Published in final edited form as:

Free Radic Biol Med. 2018 May 01; 119: 75–84. doi:10.1016/j.freeradbiomed.2018.02.013.

An Overview of the Emerging Interface Between Cardiac Metabolism, Redox Biology and the Circadian Clock

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Abstract

At various biological levels, mammals must integrate with 24-hr rhythms in their environment. Daily fluctuations in stimuli/stressors of cardiac metabolism and oxidation-reduction (redox) status have been reported over the course of the day. It is therefore not surprising that the heart exhibits dramatic oscillations in various cellular processes over the course of the day, including transcription, translation, ion homeostasis, metabolism, and redox signaling. This temporal partitioning of cardiac processes is governed by a complex interplay between intracellular (e.g., circadian clocks) and extracellular (e.g., neurohumoral factors) influences, thus ensuring appropriate responses to daily stimuli/stresses. The purpose of the current article is to review knowledge regarding control of metabolism and redox biology in the heart over the course of the day, and to highlight whether disruption of these daily rhythms contribute towards cardiac dysfunction observed in various disease states.

Keywords

Chronobiology; Heart; Metabolism; Redox Tone

Introduction

Cardiac metabolism encompasses a highly dynamic array of interlinked pathways designed to meet both the energetic demands of the heart, and provide precursor molecules essential for critical processes as diverse as the synthesis of cellular constituents to signal transduction. Metabolism must be tightly controlled, yet remain plastic, thereby affording an ability to rapidly shift flux between distinct pathways in response to environmental

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Disclosures
None declared.

perturbations while meeting energy demands and other biological processes. This is achieved through regulation at both the systemic and organ levels. In this review, we will focus predominantly on the heart to illustrate how circadian biology is integrated with metabolism and the role redox sensitive pathways play in this process. The heart is responsive to the physiological demands of the organism, which is orchestrated by changes in a range of signaling molecules that fluctuate secondarily to diet, physical demands and numerous stimuli/stresses.

Over the last decade our understanding of how reduction or oxidation (redox processes) of proteins can contribute to both physiology and pathology has undergone a paradigm shift. Previously, the field of free radical biology was dominated by the “oxidative stress paradigm”. This has been a popular and straightforward concept that promotes the central idea that there is a balance between free radicals or oxidants [commonly called reactive oxygen species (ROS) or reactive species] with antioxidants in normal physiology [1, 2]. Pathology then occurs when reactive species are produced in excess of the endogenous antioxidants, and this leads to indiscriminate damage to cellular macromolecules (proteins, lipids, and DNA) and kills cells [3]. This led to the postulate that ROS are bad and therefore any associated pathologies can be reversed by the appropriate application of antioxidants. Unfortunately, this model does not effectively include the robust literature, which has shown that ROS and nitric oxide (NO) can act as signaling molecules and that the over-expression of endogenous antioxidant enzymes can be toxic to the cell [4]. In this review we will use the general term ROS unless the studies being discussed have identified specific reduction products of oxygen [5]. To address these issues we, and others, have promoted the “redox biology” paradigm in which antioxidants play the primary role of modulating the complex networks controlling cell signaling and metabolism, such that pathology results from loss of control over specific intracellular domains [6, 7]. In this review, we use the term “redox” to refer to oxidative or reductive processes that have the potential to control cellular function. In some cases these redox events can be specifically defined at redox sensitive amino acids, such as cysteine, but in others we do not have this level of resolution. In writing this review it became clear, that apart from some pioneering studies, remarkably little is known about the interface between circadian and redox biology, particularly in the heart. Wherever possible, information is provided in a manner that allows the reader to have a more complete picture of the field, including its limitations and opportunities.

It is now becoming clear that cardiac metabolism is aligned with time of day, through both extrinsic (e.g., neurohumoral factors) and intrinsic (e.g., circadian clock) influences [8, 9]. During various disease states (including aging), metabolic plasticity is often attenuated/abolished [10, 11], and this is frequently associated with loss of control of redox homeostasis and progression of cardiac pathologies. Metabolism and redox biology are closely interlinked, and like metabolism, the redox status of specific regions in the cell are maintained within specific set points (termed redox tone), through adaptive regulation of the redox network [6, 7, 12–17]. As with cardiac metabolism, redox biology exhibits a time of day dependence, supporting the notion that the integration of metabolic and redox signaling with biological clocks is essential for normal physiology [18–21]. Furthermore, the incidence of adverse cardiovascular events (e.g., myocardial infarction, sudden cardiac death) is time-of-day-dependent, occurring more frequently in the early morning hours [22];

these rhythms occur in parallel with fluctuations in cardiac metabolism and redox tone. The purpose of the current article is to review knowledge regarding control of metabolism and redox biology in the heart over the course of the day, and to highlight whether disruption of these daily rhythms contribute towards cardiac dysfunction observed in various disease states.

