian rhythms in
expression were determined by quantitative
RT-PCR for total RNA prepared from hearts
that were isolated from rats at 3-h intervals.
Values are shown as means ± SE for 12
separate observations at each time point.
Data are represented as number of mRNA
molecules per nanograms total RNA.

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Invited Review

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MYOCARDIAL CIRCADIAN CLOCK

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apparent loss of circadian clock function in cells cultured in the absence of serum. Both sets of investigators report that culturing of fibroblasts for prolonged periods of time leads to asynchrony between individual cell-autonomous clocks and that serum reestablishes synchrony, as opposed to reactivation of the clock mechanism itself (91, 148). We (33) have also reported that challenge of isolated cardiomyocytes with norepinephrine for only 2 h (mimicking a burst of sympathetic activity at the onset of consciousness) results in significant oscillations in circadian clock genes, suggesting that norepinephrine acts as a putative zeitgeber for the circadian clock within the heart. The latter observations are consistent with those of Terazono et al. (133), who observed that adrenergic stimulation influences circadian clock gene expression in the liver.

ENVIRONMENTAL VERSUS INTRINSIC INFLUENCES ON CARDIOVASCULAR FUNCTION

Intracellular circadian clocks clearly exist within at least two major cell types of the cardiovascular system, namely, cardiomyocytes and VSMCs. This molecular mechanism undoubtedly exists within all mammalian cell types, although characterization has yet to be reported in, for example, endothelial cells. Because of our recent appreciation for peripheral and central circadian clocks, new hypotheses have arisen regarding potential mechanisms responsible for diurnal variations in cardiovascular physiology, as well as pathophysiology. Without question, circadian rhythms in environmental factors (e.g., locomotion, feeding) influence cardiovascular function. However, circadian clocks may contribute to normal diurnal variations in cardiovascular function in a number of different ways. For example, through the use of the retrograde pseudorabies virus, the presence of multisynaptic autonomic connections from the SCN to the heart have been reported (113), suggesting that the central clock may modulate cardiac function through direct (autonomic) nervous stimulation. Circadian clocks within individual cells of the cardiovascular system potentially influence cardiovascular function by allowing anticipation of the onset of neurohumoral factors (e.g., increased sympathetic nervous stimulation before awakening), thereby ensuring an appropriately rapid response. Thus, in the in vivo setting, a complex interplay between environmental influences and intrinsic mechanisms (i.e., central and peripheral circadian clocks) likely contributes to changes in cardiovascular function over the course of the day. Here we provide a brief overview of specific evidence supporting roles for environmental versus intrinsic influences on cardiovascular function.

Circadian rhythms in blood pressure have been investigated intensively (28, 29, 54, 64, 83, 141, 143, 161). In humans, blood pressure is lowest in the night, reaching a trough at around 3:00 AM, and peaks soon after waking (9:00 AM). A second peak in blood pressure is often seen early in the evening (7:00 PM) (29). The opposite is true for nocturnal animals, such as rodents, that exhibit elevated blood pressure levels at night, when the animal is most active (141). Day-to-night differences in physical and mental activity appear to be major determinates of blood pressure circadian rhythms (22, 28). In humans, shift workers show an essentially complete resynchronization of blood pressure rhythms within the first 24 h of the shift rotation (19, 128). Similarly, alterations in the light-dark cycles and/or restricted feeding induces rapid phase shifting of blood pressure, heart rate, and behavioral activity circadian rhythms in the rat (141). It has been postulated that changes in the conscious state of the animal primarily influence blood pressure through changes in sympathetic activity, suggesting that environmental factors serve as the major influence on blood pressure (76). Indeed, a strong correlation exists between diurnal variations in this cardiovascular parameter and plasma norepinephrine and epinephrine levels, suggesting that the sympathetic system drives blood pressure circadian rhythms (105, 123, 136). It should also be noted that additional cardiovascular reactive peptides (e.g., atrial natriuretic peptide, vasoressin, and components of the renin-angiotensin system) exhibit considerable diurnal variations and therefore potentially influence blood pressure circadian rhythms (43, 62, 63, 100). Whether the circadian clock within the cells of the cardiovascular system influences responsiveness to these neurohumoral factors, thereby contributing to circadian rhythmicity in blood pressure, is an attractive possibility that awaits investigation.

Although blood pressure and heart rate circadian rhythms are normally paralleled, several lines of evidence suggest that the circadian rhythms of these two cardiovascular parameters might be differentially regulated. Rhythms in heart rate appear to be more intrinsic, driven in large part by diurnal variations in autonomic nervous system activity (72, 114). Furthermore, Hu et al. (59) have recently reported that despite controlling for sleep-wake and behavior cycles in humans, circadian rhythmicity in heart rate variability persists, peaking in the early hours of the morning. Such studies expose an intrinsic component influencing normal cardiovascular function. One could hypothesize that SCN-driven diurnal variations in autonomic stimulation, coupled to the cardiomyocyte circadian clock-driven diurnal variations in responsiveness of the heart to autonomic stimulation, together act as major determinants of heart rate circadian rhythms. Whether environmental modulation of synchronization between peripheral and central clocks contributes to cardiovascular disease development remains unknown; such a loss of synchronization is possible through changes in feeding and sleep patterns, as occurs during diabetes mellitus, obesity, sleep apnea, and shift work, all of which are associated with elevated risk for cardiovascular disease (see MODULATION OF CIRCADIAN CLOCK DURING PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STATES).

