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Circadian Regulation of Cardiac Physiology: Rhythms That Keep the Heart Beating

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Abstract

On Earth, all life is exposed to dramatic changes in the environment over the course of the day; consequently, organisms have evolved strategies to both adapt to and anticipate these 24-h oscillations. As a result, time of day is a major regulator of mammalian physiology and processes, including transcription, signaling, metabolism, and muscle contraction, all of which oscillate over the course of the day. In particular, the heart is subject to wide fluctuations in energetic demand throughout the day as a result of waking, physical activity, and food intake patterns. Daily rhythms in cardiovascular function ensure that increased delivery of oxygen, nutrients, and endocrine factors to organs during the active period and the removal of metabolic by-products are in balance. Failure to maintain these physiologic rhythms invariably has pathologic consequences. This review highlights rhythms that underpin cardiac physiology. More specifically, we summarize the key aspects of cardiac physiology that oscillate over the course of the day and discuss potential mechanisms that regulate these 24-h rhythms.

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1. INTRODUCTION

Life on Earth is subjected to dramatic fluctuations in the environment over the course of a normal day. These include, but are not limited to, changes in light intensity, humidity, and temperature, as well as the presence/absence of natural predators and prey. It is therefore not surprising that organisms have evolved strategies not only to adapt to 24-h oscillations in environmental parameters, but also to anticipate them. This concept is readily illustrated at the level of behaviors, such as sleep/wake and fasting/feeding cycles. Given known influences that behaviors and environmental factors have on biological systems, it is not surprising that time of day has emerged as a significant modulator of essentially all aspects of mammalian physiology (1, 2). The cardiovascular system serves as an excellent example. Associated with the psychological and physical stresses of waking, blood pressure and heart rate increase steeply; these parameters remain elevated throughout the awake period but decrease again during the sleep period in healthy individuals (3). In doing so, daily rhythms in cardiovascular function ensure that supply and demand remain balanced (e.g., increased delivery of oxygen, nutrients, and endocrine factors to organs during the active period, as well as the removal of metabolic by-products) (4–6). Various additional cellular processes (e.g., transcription, translation, cellular signaling, metabolism) and organ physiology (e.g., hormone release, neurological activity, muscular contraction) also oscillate over the course of the day (2). Underscoring their fundamental importance, failure to maintain these physiologic rhythms invariably has pathologic consequences (7). This review, which focuses on cardiac physiology, has two primary goals: (a) summarize the extent to which cardiac physiology oscillates over the course of the day and (b) discuss recent insights regarding putative mechanisms orchestrating 24-h rhythms in cardiac physiology.

It is important to appreciate the concept that cells/organs/organisms are not only capable of adapting to stimuli/stresses but can also anticipate them. With regard to adaptation, a stimulus-response concept is typically considered, wherein a cell/organ/organism responds upon challenge to a stimulus/stress (both acutely and chronically, depending on the nature of the stimulus/stress and/or its duration). Such a concept is often utilized as an explanation for time-of-day-dependent oscillations in cardiovascular parameters. Indeed, associated with the steep rise in blood pressure and heart rate in the early hours of the morning are increases in multiple neurohumoral factors that are known to augment these parameters (e.g., norepinephrine, epinephrine) (8–10). It is therefore not surprising that classically, 24-h oscillations in heart rate and blood pressure were considered to be largely mediated by daily fluctuations in neurohumoral factors and secondary to behaviors such as sleep/wake and fasting/feeding cycles. However, in recent years studies in both humans and animal models have challenged this concept. For example, when healthy individuals are subject to a light/dark cycle of 28 h, termed forced dyssynchrony, 24-h oscillations persist for distinct cardiovascular parameters, including heart rate (11). Such observations suggest that daily rhythms in heart rate are not simply an adaptation to environmental and/or behavioral rhythms. Instead, an intrinsic timekeeping mechanism, namely the circadian clock, must be present within organisms, to drive 24-h rhythms in distinct biological processes.

Circadian clocks are cell-autonomous molecular mechanisms comprising a series of positive and negative feedback loops, with a free running period of approximately 24 h (2). Circadian clocks confer the selective advantage of anticipation, allowing a cell/organ/organism to prepare for a stimulus/stress prior to its onset, thereby facilitating a temporally appropriate response. At the core of the mammalian mechanism are two transcription factors, Clock (circadian locomotor output cycles kaput) and Bmal1 (brain and muscle ARNT-like 1), which, upon heterodimerization, bind to E-boxes in the promotor of target genes, invariably leading to induction (12, 13). Target genes include negative feedback components, such as period (*Per1/2/3*), cryptochrome (*Cry1/2*),

and Rev-erb (*Rev-erb α /* β) isoforms; Per/Cry heterodimer complexes repress Clock/Bmal1 transcriptional activity through physical interactions, whereas Rev-erb α / β represses Bmal1 at a transcriptional level (14–16). The Clock/Bmal1 heterodimer influences expression of numerous additional target genes, some of which are integral to the clock mechanism, whereas others, termed clock-controlled/output genes (CCGs), have the potential to modulate cellular function in a time-of-day-dependent manner. Circadian clocks have been described in all mammalian cells, including cardiomyocytes (2, 17). It has been estimated that between 3% and 16% of an organ's transcriptome is circadian regulated, impacting a diverse array of biological processes (18). Studies using mouse models in which the circadian clock mechanism has been genetically disrupted only in cardiomyocytes suggest that up to 10% of the cardiac transcriptome is regulated in some manner by the cardiomyocyte circadian clock; known functions of the encoded proteins range from transcription, signal transduction, cellular constituent turnover, transport, and metabolism (19). All of these processes influence contractile function of the heart, suggesting that the cardiomyocyte circadian clock enables the heart to anticipate day-night differences in contractile demand through preemptive modulation of critical cellular processes.

It is apparent, therefore, that both extrinsic (i.e., neurohumoral factors) and intrinsic (i.e., circadian clock) mechanisms have the potential to contribute toward daily fluctuations in cardiac physiology, with both contributing at multiple levels. If considered as independent factors, the effects of neurohumoral factors and circadian clocks would be additive. However, neurohumoral factors are extracellular stimuli, for which target tissue responsiveness is modulated by circadian clocks. In the case of the heart, the cardiomyocyte circadian clock has been shown to influence responsiveness to hormones (e.g., epinephrine, insulin) and nutrients (e.g., fatty acids) (20–23). Circadian clocks therefore amplify or dampen the responsiveness of the heart to neurohumoral stimuli, depending on the time of day. Moreover, the timing of the circadian clock mechanism needs to reset/entrain; for the heart, important entrainment factors (termed zeitgebers) include neurohumoral factors such as cortisol and norepinephrine (17, 24). It is also noteworthy that the levels of neurohumoral stimuli are themselves often under circadian clock control (6). For example, daily fluctuations in plasma cortisol levels are dependent on the circadian clock within the adrenal cortex (25). Taken together, a complex interplay between neurohumoral and circadian clock influences likely contributes to time-of-day-dependent oscillations in cardiac physiology.