Rhythms in Cardiac Metabolism

A great deal of information has accumulated regarding circadian control of metabolism. This section we will use the research literature in the heart, and whenever possible, will attempt to highlight potential links with redox biology (which will be developed further in subsequent sections). Over the course of the day, the myocardium exhibits profound metabolic plasticity at multiple levels, corresponding with time of day-dependent fluctuations in energetic demand and nutrient availability [23]. This is illustrated by physiological changes in myocardial glucose utilization over the course of the day. It has been established that elevation of workload and/or insulin levels rapidly promotes glucose utilization of the heart [24, 25]. It is therefore not surprising that during the awake period, a time at which physical activity and food consumption are increased, myocardial glucose utilization has been reported to be increased in both the rat and mouse heart [26–28]. This is observed at the levels of both non-oxidative (e.g., glycolysis, glycogen synthesis) and oxidative (i.e., full catabolism to CO₂) glucose disposal. For example, myocardial glucose oxidation is doubled during the active period, relative to the sleep period [27].

Evidence exists in support of the concept that additional glucose metabolism pathways may be under circadian control. One example includes the hexosamine biosynthetic pathway, which utilizes the glycolytic intermediate fructose 6-phosphate via the glutamine:fructose 6-phosphate amidotransferase reaction, ultimately resulting in post-translational modification of proteins, through O-GlcNAcylation [29]. Importantly, flux through the hexosamine biosynthetic pathway is augmented in the heart during the active period, and is associated with increased protein O-GlcNAcylation at this time [27]. Interestingly, the O-GlcNAc pathway is also responsive to increased oxidative stress and offers a potential mechanistic link between redox regulated pathways and metabolism [6, 30]. Glucose dependent metabolism is also essential for providing substrates for the pentose phosphate pathway (PPP), generating NADPH as the key reducing equivalent for maintaining thiol redox status and the controlled generation of ROS or NO through cell signaling. Interestingly, in cell models it has been shown that time dependent oscillations in the PPP result in changes in the peroxiredoxin redox state and the regulator of redox homeostasis, Nrf2 (nuclear factor (erythroid-derived 2)-like 2) [18–20]. Whether PPP flux oscillates over the course of the day in the heart (the topic of the current review article) is currently unknown.

Relative to glucose oxidation, the oxidation of fatty acids by the heart exhibits a minimal time-of-day-dependence, likely reflecting the fact that fatty acids serve an important role in basal cardiac contraction (as opposed to meeting energetic demands during elevated workload) [28, 31]. In contrast, triglyceride synthesis (and phospholipid synthesis, to a lesser extent) exhibits a marked daily fluctuation (more than 3-fold), peaking towards the end of the active period in the mouse heart [31]. Moreover, the transcriptional

responsiveness of the heart to fatty acids also exhibits a time-of-day-dependence, with greatest induction of fatty acid responsive genes (many of which promote fatty acid oxidation) during the active period (particularly at the beginning of the active period) [32]. Such observations have led to the hypothesis that prolongation of the sleep phase fast into the active period, when, for example, the animal in the wild is unsuccessful in its forage for food, allows the myocardium to rapidly increase reliance on fatty acids as a fuel (which are abundant due to continued lipolysis), while the forage for food continues. Moreover, once successful in its forage for food, increased triglyceride synthesis towards the end of the active period will likely provide an endogenous fuel source for the myocardium during the upcoming sleep period. Promotion of phospholipid synthesis at the beginning of the sleep phase may be important for replacement of damaged (e.g., oxidized) phospholipids (a concept that will be expanded upon below).