CIRCADIAN RHYTHMS IN THE INTRINSIC PROPERTIES OF THE CARDIOVASCULAR SYSTEM

An important question that remains unanswered is whether circadian rhythms in cardiovascular function are mediated solely through changes in the level of neurohumoral stimuli or whether diurnal variations in the intrinsic properties of the cardiovascular system also play a significant role. Increasing evidence suggests that specific components of the cardiovascular system respond differentially to neurohumoral stimuli at specific times of the day. For example, healthy human endothelium exhibits a marked circadian rhythm, with decreased function in the early hours of the morning (as assessed by brachial artery flow-mediated, endothelium-dependent vasodilation) (97). This functional circadian rhythm persists ex vivo, such that rat aortic rings isolated at distinct times of the day...
exhibit differences in endothelium-dependent and -independent vasodilation; diurnal variations in the responsiveness of aortic rings to pharmacological stimulation (β-adrenergic agonists, Ca²⁺ channel antagonists) also persist in vitro (46, 47). Taken together, these studies reveal circadian rhythmicity in the intrinsic properties of the vasculature.

Comparable observations have been made regarding diurnal variations in the intrinsic properties of the heart. We (158) have reported previously that diurnal variations in cardiac function persist when rat hearts are isolated and perfused ex vivo; hearts isolated in the middle of the dark phase (the active period for the rat) exhibit greater cardiac power relative to those hearts isolated in the middle of the light phase. Similarly, Yamashita et al. (151) observed significant variations in the transient outward and steady-state currents for ventricular myocytes isolated at different times of the day, thereby exposing diurnal variations in the intrinsic electrical properties of the cardiomyocyte. Additionally, Lapenna et al. (75) have reported increased susceptibility of isolated perfused rat hearts to H₂O₂ during the dark phase, as assessed by release of thiobarbituric acid-reactive substances (lipoperoxidation end products), exposing diurnal variations in oxidative stress tolerance of the heart.

Given that ex vivo experiments inherently control for acute influences of neurohumoral stimuli, diurnal variations in the intrinsic properties of the heart (and vasculature) are likely mediated by changes in gene and protein expression over the course of the day. Both hypothesis-testing and hypothesis-generating strategies have been adopted for identification of mediators of diurnal variations in the intrinsic properties of the heart. In the former case, candidate genes and proteins, as well as metabolic and signaling pathways known to influence heart function, have been investigated. For example, we (158) and others (146) have observed that gene expression of myocardial contractile proteins exhibits significant circadian rhythmicity in the normal rodent heart. Increased contractility and cardiac output during the dark phase for the rat, as observed both in vivo and ex vivo, correlate with increased expression of both myosin heavy chain (MHC) isofoms α and β (146, 158). However, it should be noted that these studies investigated gene expression only, and data concerning circadian rhythms in MHC protein levels have not been reported. Indeed, given that contractile proteins are known to possess relatively long half-lives, it is unlikely that significant changes in MHC protein expression influence contractile function over the course of a normal day.

Myocardial contractility and metabolism are inherently interlinked, such that changes in contractility affect metabolism, and vice versa. We found that increased cardiac output observed for rat hearts excised during the dark phase was associated with increased carbohydrate oxidation and oxygen consumption (with no significant change in fatty acid oxidation) (158). This is reminiscent of the observation that an acute increase in myocardial workload results in increased rates of carbohydrate oxidation, with lesser effects on fatty acid oxidation rates (42). Consistent with increased myocardial carbohydrate metabolism during the dark phase, rat hearts isolated at this time of the day possess increased gene and protein expression of the contraction- and insulin-responsive glucose transporter GLUT4, as well as increased gene expression of GLUT1 and glycogen synthase (diurnal variations in protein levels of the latter two metabolic proteins have not been reported) (158). Furthermore, circadian rhythms in the tricarboxylic acid cycle flux-generating enzymes citrate synthase and oxoglutarate dehydrogenase (at the level of gene expression) correlate with increased oxygen consumption during the dark phase (158). These observations have led to the suggestion that increased myocardial oxidative capacity may promote increased contractile function during the active phase for the mammal.

Despite the reported lack of diurnal variation in fatty acid oxidation rates for excised working rat hearts, we observe a coordinated induction of genes known to promote fatty acid oxidation during the dark phase (121, 122, 158). Table 1 summarizes observations regarding diurnal variations in 12 genes known to promote myocardial fatty acid oxidation, either directly (e.g., component of the β-oxidation pathway) or indirectly (e.g., through modulation of intracellular malonyl-CoA levels, a potent inhibitor of fatty acid oxidation). It could be hypothesized that the apparent dissociation between diurnal variations in fatty acid oxidation gene expression and rates of exogenous fatty acid oxidation reflects a lack of significant diurnal variation in myocardial fatty acid oxidation enzymes at the protein level. However, it should be noted that the fatty acid concentration (0.4 mM oleate) utilized in our previously pub-

<table>
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Rats were housed in a standard 12:12-h light-dark cycle [lights on between zeitgeber time (ZT) 0 and ZT 12; lights off between ZT 12 and ZT 24] before isolation of hearts at 3-intervals over course of day. Gene expression was measured by real-time quantitative RT-PCR for at least 12 separate observations. All trough-to-peak inductions are statistically significant (P < 0.05). References provide information regarding protein function, gene regulation, and/or diurnal variations. PDC, pyruvate dehydrogenase complex. See text for gene definitions.
lished studies measures submaximal rates of fatty acid oxidation, as opposed to fatty acid oxidative capacity (158). Future studies are therefore required to investigate whether diurnal variations in myocardial fatty acid oxidation capacity exist at the protein and metabolic flux levels.