2. DAILY OSCILLATIONS IN CARDIAC PROCESSES

As discussed above, day-night differences in cardiac physiology have been reported at multiple levels. Therefore, the purpose of the following subsections is to highlight what is known regarding circadian control of these processes, and their potential importance in cardiac physiology.

2.1. Cardiac Function

The following subsections address circadian regulation of cardiac electrophysiology and contractility.

2.1.1. Electrophysiology. In healthy subjects, time-of-day-dependent oscillations have been reported for multiple cardiac function parameters, including contractility and electrophysiology. The latter includes the R-R interval (which has an inverse relationship with heart rate), QT interval, and heart rate variability (HRV) (26, 27). In humans, both R-R and QT intervals are increased during the night, whereas HRV is increased in the morning (26–28). Classically, diurnal variations in these parameters have been explained by fluctuations in autonomic and sympathetic tone;

however, during forced dyssynchrony, 24-h rhythms in several of these electrophysiologic parameters persist, suggesting a potential contribution of the intrinsic timekeeping mechanism (11). Time-of-day-dependent rhythms in heart rate and electrophysiologic parameters have also been described in various animal models, ranging from dogs to rodents (29, 30). Interestingly, ex vivo perfused mouse hearts tend to exhibit increased heart rates in the middle of the active phase (compared to the sleep phase) (20). Moreover, 24-h oscillations in spontaneous beating have been reported in cultured neonatal rat cardiomyocytes (31). Such observations suggest that daily rhythms in heart rate may be mediated, in part, by intrinsic circadian clocks. Use of genetically modified mouse models has helped to reinforce this concept. Both germline *Bmal1* knockout and *Clock* Δ 19 mutant (a dominant negative mutant of *Clock*, due to deletion of the transactivation domain) mice exhibit a pronounced attenuation of day-night differences in heart rate; in both cases, the mesor (daily average) is lower (i.e., bradycardia) (32). Both germline *Bmal1* knockout and *Clock* Δ 19 mutant mice exhibit alterations in behaviors and systemic factors, which could contribute to the observed heart rate phenotype. To avoid these systemic perturbations, cell type-specific models have been employed. Cardiomyocyte-specific *Clock* Δ 19 mutant (CCM) mice exhibit a reduced amplitude of day-night differences in heart rate and bradycardia (20). However, inducible cardiomyocyte-specific *Bmal1* knockout mice exhibit normal day-night differences in heart rate (29). Collectively, these observations suggest that circadian regulation of extracardiac influences (such as autonomic tone) potentially play a dominant role in mediating time-of-day-dependent oscillations in heart rate, while the exact contribution of the cardiomyocyte circadian clock remains somewhat less clear.

Multiple ion channel subunits have been reported to fluctuate in atria and/or ventricles in a time-of-day-dependent manner, including integral components of K^+ (*Kchip2*, *Kcna5*, *Kcnd2*, *Kcnh2*, and *Kcnk3*), Na^+ (*Scn5a*), and Ca^{2+} (*Cacna1d*) channels (29, 33–37). Daily rhythms in distinct ion channel components are associated with predicted day-night differences in electrophysiological properties of the heart. For example, elevated cardiac *Kcna5* protein levels during the active period are associated with increased steady-state current (I_{Kur}) density in rat myocytes (37). In many instances, rhythms in these ion channel subunits are lost following disruption of circadian clocks and/or autonomic blockade (19, 20, 29, 33–37). For example, *Cacna1d*, encoding for a subunit in L-type voltage-gated ion channels, is rhythmic at both mRNA and protein expression levels in wild-type hearts, but not CCM hearts (20). Another subunit of this channel, *Cacna1d*, also appears to be regulated by the cardiomyocyte clock, not only at the level of expression, but also posttranslationally via the *Pi3k/Akt* signaling axis, which is also under clock control (19, 38). Together, these studies illustrate that the electrophysiologic properties of the heart are markedly different depending on the time of day, potentially contributing toward daily oscillations in heart rate.

2.1.2. Contractility. Similar to heart rate, contractility of the heart exhibits time-of-day-dependent oscillations at multiple levels. Echocardiographic studies in humans suggest that over a 24-h period, although systolic function does not exhibit fluctuations in healthy subjects, multiple diastolic parameters do, such as relaxation time, which peaks in the early hours of the morning (39, 40). When the intrinsic contractile properties of the heart are investigated through the use of isolated perfused mouse and rat hearts, parameters including cardiac output and rate pressure product are elevated during the active period; this was most striking for contractile reserve. When ex vivo mouse hearts are challenged with high workloads, cardiac output and rate pressure product were almost twofold higher during the active phase (compared to the sleep phase) (20, 41). Moreover, genetic disruption of the cardiomyocyte circadian clock abolishes these time-of-day-dependent rhythms in contractile function of the heart ex vivo, underscoring mediation

by this intrinsic mechanism (20). Contractility of the heart is influenced by numerous mechanisms, including metabolism, cell signaling, Ca^{2+} homeostasis, and myofilament composition, all of which are under strict circadian control (5, 42). β -Adrenergic signaling is a well-established positive inotrope, in large part through protein kinase A (Pka) activation, and Pka activity in the heart is increased at the beginning of the active period, associated with increased phospholamban phosphorylation and Serca2a activity (43). In contrast, calcineurin activity increases in the heart at the beginning of the sleep phase, thereby reversing the actions of Pka (43). Both Pka and calcineurin appear to be directly regulated by the cardiomyocyte circadian clock (19, 20), suggesting that both extrinsic and intrinsic mechanisms contribute toward time-of-day-dependent rhythms in this ionotropic signaling axis. Contractile protein components also exhibit significant day-night differences in the heart, at multiple levels. For example, the calcium sensitivity of myosin ATPase activity is augmented during the active period, and this day-night difference is absent following genetic disruption of the cardiomyocyte circadian clock (44). Numerous myofilament components also fluctuate over the course of the day, including myosin-binding protein C, desmin, tropomyosin, troponins I and T, and titin cap (44). The extent to which daily oscillations in myofilament proteins and/or posttranslational modifications of contractile components play a significant role in day-night differences of cardiac contractility remains an important, yet unanswered question.

2.2. Transcription, Translation, and Posttranslational Modifications

The following subsections address circadian regulation of transcription, translation, and posttranslational modifications in the heart.