The awake-to-sleep transition has emerged recently as an important period of growth and repair of the myocardium. Similar to increased phospholipid synthesis, protein synthesis has been reported to be increased at this time, both *in vivo* and *ex vivo* [33]. Consistent with increased protein synthesis, McGinnis et al reported a trend ($p=0.064$) for an increase in the biventricular weight to tibia length ratio at the end of the active period [33]. Figure 1 shows the assimilated data for time-of-day-dependence of murine biventricular weight from two distinct studies [33, 34]; combining data for ZT9, ZT12, and ZT15 (i.e., the 'light-to-dark' or sleep-to-awake' transition), as well as the data for ZT21, ZT24/0, and ZT3 (i.e., the 'dark-to-light' or awake-to-sleep' transition), reveals that biventricular weight is 6.9% higher at the awake-to-sleep transition period compared to the sleep-to-awake transition period (Figure 1). Unbiased transcriptome analysis support this concept, indicating a coordinated induction of genes involved in protein turnover (i.e., synthesis and degradation) at the wake-to-sleep transition [35–37]. Far less is known regarding time-of-day-dependent regulation of protein degradation in the heart, although evidence exists in support of increased autophagy during the beginning of the sleep phase (e.g., increased LC3 lipidation and autophagosomes at this time) [33, 34]. The autophagy pathway is an essential element in the antioxidant defenses of the cell since it removes oxidized proteins which can lead to proteotoxicity [38, 39]. Since the greatest rate of ROS production is anticipated in the wake phase and is associated with the greatest level of protein oxidation [18], then activation of autophagy at the beginning of the sleep phase maybe an essential step in maintaining redox homeostasis. Whether other cellular constituents also undergo quality control in the heart at this time (e.g., mitophagy and/or fission/fusion of mitochondria) remains unknown. We have recently speculated that benefits of temporal partitioning of cellular constituent turnover towards the sleep phase include: 1) replacement cellular constituents that were oxidatively damaged during the recent active period; 2) maximizing ATP availability for contractile processes during the active period (as cellular constituent replacement is energetically demanding); and 3) ensuring cellular constituents are functionally optimally upon awakening (a period of greatest change in energetic demand) [23]. Consistent with the latter, we have recently observed that mitochondrial complex activities exhibit a time-of-day-dependent oscillation in the mouse heart, peaking at the sleep-to-wake transition (Young & Darley-Usmar, unpublished observations).

Rhythms in Cellular Redox Status

Cardiac tissue and mitochondrial function are highly dependent on redox dependent processes for normal physiological function [4, 6, 12, 40–42]. Metabolic and redox processes not only need to be in synchrony with the environment, but also with each other; a mismatch between these factors could lead to disruption of redox signaling, and ultimately cellular dysfunction [6]. While it is clear that redox signaling is domain controlled it has proved technically difficult to be specific about which redox species is mediating a specific biological effect [7]. However, in the case of isolated mitochondria, a broad range of studies using sophisticated techniques allow us to be specific about the ROS involved [43]. The biology of the production of superoxide and hydrogen peroxide specifically is now reasonably well understood and comes in essentially two flavors. The first is where they are metabolic by-products of metabolism and their levels are controlled by enzymes such as catalase. This is the case with fatty acid oxidation in the peroxisomes where the enzymes oxidizing fatty acids and catalase are co-localized. The second is where the production of hydrogen peroxide or superoxide is a controlled process mediated by specific oxidases for the purposes of redox signaling. A good example of this category are the family of NADPH oxidases (the NOX and DUOX enzymes) [44].

The oxidation of substrates by the mitochondrion requires the concerted reduction of oxygen to water in a pathway involving well over 30 redox centers and proteins. Mitochondria are an interesting example of how the perspective of the role of ROS in biology has evolved over the last few decades [43, 45]. Many of the concepts related to mitochondrial redox biology were based on seminal observations in the nineteen sixties and seventies that established the paradigm that mitochondrial superoxide and hydrogen peroxide are a result of a leak of electrons along their path to full reduction to water, and are therefore simply an unwanted by-product of aerobic metabolism [46, 47]. From these data, largely based on sub-cellular fractions, the notion that mitochondria are a major source of ROS in the cell emerged. However, more recent quantitative measurements have shown that relative to enzyme systems designed solely to generate superoxide, such as the NADPH oxidases, mitochondria are not a major source of ROS in the cell [48]. Recent studies have shown that small amounts of superoxide generated from multiple specific redox centers in the mitochondrion are highly dependent on the substrates available to the mitochondrial electron transport chain [49]. Since the site and rate of generation of superoxide from these distinct sites are linked to metabolism they have the potential to play a role in cellular signaling [43, 50]. Redox active enzymes also change their location within the cell depending on activation state and physiological stimuli. For example, this has been shown for eNOS in response to the shear stress associated with blood flow [51, 52]. Based on these considerations, it is then clear that circadian changes in metabolism and signaling will generate time of day fluctuations in cellular redox status, within specific sub-cellular domains.