A total of 14 different K⁺ channels were investigated by Yamashita et al. (151) in the search for potential mediators of observed diurnal variations in the intrinsic electrical properties of the heart. Of the K⁺ channels investigated, circadian rhythms in both mRNA and protein levels were observed for Kv1.5 and Kv4.2 channels. These two K⁺ channels exhibit opposing diurnal variations in expression relative to one another in the rat heart, with the Kv1.5 channel reaching a peak level of expression during the dark phase, whereas expression of the Kv4.2 channel peaked during the light phase (151). Increased expression of Kv1.5 channels during the dark phase is consistent with increased steady-state current of cardiomyocytes isolated at this time. In contrast, peak expression of Kv4.2 channels during the light phase corresponded with increased transient outward current. Pharmacological studies in isolated perfused rat hearts further support the hypothesis that circadian rhythms in the intrinsic electrical properties are mediated, at least in part, by diurnal variations in myocardial expression of Kv1.5 and Kv4.2 channels (151).

Reactive oxygen species (ROS) play a pivotal role in myocardial physiology and pathophysiology. Similar to other highly reactive short-lived biological molecules [e.g., nitric oxide (NO)], physiological concentrations act in an essential signaling manner, whereas pathophysiological elevation (i.e., oxidative stress) is associated with multiple disease states, including heart failure (51). Oxidative stress occurs when production of ROS exceeds the antioxidant capacity of the cell. ROS originate from multiple enzymatic and nonenzymatic reactions. One potential site of ROS generation for an oxidative organ such as the heart is the mitochondrion; misdonation of electrons to molecular oxygen at complexes I and III of the electron transport chain (ETC) results in superoxide production (12, 44). The latter occurs when components of the ETC become highly reduced. Under conditions of normal oxygen availability, the likelihood of superoxide production via the ETC increases when ATP consumption is low (i.e., less active state) and when fatty acid availability is high (this highly reduced substrate supplies electrons to the ETC via both FADH₂ and NADH). Such conditions (i.e., decreased myocardial ATP consumption coupled with increased fatty acid availability) occur when the animal is resting (i.e., light phase for the rat). Given this rationale, one would hypothesize that the heart increases antioxidant capacity during the resting phase, thereby minimizing oxidative stress. Antioxidant capacity has been shown to exhibit marked diurnal variations in serum and several peripheral tissues, although little is known for the heart (6). Lapenna et al. (75) reported that glutathione peroxidase (a major antioxidant enzyme in the heart) exhibits increased activity in the rat heart during the light (resting) phase. Coincident with diurnal variations in myocardial antioxidant capacity, decreased susceptibility to H₂O₂-induced oxidative damage was observed for hearts isolated during the light phase (75).

Putative mediators of diurnal variations in myocardial metabolism and contractile function have also been investigated through hypothesis-generating approaches. Microarray analysis of hearts isolated at 3-h intervals from mice maintained in a normal 12:12-h light-dark cycle exposed diverse and extensive circadian rhythms in myocardial gene expression (80). Approximately 13% of all genes expressed in the murine heart exhibit significant circadian rhythmicity. Clustering of rhythmic genes into biological pathways/processes exposed synchronous expression profiles for genes encoding proteins involved in mitochondrial oxidative metabolism, protein synthesis, calcium binding, and GTP-binding pathways. Consistent with increased myocardial oxygen consumption during the dark phase, a coordinated induction of mitochondrial oxidative metabolism genes in the heart was observed at this time (80, 158). It was noted that for those genes identified as rhythmic in the murine heart, a subset appeared to change in response to light-dark cycle transitions. Storch et al. (125) performed a similar microarray-based study, with the exception that mice were placed in dim light for at least 42 h before isolation of the heart. The study reports that despite exposure of the animals to constant darkness, ~8% of myocardial genes exhibited rhythmicity in expression. Again, oscillations in gene expression could be clustered in a biological process-specific manner, with genes involved in metabolism, protein turnover, and G protein-coupled signaling pathways receiving particular attention (125).

It is apparent that the majority of studies investigating diurnal variations in the intrinsic properties of the heart have, to date, focused primarily on alterations in gene expression. An important next step will require examination of the heart at the proteomic level, enabling determination of the extent to which myocardial proteins oscillate over the course of a normal 24 h. The extent to which proteins exhibit a diurnal variation will depend not only on oscillations for the encoding mRNA but also on the turnover rate of the protein itself. Furthermore, diurnal variations in the activity of a specific protein will also be influenced by posttranslational modifications (e.g., reversible phosphorylation). Clearly, understanding the complex interplay between transcriptional, translational, and posttranslational events over the course of the day will improve our understanding of the mechanisms mediating diurnal variations in the intrinsic properties of the heart.