2.2.1. Transcription. Hypothesis-generating approaches designed to determine potential molecular underpinnings of day-night differences in cardiac function have yielded several important concepts. The first is that a surprisingly large number of mRNA species fluctuate in the heart over the course of a 24-h period. For example, when mice are housed under standard 12h:12h light:dark cycles, approximately 13% of genes expressed in the heart exhibit statistically significant changes over the course of the day (45). Two major expression patterns were observed for these genes: cosine (single daily peak) and biphasic (two peaks per day) (45). In the latter case, the two peaks were often observed at the light-dark transitions. Bioinformatics approaches revealed that genes exhibiting time-of-day-dependent variations in hearts clustered into diverse biological functions, ranging from cellular growth and remodeling, to transcription, translation, mitochondrial function, and cellular signaling (45). When mice were housed in constant darkness, thereby eliminating the acute effects of light stimulation, approximately 6% of the cardiac transcriptome was identified as circadian regulated (18), which was similar to the estimate (8%) reported by Storch et al. (46). Again, two primary patterns of oscillating genes were identified: cosine and biphasic (peaking at times when light:dark transitions would have had occurred if mice had not been housed in constant darkness) (18). Interestingly, the heart exhibited temporal gene expression patterns that were markedly distinct to those observed in other tissues such as kidney, aorta, skeletal muscle, white adipose tissue, or brown adipose tissue. As one example, the time-of-day-dependent oscillation of vascular endothelial growth factor alpha is essentially antiphase in heart compared to brown adipose tissue (18). Collectively, these studies highlight that the heart exhibits extensive, divergent, and unique daily fluctuations in its transcriptome.

Many questions have arisen following temporal characterization of the cardiac transcriptome, including (a) what mechanism(s) orchestrate time-of-day-dependent fluctuations in the cardiac transcriptome and (b) whether daily changes in mRNA species translate to fluctuations in

corresponding proteins and/or cardiac function. With regard to the first question, both extrinsic and intrinsic factors have the potential to mediate temporal control of the cardiac transcriptome. A plethora of neurohumoral factors are known to impact gene expression in the heart, ranging from endocrine factors (e.g., glucocorticoids, insulin, epinephrine) to nutrients (e.g., fatty acids, glucose, vitamins) and neural stimulation (e.g., norepinephrine). Many of these extrinsic stimuli fluctuate over the course of the day. For example, norepinephrine, which peaks in the early hours of the morning, can acutely increase expression of multiple immediate early genes in the heart (47). Insulin, which fluctuates with fasting/feeding cycles, also influences expression of numerous genes in the heart involved in cellular growth and metabolism, in part through regulation of FoxO transcription factors (48). Fatty acids, which increase during periods of fasting and/or following a high-fat meal, impact gene expression through numerous mechanisms, including direct binding to nuclear receptors [e.g., peroxisome proliferator-activated receptor (Ppar) isoforms] (49). Similarly, cortisol, which peaks in the early hours of the morning, binds to nuclear (glucocorticoid) receptors, modulating expression of target genes (50).

It is not surprising that the cardiac transcriptome fluctuates dramatically over the course of the day. However, intrinsic circadian clocks also modulate cardiac gene expression in a time-of-day-dependent manner; indeed, the classic mammalian mechanism is composed of a series of transcription factors (51). In mice, selective overexpression of a dominant negative Clock mutant protein (CCM) or deletion of Bmal1 (CBK), specifically in cardiomyocytes, results in a temporal suspension of the circadian clock in the heart at the beginning of the sleep phase (19, 20). Interestingly, the majority of genes that exhibit 24-h oscillations in expression in normal murine hearts also become temporally suspended in CCM and CBK hearts, indicating that as much as 10% of the cardiac transcriptome is regulated in some manner by the cardiomyocyte circadian clock (19, 20). Moreover, gene ontology analysis suggested that clock-regulated genes in the heart are involved in processes such as metabolism, cellular signaling, and electrophysiology. What is less clear is which of these genes are directly regulated by a circadian clock component (e.g., the Clock/Bmal1 heterodimer), as opposed to secondarily (e.g., through clock-controlled transcription factors, such as Klf15) (52). A number of bioinformatic filters have been applied to CCM and CBK transcriptomic data, in an attempt to identify direct target genes of the Clock/Bmal1 heterodimer; only a small number of target genes were identified from this stringent analysis, many of which are known to influence cardiac metabolism (see Section 2.3.1) (19). However, the contribution of other circadian clock components (e.g., Rev-erb isoforms) remains less clear. Interestingly, agonists to Rev-erb α/β have recently been reported to dramatically impact gene expression in the heart (53); however, whether these effects are direct (i.e., activation of Rev-erb α/β in the heart) or indirect (e.g., agonist-induced changes in neurohumoral factors) is currently unknown.

2.2.2. Translation and protein turnover. In order to influence cardiac function over the course of the day, mRNA fluctuations must translate to meaningful alterations in protein levels and subsequently function. Moreover, the levels of some proteins may fluctuate independently to changes in mRNA. The latter was highlighted in the liver, where combined transcriptomic and proteomic approaches revealed that only approximately 50% of protein expression oscillations could be explained by alterations in gene expression (54). Time-of-day-dependent proteomic (and transcriptomic) analyses have been performed for the heart; when proteomic data were compared to previously published transcriptomic analyses, it appeared that only a subset of oscillating proteins could be readily explained by fluctuations in reciprocal mRNA species (44). Collectively, these observations suggest that at the proteomic level, the heart is markedly different during the day versus night, and the likelihood exists that daily rhythms in the cardiac proteome are driven by a combination of transcriptional and protein turnover events.

Protein turnover is considered the culmination of protein synthesis (i.e., translation) and degradation (through, for example, the proteasome and/or autophagy), both of which are circadian regulated in various tissues, including the heart. Cardiac protein synthesis is lowest in the middle of the active period and peaks at the beginning of the sleep phase; these rhythms are associated with concomitant oscillations in the activation status of the mTOR/S6/4EBP1 signaling axis, an established mechanism promoting translation initiation (22). Extrinsic modulators of protein synthesis and the mTOR/S6/4EBP1 signaling axis thus might be considered as key drivers for time-of-day-dependent rhythms, such as insulin or Igf1 (55); however, a number of key observations suggest that this is not the case. For example, in ad libitum-fed mice, plasma insulin levels are highest toward the beginning of the active period, a time at which protein synthesis is low, while plasma Igf1 levels do not fluctuate over the course of the day (56, 57). Moreover, during continuous fasting, despite chronically low plasma insulin levels, the mTOR/S6/4EBP1 signaling axis in the heart remains active at the wake-to-sleep phase transition (58); similar observations have been reported in skeletal muscle (59). Such observations cannot discount the possibility that locally generated Igf1 acts in a paracrine/autocrine fashion, which persists during fasting. On the other hand, genetic disruption of the cardiomyocyte circadian clock results in loss of time-of-day-dependent oscillations in both cardiac protein synthesis and the mTOR/S6/4EBP1 signaling axis, both of which are chronically activated in CBK and CCM hearts (22). Furthermore, rapamycin (an established mTOR inhibitor) normalizes rates of protein synthesis in CBK hearts (22). Collectively, these observations highlight a critical role for the cardiomyocyte circadian clock as a mediator of time-of-day-dependent rhythms in cardiac protein synthesis.