It is therefore not surprising that time-of-day-dependent oscillations have been reported for both ROS generating and modulatory pathways, in various tissues [6, 18–20]. Indeed, a number of studies suggest that levels of endogenous antioxidants and the redox status vary in a time-of-day-dependent manner [6, 18, 53–56]. Indeed, the possibility that the PPP regulates the circadian clock through its effects on protein thiols and the NAD(P)H/NAD(P)

⁺ redox couple has been suggested [18]. These data are consistent with some early studies in human subjects in which it was shown that the lowest levels of glutathione (GSH) occur at early morning hours [54]. Interestingly, this is the period in which humans have the highest incidence and severity of coronary infarction [53–55].

One of the most important pathways linking circadian rhythms and redox biology is through mechanisms involving the signaling molecules Keap1-Nrf2 and activation of the Antioxidant Response Element (ARE) which functions in many tissues and organs [4]. For example, in the mouse liver, expression of *Keap1* and *Nrf2* mRNA present a time-of-day variation, peaking during the sleep phase (consistent with peak levels of GPx, SOD, and CAT at this time). Similarly, time-of-day-dependent regulation of NRF2 has been reported in the lung [57]. Although the importance of the KEAP1-NRF2 pathway has been demonstrated in the heart, little is known whether the pathway is circadian regulated in this tissue.

One of the best understood examples of a highly evolutionarily conserved clock mechanism is dependent upon the redox proteins peroxiredoxins, which was initially described in erythrocytes [19, 20]. It is not clear the extent to which this pathway contributes to the interface between circadian biology and redox regulation of metabolism in the heart, although it has a potential role given that the enzymes are present. Time-of-day-dependent oscillations of redox status have also been reported in a brain region known to be important in circadian biology, namely the suprachiasmatic neurons (SCN); oscillations in FAD and NAD(P)H within the SCN appear to impact neural excitation, through alterations in K⁺ channel activity [58]. Again, the extent to which daily fluctuations in cofactor redox status influence cardiac biology remains to be elucidated.

NADPH oxidases are a source of regulated superoxide formation within cells. The heart is enriched with NOX2 and NOX4, which are located in the sarcolemma and in intracellular compartments, respectively [59, 60]. NOX4 expression exhibits a rhythmic pattern in cardiovascular tissues, peaking towards the end of the sleep phase [61]. Moreover, NOX activity is dependent on NADPH availability, which is generated primarily from the PPP. Given increased myocardial glucose utilization during the active phase, it is likely that NADPH availability increases at this time, via the PPP. Interestingly, NADPH is required for both the metabolism of hydrogen peroxide through the action of peroxidases and the generation of ROS through NOX and DUOX enzymes. It is also noteworthy that nitric oxide synthase (NOS), also an NADPH dependent enzyme, is able to generate superoxide when it is uncoupled which occurs at the earliest stages of vascular dysfunction [51, 62]. Tunçtan and colleagues (2002) have reported time-of-day-dependence in aortic NOS activity (peaking at the beginning of the active period), suggesting that this enzyme may contribute towards daily oscillations in ROS generation [63].

Control of redox signaling and the intracellular redox state is controlled by a network of enzymes which control the domains in which ROS can exert their effects [6, 7]. Subramanian and colleagues (2008) have shown that all principal redox modulatory enzymes exhibit 24-hr rhythms in rat plasma, peaking at the awake-to-sleep transition (GPx and CAT) and in the middle of the sleep phase (SOD) [64]. Oscillations have also been reported in the mouse liver for GPx, SOD, and CAT which peak towards the latter half of the

sleep phase [65]. In the hippocampus, both GPx and CAT activities and mRNA expression show a circadian oscillation, again peaking during the sleep phase [20, 66]. Time-of-day-dependent differences in distinct antioxidant enzymes have also been reported in the rodent heart; hearts isolated at the beginning of the active phase exhibited increased glutathione transferase activity and a concomitant decrease in selenium-dependent GPx (in the absence of alterations in SOD and glutathione reductase activity, relative to hearts isolated at the beginning of the sleep phase) [67]. Interesting, hearts were more susceptible to hydrogen peroxide induced damage at the beginning of the active phase, consistent with a more reduced redox status at this time (which may have significant consequences during adverse pathologic events, as discussed below).