CIRCADIAN CLOCK VERSUS NEUROHUMORAL FACTORS AS MEDIATORS OF DIURNAL VARIATIONS IN INTRINSIC PROPERTIES OF HEARTS

Circadian rhythms in the intrinsic properties of the heart (and vasculature) are potentially mediated by intracellular (i.e., circadian clock within the cardiomyocyte) and/or extracellular (i.e., neurohumoral) influences (Fig. 3). It is reasonable to hypothesize that diurnal variations in one or more circulating hormones (e.g., thyroid hormone) mediate diurnal variations in myocardial gene and protein expression (e.g., GLUT4) before examination of the heart ex vivo, independent of the circadian clock within the cardiomyocyte. Therefore, to distinguish between intracellular versus extracellular influences, one must investigate each independently of the other. One strategy for investigating the role of the intracellular circadian clock, in the absence of confounding neurohumoral influences, is the utilization of cultured cells. Because of its intrinsic nature, the circadian clock persists in cultured cells, whereas any potential influences by diurnal variations of neurohumoral factors in the in vivo setting are abolished (90). Such a strategy has been utilized successfully in the identification of circadian clock-
regulated genes in several cell types. For example, genes encoding for insulin and ppara exhibit significant 24-h oscillations in cultured pancreatic β-cells and hepatocytes, respectively, suggesting that these genes are regulated by the circadian clock (1, 96). Through the use of adult rat cardiomyocytes, we (33) have recently identified two metabolic genes as novel circadian clock-regulated genes in the heart. We observed significant circadian oscillations in the gene expression of pyruvate dehydrogenase kinase 4 (pdk4) and uncoupling protein 3 (ucp3) in isolated adult rat cardiomyocytes cultured under conditions associated with maintenance of the circadian clock (i.e., 2.5% FCS). In contrast, in the absence of serum, oscillations in both circadian clock and metabolic genes were either severely attenuated or abolished. The timing of metabolic gene oscillations was similar in vivo and in vitro when compared with circadian clock gene oscillations. For example, oscillations in pdk4 and ucp3 mRNAs were similar to one another, and antiphase with respect to rev-erbaa mRNA, both in vivo and in vitro. Taken together, these observations strongly suggest that the circadian clock within the heart regulates myocardial metabolism (33).

Experiments utilizing isolated cardiomyocytes, under conditions in which cell autonomous clocks remain synchronized, will allow identification of multiple circadian clock-regulated genes in the heart. Such a system will enable “follow-up” investigation of putative circadian clock-regulated genes identified initially through either hypothesis-testing (i.e., candidate gene) or hypothesis-generating (i.e., microarray) strategies. For example, whether oscillations in Kv1.4 and/or Kv4.2 channels persist in isolated cells has yet to be investigated; if these oscillations persist, this will provide strong evidence in support for the hypothesis that the circadian clock within the cardiomyocyte regulates diurnal variations in the electrical properties of the heart. Similarly, if oscillations in antioxidant enzymes persist in isolated cardiomyocytes, this would suggest that the circadian clock mediates diurnal variations in susceptibility of the heart to oxidative stress. Studies addressing such questions currently await completion.

It is worthy to note that considerable evidence suggests plasminogen activator inhibitor 1 (PAI-1; a key factor in fibrolytic activity) is regulated directly by the circadian clock. Expression of pai-1 exhibits striking circadian rhythmicity in both the heart and vasculature, as does plasma PAI-1 activity (86, 93). Maemura et al. (77) have shown that the bHLH/PAS transcription factor cycle-like factor (CLIF) forms a heterodimer with CLOCK and binds to specific E-boxes within the pai-1 promoter, subsequently inducing pai-1 gene expression. Such observations have led investigators to hypothesize that the circadian clock plays a significant role in mediating the onset of myocardial infarctions in the morning, through induction of pai-1 (78). Consistent with pai-1 being a circadian clock-regulated gene, myocardial pai-1 expression peaks near the light-to-dark phase transition in the rat, similar to that of dbp, another clock output gene (86). However, pai-1 is undoubtedly regulated by an array of neurohumoral factors, in addition to the circadian clock. These include glucose, insulin, glucocorticoids, and transforming growth factor-β (15, 30, 65, 129). Indeed, a routine change of serum-free medium for cultured adult rat cardiomyocytes causes an immediate induction in pai-1 expression (2.5-fold within 3 h; D. J. Durgan and M. E. Young, unpublished observations). Such a high sensitivity of the pai-1 promoter to changes in the environment severely hinders investigations of potential pai-1 gene oscillations in vitro.

As exemplified by pai-1, reliance on isolated adult rat cardiomyocytes for identification of circadian clock-regulated genes has limitations. The use of adult rat cardiomyocytes is also somewhat hindered by the phenomenon of dedifferentiation that occurs as the culture is maintained. This process may impair identification of specific circadian clock-regulated genes. For these reasons, development of alternative models is essential for identification of circadian clock-regulated genes (and in turn circadian clock-regulated processes). One strategy for dissociating between the influence of the circadian clock versus that of neurohumoral factors is to eliminate the former. A ubiquitous, constitutive clock null mouse exists, which has proved useful in expanding our knowledge regarding the intricacies of the circadian clock mechanism (145). However, as the circadian clock is impaired in all cells of clock mutant mice, diurnal variations in neurohumoral factors are abnormal, preventing dissociation of intracellular (i.e., circadian clock) versus extracellular (i.e., neurohumoral) influences on circadian rhythms in myocardial gene expression and function. An alternative strategy is therefore to manipulate the circadian clock only within the cardiomyocyte. Such a heart-specific circadian clock-impaired mouse model, which has recently been generated in our laboratory, will undoubtedly improve our understanding of the role(s) of this molecular mechanism within the heart.