Damaged and short-lived proteins are targeted for degradation via the ubiquitin-proteasome system (UPS). This includes circadian clock components, which oscillate in a UPS-dependent manner (60–62). Consistent with an obligate relationship between circadian clock and the UPS, several E3 ligases and proteasome subunits are directly regulated by cell-autonomous circadian clocks (54, 63). Although circadian control of the UPS system has not been investigated directly in the heart, transcriptomic analyses have revealed that a number of UPS components exhibit time-of-day-dependent oscillations; these include various proteasome subunits, such as Psmb9, Psmd5, and Psmd10 (19). Proteins, as well as other cellular constituents, are also targeted for degradation via autophagic pathways. Evidence has accumulated suggesting circadian regulation of autophagy in multiple organs, including the heart, as discussed in more detail below.

2.2.3. Posttranslational modifications. The posttranslational modification of proteins is an important mechanism responsible for fine-tuning protein function, localization, and stability, and appreciable evidence has accumulated, highlighting that posttranslational modification of proteins exhibits time-of-day-dependent oscillations in multiple tissues, including the heart. Many studies have focused on core circadian clock components, but such changes extend far beyond the clock mechanism. These posttranslational modifications include phosphorylation, acetylation, O-GlcNAcylation, ubiquitination, SUMOylation, ADP-ribosylation, methylation, and S-nitrosation (**Table 1**) (64–68). Here, we have focused on phosphorylation, acetylation and O-GlcNAcylation, which together represent key connections between the circadian clock and cardiac processes, such as signaling and metabolism.

Phosphorylation is the most widely studied circadian-regulated posttranslational modification. In the liver, over 20,000 phosphorylation sites have been reported to oscillate in a time-dependent manner, including those on core clock proteins (69). Interestingly, the magnitude of changes in amplitude of phosphorylation levels is substantially greater than that in transcript or protein levels; the phase distributions between peaks in phosphorylation and protein levels were also divergent (69). Moreover, different kinase pathways, responsible for regulating different physiological

Table 1 Known posttranslational modifications of the mammalian circadian clock^a

Protein	Posttranslational modifications
Clock	P, O-GlcNAc, ADPrib, Me,
Bmal1	P, O-GlcNAc, Ac, Ub, S-Ni, SUMO
Per1/2	P, O-GlcNAc, Ac, Ub, Me
Cry1/2	P, Ub
Rev-erba/β	P, Ub

^aInformation from Reference 64 and PhosphoSitePlus (<https://www.phosphosite.org/>).

pathways, were partitioned into day and night phases. Overall, these findings provide strong evidence demonstrating the importance of phosphorylation in regulating biological functions over the course of the day. One such function includes the circadian clock itself. In mammals, casein kinase (CK)1δ and 1ε play a central role in the regulation of core clock components (68). Per1/2 proteins, which repress Clock and Bmal1, are phosphorylated by CK1δ/ε, regulating their nuclear translocation and ubiquitin-mediated degradation (68). In doing so, CK1δ/ε is critical for maintaining the ~24-h periodicity of the clock (68). This is evident by the fact that the *tau* mutant hamster, which has a point mutation in CK1ε, possesses a clock with a free running period of 20 h (70). The complexity surrounding CK1δ/ε-mediated Per1/2 phosphorylation is demonstrated by the fact that in some cells it promotes cytoplasmic accumulation, whereas in other it facilitates nuclear translocation (**Figure 1a**) (68).

Cry1/2, repressor of Clock/Bmal1 transactivation, is also a known target of AMPK; AMPK-dependent phosphorylation of Cry1/2 promotes its proteasomal degradation (67). AMPK has also been shown to phosphorylate CK1ε, increasing its activity and subsequently decreasing Per2 stability (71). Following phosphorylation by dual-specificity tyrosine-phosphorylation-regulated kinase (Dyrk1a), Cry2 (but not Cry1) is phosphorylated by Gsk3β, which accelerates its degradation. Gsk3β also phosphorylates Rev-erba, a negative regulator of Bmal1, increasing its stability (67, 72). Importantly, both AMPK and Gsk3β have been shown to exhibit circadian rhythms in the heart, which likely play a role not only in control of the cardiomyocyte circadian clock but also as modulators of cardiac physiology.

Although protein levels of Bmal1 and Clock exhibit relatively modest time-of-day-dependent fluctuations in many tissues relative to mRNA levels, both demonstrate more robust rhythms in phosphorylation levels, which is important for regulation of their stability and consequently their transcriptional activity (66). Bmal1 phosphorylation by CK1δ/ε occurs on a highly conserved Ser90 residue, required for dimerization with Clock; consequently, mutation of this site to alanine prevents phosphorylation and results in disruption of circadian clock function (66). Multiple posttranslational modifications are dependent on Bmal1:Clock heterodimerization and/or Ser90 phosphorylation (66). Moreover, the number of kinases reported to phosphorylate Bmal1 is growing and includes Erk, Jnk, Gsk3β, cyclin-dependent kinase 5 (Cdk5), Pkcγ, and calmodulin-dependent protein kinase II (CamkII) (66, 73). In addition to Bmal1, Clock is phosphorylated by both Gsk3β and Cdk5. In general, phosphorylation-deficient forms of both Bmal1 and Clock exhibit increased stability or reduced activities (74). The cyclic control of clock components, as well as downstream processes, through phosphorylation, requires not only kinases, but also phosphatases; protein phosphatase-1 (Pp1) and Pp2a are the best characterized in terms of circadian regulation. In the case of Pp1, previous studies suggest that activity of this phosphatase likely oscillates over the course of the day in the heart, secondary to circadian regulation of inhibitor-1 (an established inhibitor of Pp1). Interestingly, the protein phosphatase 2 regulatory subunit B gamma (*Ppp2r2c*) gene has been linked to cross talk between the circadian clock and