Extrinsic versus Intrinsic Mediators

An important question relates to the identity of the mechanisms mediating daily fluctuations in cardiac metabolism and redox tone. Numerous possibilities exist. A simple explanation is that neurohumoral perturbations secondary to daily behavioral cycles acutely modulate signaling axes critical for metabolic and redox regulation. Evidence has emerged suggesting that this may not be the sole explanation. For example, elegant studies by Scheer and Shea have dissociated 24-hr oscillations in various neurohumoral factors from sleep/wake and feeding/fasting cycles [68–70]. Moreover, many of the studies investigating diurnal variations in cardiac metabolism were performed in an ex vivo setting (i.e., isolated working rat/mouse hearts), during which both the workload and milieu were constant; given that hearts were allowed to reach a steady state prior to assessment of metabolism, acute neurohumoral influences were largely eliminated [26, 27, 31]. Such observations suggest that extrinsic influences are not the only contributor to time-of-day-dependent oscillations in biological processes. In the subsequent sub-sections, candidate extrinsic (i.e., neurohumoral factors) and intrinsic (i.e., circadian clocks) influences on various aspects of cardiac metabolism and redox tone will be discussed.

Extrinsic Mediators

Various neurohumoral factors exhibit 24-hr rhythms, tracking closely with environmental cues and physiological factors/behaviors (e.g. sleep/wake and feeding/fasting cycles) [71]. Many of these factors are known to influence myocardial metabolism and/or redox tone. Here, we will focus on distinct neurohumoral factors that may contribute to time-of-day-dependent oscillations in these two cardiac parameters. Please note that the circadian clock (discussed in subsequent sections) likely contributes towards 24-hr rhythms in many neurohumoral factors; this concept has been reviewed elsewhere [71].

The renin-angiotensin-aldosterone system (RAAS) is responsible for the synthesis of angiotensin I and II (AngI and II), catalyzed by renin and angiotensin-converting enzyme (ACE), respectively. In the rat heart, *Ace* and *Ace2* exhibit a 24-hr oscillation, peaking towards the beginning of the active period [72–74]. AngII is known to induce ROS in a NOX-dependent manner, a process that is important in modulating vascular tone [75]. Interestingly, ACE-dependent ROS generation antagonizes NO effectiveness in rodent carotid arteries [76]. In cardiac tissue, AngII mediates NOX-dependent ROS formation via

AT1 receptors [77, 78]. These data suggest that ACE/AngII may modulate cardiac ROS formation over the course of the day in a NOX-dependent fashion. With regards to cardiac metabolism, AngII appears to attenuate glucose oxidation, likely through induction of PDK4 (a known inhibitor of pyruvate dehydrogenase) [79]. It is also noteworthy that consistent with the known role of the RAAS in cardiac remodeling, ACE inhibition during the sleep phase appears to attenuate cardiac hypertrophy [80]. Additional studies are required to understand the full extent to which the RAAS contributes to daily rhythms in cardiac metabolism and redox tone.

The systemic production of norepinephrine (NE)/epinephrine (EPI) occurs via the adrenal medulla (specifically in chromaffin cells), while postganglionic neurons from the sympathetic division of the autonomic nervous system also release NE locally [81]. NE/EPI synthesis oscillates during the 24-h period, leading to elevated plasma levels in the early hours of the morning in humans [82]. NE/EPI bind to G-protein coupled receptors; subtypes $\alpha 1$ and $\alpha 2$ are coupled to Gq and Gi proteins, respectively, while β subtypes (1, 2 and 3) are Gs-coupled protein receptors. In the heart, adrenergic stimulation influences a plethora of processes, including contractility, ion homeostasis, metabolism, and even remodeling [81]. In the case of metabolism, adrenergic stimulation acutely promotes glucose uptake, glycogenolysis, glycolysis, and lipolysis. With respect to redox tone, elevated levels of NE induce ROS generation cultured H9c2 cardiac myoblasts [83].