**POTENTIAL ROLES FOR CIRCADIAN CLOCK WITHIN HEARTS**

It is clear that the heart possesses an intrinsic, fully functional circadian clock. What is less apparent is the identity of the processes that this mechanism influences. Given that circadian clocks confer the selective advantage of anticipation,
the question regarding the nature of the environmental stimuli/events that the heart anticipates should be addressed. Anticipation of diurnal variations in cardiac workload would be a logical candidate. The circadian clock within the heart could potentially influence diurnal variations in cardiac output and efficiency through a host of molecular mechanisms, including modulation of myocardial 1) responsiveness to sympathetic stimulation; 2) electrical properties; 3) calcium homeostasis; 4) contractile protein composition; 5) antioxidant capacity; 6) signaling cascades; and 7) metabolism (both oxidative and nonoxidative). Diurnal variations in gene (and, in certain cases, protein) expression of key components of all these processes have been identified in the heart through hypothesis-testing and hypothesis-generating approaches (75, 80, 121, 122, 125, 151, 158). However, to date, very few studies have linked the circadian clock within the cardiomyocyte directly to diurnal variations in myocardial processes. Clearly, with the identification of circadian clock-regulated genes in the heart, those processes directly influenced by this molecular mechanism will unfold.

We (33) have recently reported strong evidence that the circadian clock within the cardiomyocyte influences myocardial metabolism (see discussion above). Our data support the hypothesis that the circadian clock mediates increased myocardial carbohydrate metabolism after the light-to-dark phase transition in the rat (and a reciprocal decrease in fatty acid oxidative capacity at this time). This has led us to speculate that the circadian clock within the heart allows anticipation of diurnal variations in workload, substrate availability, and/or the energy supply-to-demand ratio (154). However, to date, very few studies have linked the circadian clock within the cardiomyocyte directly to diurnal variations in myocardial processes. Clearly, with the identification of circadian clock-regulated genes in the heart, those processes directly influenced by this molecular mechanism will unfold.

During a sudden increase in workload, the heart responds to the increase in energetic demand by increasing carbohydrate oxidation, with a lesser effect on fatty acid metabolism (42). At the onset of the active phase (light phase for humans, dark phase for rodents), a sudden increase in sympathetic activity and workload on the heart occurs (105, 123, 136, 141). By increasing its capacity for carbohydrate oxidation, the heart would anticipate the sudden increase in workload at this time. The oxidative decarboxylation of pyruvate to acetyl-CoA is a primary site of regulation for carbohydrate oxidation in the heart, a reaction catalyzed by the pyruvate dehydrogenase complex (PDC) (118, 127). PDC is regulated at multiple levels, including reversible phosphorylation (126). Pyruvate dehydrogenase kinase (PDK, of which four isoforms have been identified in humans) phosphorylates and inactivates PDC, thereby reducing carbohydrate oxidation. We (33, 122, 158) have found that gene expression of the inducible isoform of PDK, namely, pdk4, exhibits a trough in the rat heart just before the onset of the dark phase and that these oscillations persist in isolated cardiomyocytes, suggesting direct regulation by the circadian clock within the heart. Consistent with the assumption that diurnal variations in PDK4 protein expression will exhibit a slight temporal delay relative to those of pdk4 mRNA, we have found that the percentage of PDC in the active form is significantly higher at the light-to-dark phase transition, compared with the dark-to-light phase transition (Fig. 4). The circadian clock within the heart therefore likely increases myocardial carbohydrate oxidative capacity in anticipation of increased workload on awakening of the animal. Increased circulating glucose levels at this time (due to a sudden increase in hepatic glucose output, the so-called dawn phenomenon) would provide an appropriate substrate for increased cardiac output at the end of the overnight fast (9, 74). The dawn phenomenon also appears to be mediated by intracellular circadian clocks (74).

Dissociation between diurnal variations in circulating fatty acids and the capacity for myocardial oxidative metabolism exists in the ad libitum-fed laboratory rat (122, 158). Rates of