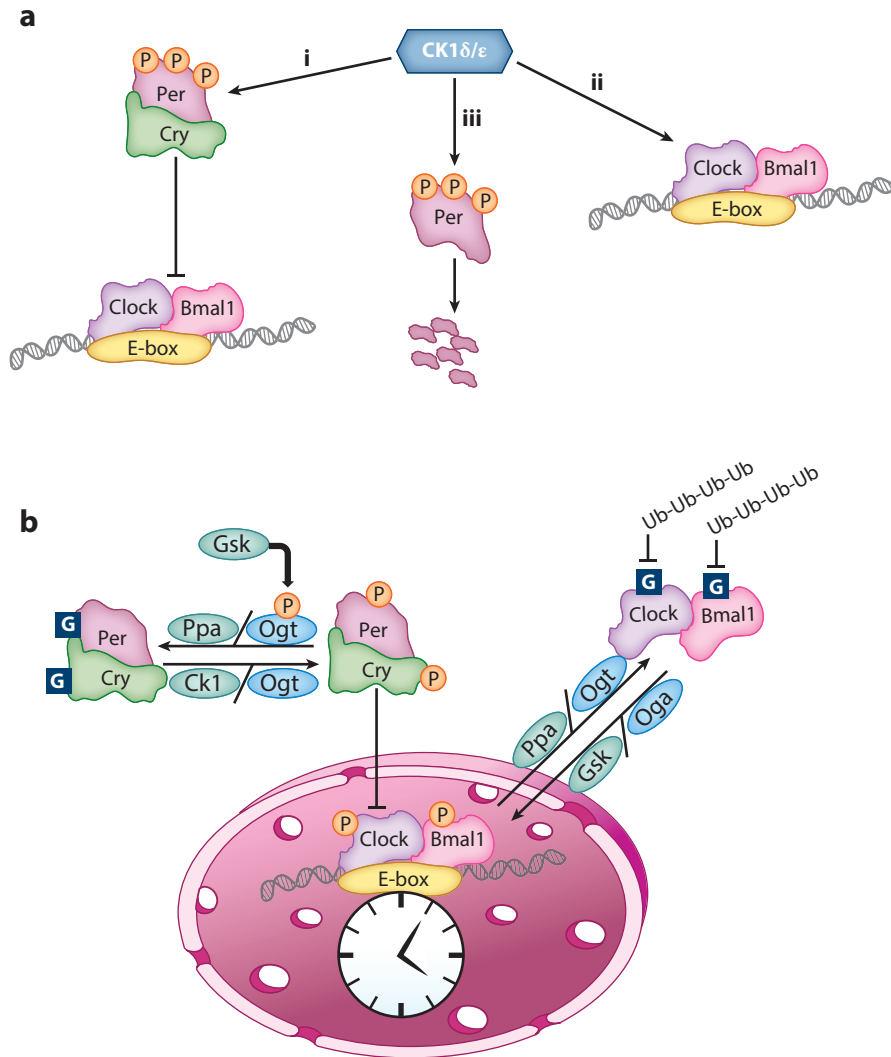


Figure 1

(a) Functions of casein kinase (CK) 1 δ/ϵ in the mammalian clock. (i) Interaction with Cry stabilizes Per, allowing it to be phosphorylated by leading to its nuclear translocation and repression of Clock/Bmal1 transcription; (ii) CK1 δ/ϵ phosphorylation of Per in the cytosol leads to proteasomal degradation; (iii) CK1 δ/ϵ phosphorylation of Bmal1 increases its transcriptional activity. (b) Cross talk between phosphorylation (P) and O-GlcNAcylation (G) in the regulation of the circadian clock. Gsk3 β phosphorylation of Ogt results in its activation, leading to O-GlcNAcylation of Per and Cry, which prevents their phosphorylation, thereby repressing their effects on Clock/Bmal1 transcription. O-GlcNAcylation of Clock also represses the effects of Per. Phosphorylation and O-GlcNAcylation also play a role in regulating the ubiquitination and subsequent degradation of Clock and Bmal1.

heart disease (75). The interactions between kinases/phosphatases with the mammalian circadian clock are summarized in **Table 2**.

The role of acetylation in regulating the circadian clock was first recognized when rhythms in H3 histone acetylation in mouse liver were shown to accompany Clock-Bmal1 transcriptional

Table 2 Interactions between kinases and phosphatases and the mammalian circadian clock^a

Kinase/phosphatase	Clock target
Pp1	Per1/2
Pp5	CK1
CK1	Per1/2, Cry1/2
CK2	Per1/2, Bmal1
Gsk3 β	Cry1/2, Bmal1, Clock, Rev-erba/ β
AMPK	Cry1/2

^aBased on Reference 73, figure 1.

activity and that Clock exhibited histone acetyltransferase activity (76). In addition to specific histone targets, Clock also acetylates Bmal1, and Bmal1 acetylation demonstrates time-of-day-dependent rhythms. Importantly, Bmal1 acetylation is required for normal clock function and is necessary to inactivate Bmal1 activity via the recruitment of Cry proteins to the Clock-Bmal1 complex (67). Sirt1, an NAD⁺-dependent deacetylase, antagonizes Clock-mediated acetylation; Sirt1 directly interacts with Clock in a circadian-dependent manner and is responsible for deacetylation of Per2, which stimulates Per2 degradation. Conversely, knockdown of Sirt1 increases Per2 acetylation and its stability (77). The precise mechanism by which acetylation stabilizes Per2 is not known; however, the fact that it occurs on lysine residues that can also be ubiquitinated could be a factor. In the mouse heart, rhythms in the acetylation of the Per1/2 promoter preceded those of Per1/2 mRNA, consistent with regulation of the cardiomyocyte circadian clock by acetylation (78).

The activity of Sirt1 is dependent on NAD⁺, and nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting step in the NAD⁺ salvage pathway. Nampt itself is directly regulated by the circadian clock, resulting in rhythms in both NAD⁺ levels and Sirt1 activity (79–81). The importance of this pathway in circadian regulation is demonstrated by chronically elevated NAD⁺ levels that, due to systemic knockout of the NAD hydrolase Cd38, resulted in disruption of circadian behavior and dysregulation of peripheral clock genes (82). Pharmacological inhibition of Nampt also altered the circadian expression of Per2, resulting in an earlier onset of its peak expression and increased Bmal1 acetylation, similar to that seen in response to loss of Sirt1 (81). The circadian rhythms of protein acetylation in the heart has not been examined fully; however, as in other tissues, the heart exhibits time-of-day-dependent changes in both Nampt and NAD⁺ levels. Moreover, Nampt and NAD⁺ levels are both chronically low in CCM hearts (56, 83). Contrary to what might be anticipated, CCM hearts exhibit chronically low protein acetylation, suggesting that other histone acetylases and histone deacetylases (Hdacs) are likely clock controlled in the heart. Given the importance of NAD⁺, Sirt1, and acetylation in regulation of cardiac processes, this axis likely represents an important link between the cardiomyocyte circadian clock and cardiac physiology.