3,5,3'-Triiodothyronine (T3) impacts various cardiac processes, through both genomic and non-genomic mechanisms. For example, T3 modulates expression of SERCA2, β adrenergic receptors, and myosin heavy chain isoforms, ultimately causing perturbations in contractility [84, 85]. Regarding cardiac metabolism, T3 has been reported to promote translocation of glucose transporters (e.g. GLUT4) and glycogen synthesis [86], in addition to modulating expression of metabolic enzymes such as carnitine palmitoyltransferase 1 (CPT1) and pyruvate dehydrogenase kinase (PDK) 2 [87]. In the heart, T3 may contribute towards time-of-day-oscillations in uncoupling protein 3 (*Ucp3*), *Pdk4* and *Glut4* mRNAs [88]. Plasma T3 presents a daily rhythm, reaching maximum values around the latter half of the sleep phase in rodents [89, 90]. T3 indirectly affects ROS formation, through regulation of sex hormone-binding globulin (SHBG) synthesis and secretion in the liver [91, 92]. T3 may also influence redox tone via induction of SIRT1 (which in turn influences histone acetylation, and expression of ROS-related enzymes) [93].

The major pineal gland hormone, melatonin, presents a circadian rhythm, being exclusively synthesized during the dark phase [94]. Melatonin is perceived by tissues due to its interactions with both membrane (metabotropic Gi/Gq-coupled protein receptors) and nuclear receptors [95–97]. Acting as a photoperiod-signaling molecule, melatonin entrains the organism by modulating metabolism, gene expression and distinct behaviors [98–100]. It is noteworthy that melatonin functions directly as well, including binding to specific enzymes, as well as exerting antioxidant properties (e.g., scavenging reactive species, such as hydroxyl radicals). Melatonin also attenuates electron leakage from electron transport chain (ETC), thus reducing free radical production. At the same time, melatonin modulates expression of numerous antioxidant enzymes, such as SOD, CAT, GPx, and glutathione reductase (GR), as well as suppression of NOX isoforms [101–108].

It is noteworthy that glucocorticoids are also produced and released by the adrenal cortex in a time-of-day-dependent manner, peaking at the onset of the awake phase [109, 110]. Glucocorticoids bind to nuclear receptors, altering expression of various target genes, known to influence fatty acid delivery to the cardiomyocyte (e.g., LPL) and β -oxidation (e.g., AMPK), as well as glucose homeostasis (e.g., pyruvate dehydrogenase subunits) [111]. The contribution of glucocorticoids to daily rhythms in cardiac metabolism and redox tone requires further investigation.

Intrinsic Mediators

Recent studies have challenged the dogma that neurohumoral factors are the sole mediators of rhythms in metabolism and redox biology. Revision of this concept has occurred in large part following appreciation that essentially all mammalian cells possess autonomous circadian clocks, a transcriptionally-based timekeeping mechanism that temporally partitions biological processes [112]. Two major proteins involved in this mechanism include BMAL1 and CLOCK, which upon heterodimerization, modulate transcription through binding to E-box elements within target gene promoters [113, 114]. Notable target genes include multiple Period (*Per1/2/3*) and Cryptochrome (*Cry1/2*) isoforms, as well as Rev-erba (*Nr1d1*), which form negative feedback loops by repressing the BMAL1/CLOCK heterodimer [115–117]. These, as well as additional transcriptional and posttranslational events, result in oscillations in clock components with a periodicity close to 24 hours (a little shorter for rodents, and a little longer for humans) [112, 118]. This intrinsic mechanism modulates cellular function with respect to time-of-day by influencing thousands of genes that are not direct components of the circadian clock mechanism. The protein products of these genes have known functions in diverse processes, including transcription, translation, transport, signal transduction, and metabolism. How the cardiomyocyte circadian clock influences cardiac metabolism and redox tone will now be reviewed.