**Fig. 4. Potential mechanism by which the circadian clock within the cardiomyocyte influences myocardial metabolism in the rat.** After induction of myocardial pyruvate dehydrogenase kinase 4 (pdk4) expression during the dark phase, pyruvate dehydrogenase complex (PDC) is less active at the dark-to-light phase transition, in comparison to the light-to-dark phase transition (A); pdk4 gene expression was measured by quantitative RT-PCR, whereas PDC activity was measured by following the oxidative decarboxylation of [14C]pyruvate, as described previously (53). Total PDC activity does not exhibit a diurnal variation (data not shown). Because circadian oscillations of pdk4 persist in isolated adult rat cardiomyocytes, pdk4 is likely regulated directly by the circadian clock within the heart. As the dark phase progresses (B), circadian clock-mediated induction of pdk4 will slow carbohydrate oxidation; the latter will promote fatty acid oxidation at this time, as will potential circadian clock-mediated induction of fatty acid oxidation (FAO) enzymes. In contrast, as the light phase progresses (C), circadian clock-mediated repression of pdk4 will accelerate carbohydrate oxidation; the latter will repress fatty acid oxidation at this time, as will potential circadian clock-mediated repression of FAO enzymes. TCA, tricarboxylic acid.
myocardial oxygen consumption and expression of mitochondrial oxidative metabolism genes, as well as other genes encoding for proteins promoting fatty acid oxidation, peak during the dark phase for the rat, a time at which circulating fatty acids are present at their lowest level (122, 158). Responsiveness of the heart to fatty acids also appears to be out of synchronization with circulating fatty acids levels (122). We (122) have recently reported that the heart exhibits dramatic diurnal variations in its responsiveness to fatty acids. Through a series of in vivo models, we (122) found that fatty acids induce the expression of myocardial fatty acid oxidation genes to the greatest in the middle of the dark phase. Fatty acids rapidly induce gene expression through direct activation of a family of nuclear receptors known as peroxisome proliferator-activated receptors, PPARs (116). All three PPAR family members (α, β/δ, and γ) identified to date are expressed within cardiomyocytes to varying extents, with expression of PPARα and PPARβ/δ predominating over PPARγ (5, 20, 147). On heterodimerization with RXR family members, the PPAR/RXR dimer binds to fatty acid response elements (FAREs) located within the promoter of various target genes (66). Known PPARα (and PPARβ/δ) target genes include those promoting fatty acid oxidation; indeed, all the genes listed in Table 1 are known to be PPARα regulated (20, 121, 156). Similar to diurnal variations in the responsiveness of the heart to fatty acids, the heart was more sensitive to the PPARα-specific agonist WY-14,643 during the dark phase, suggesting that sensitivity of the PPARα system exhibits circadian rhythmicity (122). Consistent with these observations, we (122) found a coordinated induction of transcriptional activators of the PPARα system (ppara, rxra, pgc1, and p300) during the night. Interestingly, Kassam et al. (61) have shown that the circadian clock component REV-ERBAα antagonizes PPARα through binding of overlapping sequences in the promoter of target genes. Consistent with its antagonistic function, oscillations in rev-erbaa expression are antiphase to those of ppara (122).

Diurnal variations in the responsiveness of the heart to fatty acids are potentially mediated by changes in neurohumoral influences and/or the circadian clock within the cardiomyocyte. The in vivo studies performed to date cannot dissociate between these potential influences. It is possible that diurnal variations in the sensitivity of the PPARα system are due to neurohumoral-mediated changes in gene and protein expression, and/or phosphorylation status, of any of the components of the PPARα system (e.g., PPARα, RXRα, PGC1, P300), independent of the intramyocellular circadian clock. However, given that circadian rhythms in ppara and rev-erbaa are antiphase to one another, that these rhythms are consistent with diurnal variations in the responsiveness of the heart to fatty acids, and that both these genes are directly regulated by the circadian clock, it is tempting to hypothesize that the circadian clock within the cardiomyocyte directly influences diurnal variations in the responsiveness of the heart to fatty acids, as depicted in Fig. 5. Here, increased expression of REV-ERBAα near the end of the light phase functions not only as an integral component of the circadian clock but also to reduce the expression of PPARα-regulated genes (and responsiveness of the heart to circulating fatty acids). In contrast, repression of REV-ERBAα, and a concomitant induction of PPARα, RXRα, P300, and/or PGC1, during the dark phase will increase expression of PPARα-regulated genes (and responsiveness of the heart to circulating fatty acids). This hypothesis is currently under investigation.

Given that the primary role of the circadian clock is to synchronize the cell/organ with environmental stimuli, it is not obvious why a lack of synchronization exists between diurnal variations in fatty acid availability and responsiveness of the heart to fatty acids (as well as fatty acid oxidative capacity). Multiple possibilities exist. One is that the laboratory rat fed a standard rodent chow does not mimic the evolutionary selective pressures placed on the animal in the wild. Rodent chow is typically low in fat (often 10% calories from fat, or less), whereas the animal in the wild may consume a mixed diet consisting of a greater proportion of calories from fat. Indeed, when rats are fed a high-fat diet, circulating fatty acids spike postprandially, such that diurnal variations in the responsiveness of the heart to fatty acids are synchronized with fatty acid availability (i.e., both are elevated during the dark phase) (122). An additional explanation for the apparent discrepancy between fatty acid availability and responsiveness of the heart to fatty acids in the ad libitum-fed rat stems from the previously described “thrifty gene” hypothesis. This hypothesis, which states that organisms have evolved to anticipate periods of prolonged fasting, was devised as an explanation for the increasing development of obesity in Western society and relates more to seasonal fluctuations in food availability, as opposed to day/night fluctuations (24). However, such a hypothesis can be modified to accommodate shorter periods of food deprivation (i.e., <24 h). We have hypothesized that diurnal variations in fatty acid responsiveness may be the result of anticipation of prolongation of fasting, if the animal in the wild is initially unsuccessful in its forage for food. Indeed, we observe a rapid and dramatic induction of myocardial pdk4 expression at the light-to-dark transition in the fasted rat (122). This would transiently promote utilization of fatty acids at the initiation of the active phase, when availability of this substrate is increased (as the forage for food continues). More recently, we have found that manipulation of the light-dark cycle severely impairs the ability of the heart to adapt to fasting and that the rate of resynchronization of the circadian clock within the heart (after the light-dark cycle manipulation), correlates
Diurnal variations in the responsiveness of the heart to fatty acids can be viewed not only as increased sensitivity during the dark phase but also as decreased sensitivity during the light phase. More specifically, we (122) observe lowest responsiveness of the heart to fatty acids (as well as lowest fatty acid oxidative capacity) 3 h before the light-to-dark phase transition in the rodent (i.e., just before awakening of the animal). The perception is therefore that the heart deliberately ignores fatty acids at a time when they are most abundant in the circulation. When the organism awakens, elevation of heart rate and cardiac output increases the energetic demand on the heart. However, at the same time, vascular resistance increases, which has been attributed to an elevation in vasoconstrictive factors (98). This paradox of increased energy demand of the heart, at the same time as restrained coronary blood flow, becomes clinically noticeable when the severity of ischemia reaches a critical threshold, resulting in angina or myocardial infarction, as is often observed in coronary artery disease patients (27, 103). Given that the heart faces varying degrees of ischemia during the day, it is therefore logical to hypothesize that this organ has evolved mechanisms allowing anticipation of such events, thereby minimizing impairment of cardiac function. By decreasing responsiveness to fatty acids when the animal awakens, the heart will increase reliance on carbohydrate oxidation. The latter is a more efficient fuel, in terms of ATP generated per O2 molecules consumed, when compared with fatty acid oxidation (120). Therefore, by modulating myocardial fuel selection, the circadian clock may allow the heart to anticipate diurnal variations in the energy supply-demand ratio.