Another link between regulation of cardiac physiology and the circadian clock is modification of serine and threonine residue proteins by *O*-linked-*N*-acetylglucosamine (*O*-GlcNAc) (**Figure 1b**). *O*-GlcNAc is a reversible posttranslational modification, which is formed by the metabolism of UDP-*N*-acetylglucosamine (UDP-GlcNAc) by *O*-GlcNAc transferase; *O*-GlcNAc is removed from proteins by *O*-GlcNAcase. UDP-GlcNAc is synthesized by the hexosamine biosynthesis pathway, which requires fructose-6-phosphate, glutamine, acetyl-CoA, and UTP; consequently, *O*-GlcNAc modifications are typically consider to be nutrient regulated posttranslational modifications (84). Durgan et al. (83) first reported a link between protein *O*-GlcNAcylation and the cardiomyocyte circadian clock, when they observed that *O*-GlcNAc levels and Ogt protein had a time-of-day-dependent rhythm in wild-type hearts that was absent in CCM hearts. Although

Oga levels did not show any rhythmic changes, it was chronically repressed in CCM hearts. Both Bmal1 and Per1 were shown to be *O*-GlcNAcylated; Bmal1 *O*-GlcNAcylation peaked in the middle of the dark phase (83). Subsequent studies in *Drosophila* further substantiated a link between *O*-GlcNAc and the circadian clock, demonstrating that changes in Ogt expression altered the length of circadian rhythms, secondary to Per *O*-GlcNAcylation (85). *O*-GlcNAcylation of Bmal1 and Clock was found to inhibit their ubiquitination, thereby increasing their stability (86, 87), while changes in Ogt expression resulted in alterations in Bmal1/Clock target genes (86). Another study identified specific sites of *O*-GlcNAcylation on Per and showed that *O*-GlcNAc modification of Ser492 attenuated the interaction between Per and Clock (88). Interestingly, *O*-GlcNAcylation of Per2 occurs in a region that is known to regulate sleep phase in humans and competes with phosphorylation in the same region (89).

Though Rev-erb α has not been shown to be an *O*-GlcNAc target, it interacts directly with Ogt in both the cytosol and nucleus, where it stabilizes Ogt protein levels (90). As a result of influencing Ogt stability, Rev-erb α indirectly regulates cellular *O*-GlcNAc levels; consequently, changes in Rev-erb α protein levels over the course of the day could represent a mechanism leading to the rhythmic changes in *O*-GlcNAc observed in the heart (83). In cell culture, Rev-erb α , Ogt, and *O*-GlcNAc levels all peaked 24 h following serum shock, and Rev-erb α was found to regulate *O*-GlcNAc levels of over 200 proteins (90). One of those proteins was Akt, and Rev-erb α influenced both basal- and insulin-stimulated Akt phosphorylation. These studies suggest that Rev-erb α could be an important link between time-of-day-dependent changes in cardiac *O*-GlcNAc levels (83) and insulin sensitivity (22).

2.3. Metabolism, Mitochondria, and Redox Biology

The following subsections address circadian regulation of cardiac metabolism, mitochondrial function, and redox biology.

2.3.1. Metabolism. To be able to continuously adapt to changing workload demands throughout the day, the heart requires a high level of metabolic flexibility. Consequently, the heart is capable of using a wide array of substrates for energy production, including glucose, fatty acids, lactate, pyruvate, ketone bodies, and distinct amino acids (91). Both substrate availability and hormone levels fluctuate with fasting/feeding cycles, which undoubtedly contribute toward daily rhythms in cardiac metabolism. Changes in physical activity across sleep/wake cycles also impact cardiac metabolism. However, in addition to these extrinsic factors, there is substantial evidence that the cardiomyocyte circadian clock plays a significant role in regulating myocardial metabolism. For example, rhythms in glycogen and protein synthesis persist even during prolonged fasting, suggesting that feeding behavior is not the main driving factor (91). Moreover, time-of-day-dependent metabolic oscillations persist in *in vitro* models, such as the isolated perfused heart (91). The importance of these intrinsic factors is underscored by observations that genetic disruption of Clock and/or Bmal1 abolishes time-of-day-dependent rhythms in myocardial glucose and fatty acid metabolism (20, 21, 91). In the case of fatty acid metabolism, Ppars play an important role in transcriptional regulation of cardiac metabolism. Consistent with this at the beginning of the active phase, there is a coordinated regulation of Ppar α modulators, such that Ppar α target genes, which regulate fatty acid metabolism, are induced in the heart (23). Fasting, which increases circulating fatty acids, induces transcriptional activation of fatty acid metabolism in a Ppar α -dependent manner in the heart, and this is markedly attenuated in CCM hearts (23). CCM hearts exhibit a sustained increase in fatty acid oxidation compared to control littermate hearts independent of the time of day, while triglyceride synthesis is chronically low. Similar metabolic inflexibility of CCM

hearts was observed during chronic alteration of fatty acid availability, induced by either diabetes or high-fat feeding (56, 92). Although AMPK-mediated phosphorylation of acetyl-CoA carboxylase beta (Acc β) is a key mechanism for increasing fatty acid oxidation it does not appear involved in clock-dependent regulation of cardiac fatty acid metabolism (56). However, posttranslational modification of carboxylases, such as Acc β , with biotin is essential for enzymatic activity (93), and recent studies have shown that biotinylation was regulated by the circadian clock in the heart (94). Total biotinylation, as well as Acc β biotinylation, were decreased in CBK hearts, consistent with higher fatty acid oxidation rates. Moreover, dietary supplementation with biotin normalized Acc β biotinylation and reduced fatty acid oxidation to control levels (94).

Although AMPK is not responsible for time-of-day-dependent changes in fatty acid oxidation, AMPK phosphorylation and activity peak in the middle of the active phase in the heart, which is associated with an increase in glucose metabolism; these rhythms are absent in CCM hearts (83, 92). NAD⁺ represents a key link between metabolism and circadian clocks (95) and, as discussed earlier, Nampt, which is involved in NAD⁺ biosynthesis exhibits a diurnal rhythm (83) that is absent in CCM hearts. Circadian regulation of cardiomyocyte NAD⁺ levels could have wide-ranging effects given its central role in regulating numerous metabolic processes (83).

Akt phosphorylation, a key component in insulin signaling, is dependent on Pi3k; *Pik3r1*, encoding for the p85 α subunit of Pi3k, exhibits time-of-day-dependent rhythms in the heart, which are absent in both CCM and CBK hearts (19). Disruption of the cardiomyocyte clock resulted in augmented phosphorylation of Akt at both ser473 and thr308 in response to insulin treatment, which was most pronounced at the beginning of the active period (22). Surprisingly, despite higher Akt phosphorylation, insulin-mediated glycolysis and glucose oxidation were attenuated in CBK hearts, suggesting a disconnect between Akt phosphorylation and subsequent activation of Glut4 translocation (22). Insulin-mediated phosphorylation of AS160, a downstream target of Akt responsible for regulating Glut4 trafficking and glucose metabolism, was also blunted in CBK hearts. Similarly, insulin-mediated phosphorylation of Gsk3 β (another Akt target) was attenuated throughout out the day in both CBK and CCM hearts. Collectively, these observations raised the possibility that increased phosphatase activity might be counteracting the effects of Akt (22). Pp1 expression, which regulates both As160 and Gsk3 β phosphorylation, is not affected by disruption of the circadian clock in heart; however, inhibitor-1, which represses Pp1 activity, was ~50% lower in both CBK and CCM hearts. Inhibitor-1 mRNA and protein levels also exhibited time-of-day-dependent changes, which were again significantly blunted in CBK hearts. These data suggest that disruption of the cardiomyocyte circadian clock leads to impaired insulin-mediated glucose metabolism due to chronic activation of Pp1, resulting from lower inhibitor-1 levels (22).