Various murine models of genetic ablation of the cardiomyocyte circadian clock have been utilized to identify critical roles for this mechanism. These models include cardiomyocyte-specific CLOCK⁻¹⁹ mutant (CCM) and cardiomyocyte-specific BMAL1 knockout (CBK) mice; consistent with the cardiomyocyte-restricted nature of these models, behaviors and neurohumoral factors investigated in these mice are comparable to littermate control mice (as is clock function in extra-cardiac tissues) [28, 37]. Given the transcriptional nature of the circadian clock mechanism, unbiased microarray approaches have been employed; approximately 10% of the cardiac transcriptome is differentially expressed in CCM/CBK hearts relative to littermate controls. A stringent *in silico* analysis of these data subsequently identified nine genes as being direct BMAL1/CLOCK target genes in the heart (although more undoubtedly exist). Of these genes, 4 have known metabolic functions; *Nampt* (Nicotinamide Phosphoribosyl transferase, involved in NAD metabolism), *Dgat2* (diacylglycerol O-acyltransferase 2, involved in triglyceride synthesis), *Usp2* (ubiquitin-specific peptidase 2, involved in protein turnover), and *Pik3r1* (phosphoinositide-3-kinase regulator subunit 1, involved in insulin signaling, a pathway that affects multiple aspects of metabolism) [37]. Collectively, these studies reinforce the concept that the cardiomyocyte circadian clock regulates cardiac metabolism.

Metabolic flux studies focused on clock control of glucose utilization revealed abolished/attenuated time-of-day-dependent oscillations in glycolysis, glycogen synthesis, and glucose oxidation, in *ex vivo* perfused CCM hearts; a similar depression of glucose utilization was observed in the middle of the active period (corresponding to the normal peak in these parameters) for CBK hearts [27, 28, 37]. Interrogation of the putative mechanistic links between the cardiomyocyte circadian clock and myocardial glucose disposal, revealed a depression in glucose uptake in CCM hearts, associated with attenuated rhythms in the phosphorylation status of AMP-activated protein kinase (AMPK) and its downstream target AS160 (known modulators of glucose uptake) [27, 31, 33]. Diminished AS160 phosphorylation has also been reported in CBK hearts, as have decreased protein levels of glucose transporter isoforms 1 and 4, as well as the glycolytic enzyme hexokinase 2 [33]. Similarly, GSK3 β phosphorylation is lower in both CBK and CCM hearts, which may contribute towards diminished glycogen synthesis rates [33]. Paradoxically, the phosphorylated/activated form of the insulin signaling component, AKT (a known upstream kinase of both AS160 and GSK3 β), is chronically increased in CCM and CBK hearts (consistent with decreased Pik3r1) [33], suggesting that clock control of other upstream kinases (e.g., AMPK) and/or phosphatases may play important roles. Collectively, these observations suggest that the cardiomyocyte circadian clock promotes myocardial glucose utilization during the active period, likely through augmentation of glucose uptake at this time. It is noteworthy that these studies also revealed an interrelationship between protein O-GlcNAcylation and the cardiomyocyte circadian clock, wherein clock mediated augmentation of O-GlcNAcylation during the active period (through increased glucose uptake, as well as circadian regulation of O-GlcNAc transferase; OGT), results in direct O-GlcNAcylation of several circadian clock components (e.g., BMAL1, *Per1*), which in turn impacts their transcriptional activity [27]. Thus, metabolism can be considered an integral cog in the mammalian circadian clock machinery.

As with glucose utilization, the cardiomyocyte circadian clock appears to influence myocardial fatty acid utilization. For example, time-of-day-dependent oscillations in triglyceride synthesis are absent in CCM hearts, revealing a critical role for the clock mechanism in this process [31]. Although the precise mechanisms by which the cardiomyocyte clock drives oscillations in myocardial triglyceride synthesis are not fully understood, daily fluctuations in key proteins such as DGAT2 and HSL (hormone sensitive lipase) are attenuated in CCM hearts [31, 37]. Interestingly, despite only a modest daily oscillation, both CCM and CBK hearts exhibit slightly elevated fatty acid oxidation rates [28, 37]. These observations are in some ways counterintuitive, for several reasons. First, evidence suggests that CBK hearts exhibit mitochondrial dysfunction (which would be anticipated to diminish oxidative metabolism) [119]. Second, studies in the liver suggest that disruption of the hepatocyte circadian clock impairs fatty acid oxidation through mitochondrial protein acetylation (secondary to repressed NAMPT) [120]. Interestingly, unlike the liver, acetylation appears to promote fatty acid oxidation in the heart [121]. However, protein acetylation is decreased in CCM hearts, inconsistent with increased fatty acid oxidation [122]. Furthermore, acetyl-CoA carboxylase phosphorylation (which influences malonyl-CoA levels, a key regulator of fatty acid oxidation) is unaltered in CCM hearts [122]. The possibility remains that diminished rates of triglyceride synthesis, in

combination with decreased glucose utilization, promotes fatty acid oxidation in CCM/CBK hearts (i.e., substrate competition effects).