MODULATION OF CIRCADIAN CLOCK DURING PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STATES

By definition, the role of intracellular circadian clocks is to allow anticipation of alterations in the environment. If such a mechanism were impaired, one would hypothesize that the cell may not respond to its environment in an appropriate manner, in terms of promptness and/or amplitude of adaptation. This may manifest ultimately at the level of myocardial dysfunction. For example, if the circadian clock within the cardiomyocyte allows anticipation of increased fatty acid availability after prolongation of an overnight fast, impairment of this molecular mechanism may result in accumulation of intramyocardial fatty acid derivatives; appreciable evidence suggests that the latter contribute toward the development of contractile dysfunction associated with dyslipidemia, as observed during diabetes mellitus and obesity (140, 156). Before the mechanisms involved in alteration of the circadian clock during disease states are addressed, those factors known to influence this molecular mechanism during physiological conditions should be discussed. Multiple environmental events modulate the timing of circadian clocks, such as diurnal variations in light intensity, locomotion, and feeding (25, 34, 55, 87). In turn, these environmental influences affect intracellular circadian clocks via alterations in temperature, neurotransmitters (e.g., glutamate, norepinephrine), nutrients (e.g., glucose), ligands for nuclear receptors (e.g., retinoic acid), and cellular redox state (i.e., NAD+/NADH ratio), as well as autocrine, paracrine, and endocrine factors (e.g., PGE2, glucocorticoids, adrenaline, angiotensin II) (3, 14, 31, 33, 56, 82, 94, 108, 133, 135). What is also becoming increasingly clear is that zeitgebers likely act in cell type-specific manners. Yamazaki et al. (152) have shown that manipulation of the light-dark cycle is associated with tissue-specific rates of circadian clock resynchronization, with the most rapid rate of resynchronization observed for the liver (cardiovascular system components were not investigated here). Furthermore, parabiosis linkage of SCN-lesioned mice restores circadian clock rhythmicity in the liver and kidney, but not the heart, skeletal muscle, or spleen, suggesting that specific blood-borne cues are sufficient to reset some, but not all, peripheral circadian clocks (48). More recently, Davidson et al. (26) have reported that the phase of per1 oscillations differed significantly between arteries and veins of distinct anatomical location, suggesting that tissue-specific regulation of peripheral circadian clocks also occurs within the cardiovascular system.

Given the suggestion that circadian clocks are differentially regulated in a cell-specific manner, the question arises whether certain physiological and/or pathological states result in an asynchrony between peripheral circadian clocks. In the case of the cardiovascular system, one could envisage situations in which dyssynchronization between circadian clocks located within endothelial cells, VSMCs, and cardiomyocytes could contribute toward disease states. For example, the circadian clock within the endothelial cell may modulate diurnal variations in NO production, whereas the circadian clock within VSMCs may influence diurnal variations in NO-mediated vasodilation. Loss of synchronization between circadian clocks within these two cell types would therefore attenuate blood pressure diurnal variations (i.e., nondipping). If the circadian clock within the cardiomyocyte allows anticipation of blood pressure diurnal variations, then a dyssynchronization between the cardiomyocyte clock and this environmental factor may prevent normal adaptation (i.e., increased cardiac output), ultimately contributing to contractile dysfunction and failure. A loss of synchronization between circadian clocks within the same cell type, but located within different regions of the same organ, may also occur in pathological states. For example, given that changes in redox state are known to influence the timing of the circadian clock, myocardial infarction will likely produce a phase shift in circadian clocks within the ischemic region, relative to the nonischemic region. Whether such an intraorgan asynchrony contributes toward cardiovascular disease progression awaits elucidation.