In contrast to carbohydrate and lipid metabolism, limited information on circadian regulation of ketone body metabolism in the heart is available. Circulating ketone body concentrations can vary widely in response to different physiological states, from approximately 50 μ M under normal conditions to the low millimolar range in response to fasting (and even higher in pathological conditions such as diabetic ketoacidosis) (96). Ketone bodies are readily oxidized by the heart in direct proportion to their availability and are used preferentially over carbohydrates and fatty acids for energy production (96). There are reports of diurnal variations in circulating ketone bodies in humans (97, 98) and rats (99). In rats these rhythms were maintained during 24 h of fasting (99), which suggests that they could be regulated at least in part by circadian clocks. In the heart, β -hydroxybutyrate dehydrogenase 1, which plays a key role in oxidation of ketone bodies (96), is markedly repressed in CCM and CBK hearts, consistent with it being regulated by the cardiomyocyte circadian clock (19). This was associated with a >90% decrease in activity and >60% decrease in β -hydroxybutyrate oxidation (19). The growing evidence that ketone body

metabolism is increased in the failing heart (100) suggests that studies to better understand the mechanisms underlying the circadian clock regulation of cardiac ketone body metabolism are needed.

Our understanding of the time-of-day-dependent regulation of amino acid metabolism in the heart is not well developed; however, there are rhythmic changes in amino acids levels and the expression of enzymes that regulate amino acid metabolism (20, 45, 46, 101, 102). Glutamine synthetase, which plays a key role in cardiac glutamine metabolism, exhibits a clear change over the course of day at both the gene and protein level, which was repressed in CCM hearts (83). In addition, oxidation of the branched chain amino acid leucine was attenuated in CBK hearts (94), whereas leucine incorporation into proteins was higher in CBK hearts. This observation is consistent with studies reporting that time-of-day-dependent rhythms in protein synthesis are mediated by the cardiomyocyte circadian clock (22, 103). Section 2.2.2 describes in greater detail the mechanisms by which circadian clocks impact protein synthesis (and degradation).

2.3.2. Mitochondria. A key cornerstone of cardiac physiology is mitochondria function. Mitochondria are essential not only for ATP synthesis, but also for calcium homeostasis, redox signaling, and metabolite provision. Circadian regulation of mitochondrial function has been reported in a number of cells (e.g., fibroblasts) and tissues (e.g., liver). However, less is known for the heart. Time-of-day-dependent regulation of cardiac mitochondrial function was first suggested in 2001, when rat myocardial oxygen consumption was found to be higher in the middle of the night (compared to in the middle of the day) (104). To date, we are unaware of a study that has directly assessed mitochondrial function in the heart at different times of the day. However, we have recently measured the activity of mitochondrial complexes in mouse hearts at 6-h intervals over a 24-h period, revealing that complex I activity is highest at the beginning of the active phase (J. Zhang, J. Chatham, M. Young, unpublished observations). More is known about the impact of genetic disruption of the cardiomyocyte circadian clock on cardiac mitochondrial parameters. For example, oxygen consumption has been measured in isolated subsarcolemmal and intramyofibrillar mitochondria from CCM and control littermate hearts. In subsarcolemmal, but not intramyofibrillar mitochondria, state 3 respiration was decreased in CCM hearts, with glutamate, pyruvate, palmitoylcarnitine, palmitoyl-CoA, or acetyl carnitine as substrates (but not succinate, dihydroquercetin, or octanoyl-CoA). This was in the absence of changes in mitochondrial DNA content, number, citrate synthase activity, gross morphology, or volume density between CCM and control hearts (20). Kohsaka et al. (105) have reported a more severe mitochondrial phenotype in CBK hearts, which exhibit decreased mitochondrial protein content, diminished complex I and IV activities, and more divergent mitochondrial sizes.

As discussed in previous sections, multiple omics approaches support the concept of circadian regulation of mitochondrial function (18, 46, 101, 106–108). Microarray studies performed on extracardiac tissues (e.g., suprachiasmatic nucleus, liver) isolated from mice housed in constant darkness revealed that many genes related to mitochondrial function and metabolism oscillate over the course of the day, including those encoding electron transport chain proteins (*Ndufa2*, *Ndufb5*, *Ndufc1*, *Ndufv2*, *Cox7b*, *Cox4a*, *Cox6a1*, *Cox6c*, *Cox7a3*, *Cox7c*, *Atp5g2*, *Atp5l*, and *Atp5c1*) (18, 108). A similar microarray study investigating the heart reported that, of the 462 genes oscillating, 5 were mitochondrial, including a mitochondrial uncoupling protein, a membrane carrier, and a subunit of cytochrome *c* oxidase (46). When transcriptomic studies are performed in hearts from animals as diverse as mice to nonhuman primates and humans, genes involved in ATP generation, oxidative metabolism, and mitochondrial function are consistently identified as oscillating over the course of the day (101, 106, 107). Using genetically modified mouse models with circadian gene disruption,

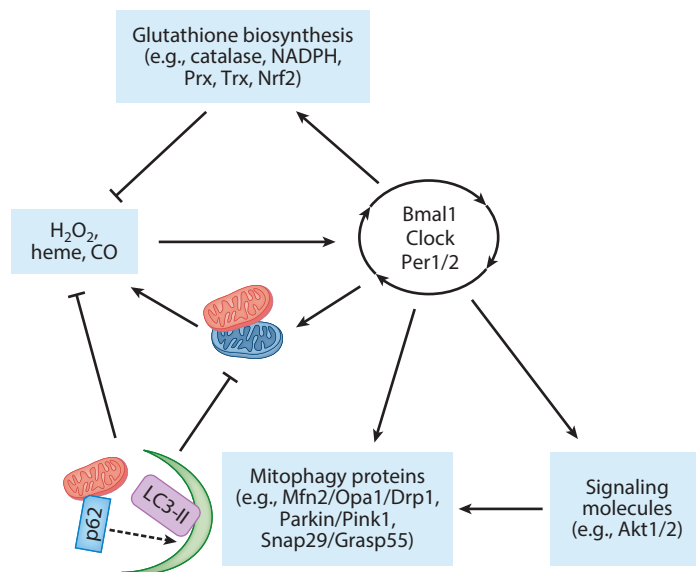


Figure 2

Cross talk between circadian clock components and reactive species. Both reactive species and antioxidants are circadian regulated. Moreover, the activities of circadian clock components can also be modulated by reactive species. To what extent this cross talk occurs in the heart requires full elucidation.

transcripts encoding distinct components of electron transport chain complexes, such as *Ndufs7*, *Sdhc*, *Cox7b*, *Atp5g2*, and *Uqcrc1*, are chronically repressed in the heart (and oscillate in control hearts) (105). mRNAs encoding for the mitochondrial biogenesis protein *Pgc1 α* , as well as fusion proteins (*Mfn2* and *Opa1*), are also decreased in CBK hearts (105). Interestingly, *Mfn2*, *Opa1*, and *Drp1* have all been shown to be circadian regulated in the liver, and that liver cell mitochondria are enlarged/swollen in hepatocyte-specific *Bmal1* knockout mice (109). Taken together, these observations suggest that cardiac mitochondrial function and morphology likely fluctuate over the course of the day, through processes governed by the cardiomyocyte circadian clock. Despite these studies, the extent to which mitochondrial function is regulated by the circadian clock in the heart remains unclear.

2.3.3. Cellular redox status. Dysregulation of redox status has been shown to affect cardiac health and response to stress (110–112). Consequently, it is important to understand whether redox signaling in the heart is circadian dependent; moreover, redox signaling may modulate circadian clock function (Figure 2). It is also possible that antioxidant defenses may also be circadian regulated and could mediate time-of-day-dependent responses of the heart to stress. Significant progress has been made toward a better understanding of the cross talk between redox biology and the circadian clock (113, 114). As early as the 1980s, glutathione and lipid peroxidation products were found to be circadian regulated in rat blood, liver, stomach, kidney, and heart, with glutathione levels higher in the dark phase (115). In human red blood cells kept in complete darkness, the oxidized/hyperoxidized PRX-SO_{2/3} dimer peaks at circadian time 16, which was associated with circadian regulation of hemoglobin autooxidation, NADH, and NADPH (116). Although peroxiredoxin dimer oscillations are transcription independent in red blood cells, these oscillations are lengthened in embryonic fibroblast cells from *mCry1/2*^{-/-} mice compared to those from wild-type mice, indicating that both transcription-dependent and -independent redox oscillations

exist (116). Peroxiredoxin oscillations also occur in synchronized mouse 3T3 cells, the unicellular pico-eukaryotic alga, heads of the fruit fly *D. melanogaster*, and the filamentous fungus *Neurospora crassa*, suggesting that this is a highly conserved mechanism (116–118). Importantly, peroxiredoxin oscillations are also observed in the heart (119), supporting the concept of time-of-day-dependent changes in cardiac redox status.

In the rat heart, higher levels of lipid peroxidation and lower levels of selenium-dependent glutathione peroxidase activity have been reported during the dark phase (120). Moreover, microarray analysis demonstrated that several isoforms of glutathione transferase are repressed in CBK hearts (19). In aortas of germline Bmal1 knockout mice, superoxide is increased, and BH4 and BH2 levels are imbalanced (121). Both L-NAME (NOS inhibitor) and BH4 (eNOS cofactor) decreased superoxide in Bmal1 knockout mice, suggesting an uncoupling of eNOS activity (121). The enzymes involved in bipterin metabolism and eNOS coupling, Gtpch-1 and Dhfr, have also been shown to be circadian regulated in aortas (121). In human aortic smooth muscle and endothelial cells, NADPH oxidase 4 (*NOX4*) gene expression is circadian regulated; genetic disruption of Bmal1-induced *Nox4* gene expression both in vivo and in vitro (122). These observations provide strong support for circadian clock-dependent regulation of redox signaling in the heart and vascular system.

Circadian clock transcription factor function is also influenced by reactive oxygen species. For example, exposing a cell line from zebrafish embryos to H₂O₂ resulted in elevated *zCry1a* and *zPer2* gene expression and also induced *zCry1a*, *zPer2*, *zPer1*, and *zCat* gene oscillation (123). Interestingly the rhythms in catalase activity were antiphase relative to *zPer2* and *zCry1a* expression; ectopic expression of *zCat* resulted in attenuated light induction of *zCry1a* and *zPer2* gene expression (123). The binding and regulation of heme seems to be a common feature shared among Npas2, Per2, Rev-erb α , and Rev-erb β , suggesting a possible mechanism for reactive oxygen species-mediated regulation of circadian rhythms (124–128). Moreover, NADPH is circadian regulated not only in the human red blood cells but also in human osteosarcoma cells (116, 129); inhibition of the pentose phosphate pathway (PPP; critical for NADPH generation) increased *BMAL1* reporter gene expression (129). Moreover, inhibition of PPP in organotypic slice culture of the suprachiasmatic nucleus and the liver lengthened the oscillation of *Per2:Luc* gene expression (129). Taken together, significant cross talk between redox homeostasis and the circadian clock exists (**Figure 2**); however, to what extent this cross talk occurs in the heart, and how its perturbation may contribute to heart diseases, remain to be determined.

2.3.4. Mitochondrial quality control. Given the importance of mitochondria for cellular functions, it is essential that there are mechanisms to ensure mitochondrial quality control. This is especially important in the heart, because oxidative phosphorylation is the main source of energy in the mammalian heart (130), and ~30% of a cardiomyocyte's volume consists of mitochondria (131). Using pulse chase as the primary method of assessment, it was estimated that the half-life of heart mitochondria in rodents was ~16 days (132). Despite their long half-life, it is essential that damaged mitochondria are replaced (i.e., degraded and synthesized). Because there is a strong circadian regulation of mitochondrial protein levels, and likely a circadian regulation of mitochondrial function, there is a strong possibility that cardiac mitochondria are more likely replaced at specific times of the day. A key pathway for removal of damaged mitochondria involves mitophagy (a specialized form of autophagy). Diurnal changes of autophagy were observed as early as in the 1970s in kidney tubules of normal rats (133). Circadian regulation of autophagy has also been investigated in other tissues, including the liver and the heart. The use of both candidate testing and hypothesis-generating studies suggests that genes and proteins known to be involved in mitochondrial dynamics and mitophagy may be circadian controlled in the heart (19, 22, 58). As

of future study may include (a) determining the exact mechanisms driving daily fluctuations in cardiac processes and/or whether perturbation of these mechanisms plays a causal role in the etiology of common cardiac diseases; (b) improving our understanding of the circadian control of organelles (e.g., lysosomes, mitochondria) in the heart; and (c) unveiling the complex interplay between extrinsic (i.e., neurohumoral) and intrinsic (i.e., circadian clock) influences contributing to the circadian control of cardiac physiology.

DISCLOSURE STATEMENT

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