Circadian clocks are altered in various animal models of cardiovascular disease. We have found that rhythmic expression of circadian clock-regulated genes (e.g., dbp, hlf, tef, pdk4, and ucp3) is significantly attenuated in the rat heart during pressure overload-induced hypertrophy (158, 159). Consistent with these observations, Mohri et al. (86) report that oscillations in circadian clock genes are severely attenuated in hypertrophied hearts isolated from high-salt-fed Dahl rats (an animal model of hypertension). In contrast, Naito et al. (92) report an augmentation of circadian clock gene oscillations in aorta and hearts isolated from a different rat model of hypertension (the spontaneously hypertensive rat), which is associated with amplified rhythms in pai-1. Diabetes mellitus, a major risk factor for the development of
heart disease in humans, is associated with a phase shift in the circadian clock within the heart (95, 160). Shift workers also exhibit increased incidence of cardiovascular disease (52, 68, 70). Intuitively, alterations in behavior, such as those exhibited in shift workers, would be expected to influence intracellular circadian clocks. Kobayashi et al. (69) reported that rat heart per2 gene expression oscillations take at least 3 days to resynchronize after reversal of the light-dark cycle; we find that at least 5 days are required for complete resynchronization of the major circadian clock components (bmal1, rev-erba, dbp; unpublished observations). In contrast, circadian rhythms in behavior and heart rate resynchronize within 1–2 days, in both rodents and humans, after reversal of the light-dark cycle (19, 128, 141). These observations suggest that the circadian clock within the heart is out of synchronization with the environment for between 1 and 4 days after reversal of the light-dark cycle. It is tempting to speculate that repetitive reversal of the light-dark cycle in shift workers significantly attenuates anticipation of the heart (as well as other components to the cardiovascular system) to changes in its environment, ultimately contributing to the development of heart disease. Sleep apnea, like shift work, is a cardiovascular risk factor that would be expected to impair intracellular circadian clocks (10, 11). Additionally, given that food intake appears to be a primary zeitgeber for peripheral circadian clocks, one would expect that development of obesity (an additional cardiovascular disease risk factor) could also alter the timing of intracellular circadian clocks. Consistent with the idea that impairment of the circadian clock likely increases risk of cardiovascular disease incidence, Turek et al. (137) have recently shown that the clock null mouse is predisposed toward the development of obesity and the metabolic syndrome.

Circadian rhythms in pathophysiological cardiovascular events are well documented. The onset of myocardial infarction and sudden death both show increased incidence in the early hours of the morning (2, 17, 88, 89). The timing of a cardiovascular event, such as myocardial infarction, also affects the severity of the insult; a greater incidence of heart failure development has been observed for subjects that experience a myocardial infarction during the night, as opposed to the morning (88). Similar to physiological cardiovascular circadian rhythms, the timing of pathological events has been attributed primarily to a sudden rise in stimuli triggering increased blood pressure and heart rate, such as sympathetic and autonomic activity, in addition to increased prothrombotic tendency and platelet aggregability (89). As for studies investigating circadian variability in physiological cardiovascular parameters, those investigating pathological events have largely ignored diurnal variations in the sensitivity of the cardiovascular system to neurohumoral stimuli. We propose that impairment in the ability of the cardiovascular system to anticipate diurnal variations in neurohumoral stimuli contributes to the development of cardiovascular disease. If true, future interventions for cardiovascular disease may target normalization of circadian clock timing. Clearly, substantial work is required to elucidate fully the role of altered circadian clock function in the progression of cardiovascular disease development.

BENCHSIDE IMPLICATIONS

The existence of circadian clocks within components of the cardiovascular system has far-reaching implications, which extend beyond the clinical setting. Given the diversity of diurnal variations in the intrinsic properties of the cardiovascular system, which manifest at multiple levels (gene expression, protein expression, and cellular and organ function), extreme caution should be taken to consider time of day issues during design of experimental strategies, for both in vivo and in vitro studies. Performance of experiments at an inappropriate time of the day and/or omission of suitable time controls may lead to erroneous conclusions being drawn. Such temporal considerations will undoubtedly aid in reducing discrepancies between studies performed in different laboratories and discrepancies between gene and protein expression measurements, as well as discrepancies between animal models and humans. Indeed, the majority of clinical studies are undoubtedly performed during the daylight hours, when the subjects are awake. For the sake of convenience, the majority of animal studies are also performed during the daylight hours, although this is a time at which rodents are asleep. Given the increased reliance on genetically manipulated mouse models for studies investigating the molecular mechanisms underlying cardiovascular disease in humans, it seems timely to reconsider the standards for utilization of such models, to include the most appropriate moment during the light-dark cycle at which such studies should be performed. Undoubtedly, studies designed to investigate myocardial gene expression in rodents are less likely to generate erroneous conclusions when performed during the dark phase (122, 154). Simple manipulation of the light-dark cycle within the housing facility can overcome issues regarding convenience.

SUMMARY

In conclusion, circadian clocks exist within multiple components of the cardiovascular system, including cardiomyocytes and VSMCs. These clocks have the potential of impacting multiple cellular processes and therefore hold the promise of modulating various aspects of cardiovascular function over the course of the day. Future directions within this field undoubtedly include identification of genes directly regulated by the circadian clock within cardiomyocytes, VSMCs, and endothelial cells. In doing so, those processes influenced by this molecular mechanism will unfold. Indeed, the recent identification of metabolic genes as circadian clock-regulated genes in the heart suggests that the clock modulates myocardial metabolism. Whether impairment of intracellular circadian clocks contributes toward cardiovascular morbidity and mortality development is an attractive possibility that requires further investigation.

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Invited Review

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