Protein quality control processes, such as autophagy and proteasome activity, as well as protein synthesis, appear to be regulated by the cardiomyocyte circadian clock. For example, time-of-day-dependent oscillations in myocardial protein synthesis observed *in vivo* are absent in CBK mice, as are rhythms in myocardial mass [33]. Furthermore, CBK hearts exhibit impaired autophagy, as indicated by accumulation of p62 and autophagolysosomes [33]. More recently, we have observed an elevation of proteasome activity at the beginning of the sleep phase in wild-type hearts, which is absent in CCM hearts (Young et al, unpublished observations). Currently, the mechanistic links between the cardiomyocyte circadian clock and protein turnover are unknown; whether USP2, a direct BMAL1/CLOCK target gene, plays a role requires further elucidation. Interestingly, a recent candidate approach suggested a possible role for mTOR (a critical nutrient sensor known to modulate both protein synthesis and autophagy). Downstream links between mTOR and protein synthesis, namely S6 and 4EBP1, exhibit time-of-day-dependent oscillations in the mouse heart that parallel protein synthesis; these oscillations are absent in CBK hearts [33]. Chronic activation of mTOR in CBK hearts is consistent with repression of *Pik3r1* (and subsequent activation of AKT). Furthermore, treatment of CBK mice with rapamycin, an established mTOR inhibitor, normalizes myocardial protein synthesis and mass within 10 days [33]. More recently, we have reported that protein synthesis, p-mTOR, p-S6, and LC3-II all exhibit time-of-day-dependent oscillations in the heart during fasting, peaking around the awake-to-sleep transition [34]. Collectively, these observations suggest that protein turnover is increased in the heart at the beginning of the sleep period, and that these rhythms are likely governed by the cardiomyocyte circadian clock (as opposed to feeding/fasting cycles).

Relative to cardiac metabolism, less is known regarding whether the cardiomyocyte circadian clock influences redox tone of the heart, either directly or indirectly. Microarray analysis of CBK hearts revealed perturbations in various genes involved in the glutathione system, including 11 distinct isoforms of glutathione transferase (Gst) [123]. Of these genes, 8 isoforms were repressed in CBK hearts (relative to control hearts); for example, *Gsta1*, *Gsta2*, and *Gstt1* were repressed by 70%, 65%, and 43%, respectively. To date, neither the mechanisms by which the cardiomyocyte clock regulates these genes, nor the functional consequence, are known. However, recent studies in extra-cardiac tissues suggest that NRF2 is directly regulated by the cell autonomous clock, which in turn would influence redox tone [57]. Interestingly, recent studies have also proposed that the HMP regulates the clock through modulation of NADPH, suggesting that redox state is likely an integral component of circadian clocks [18].

Pathologic Consequences

As mentioned briefly above, time-of-day-dependent rhythms in cardiovascular pathology are well established. In the early hours of the morning, there is an increased risk of adverse ischemic (e.g., myocardial infarctions, stroke) and electrophysiologic (e.g., arrhythmias) events [22]. Classically, these rhythms have been attributed to dramatic surges in

neurohumoral factors (e.g., norepinephrine, cortisol) and blood pressure (i.e., shear stress) occurring at the sleep-to-wake transition [22]. Recent human-based studies suggest that an endogenous timekeeping mechanism drives daily fluctuations in a number of these ‘triggers’; 24-hr rhythms in plasma cortisol and PAI-1 levels, as well as platelet activation, persist in healthy volunteers forced to live a 28-hr day [68–70, 124]. Furthermore, the PAI-1 gene has been shown to be regulated directly by the BMAL1/CLOCK heterodimer, resulting in robust oscillations in numerous tissues (including the heart) [125, 126]. Moreover, inflammation plays an important role in vulnerable plaque rupture, and both immune cell number and reactivity are elevated in the early hours of the morning; these oscillations in inflammatory mediators have been linked to circadian clocks [127]. Collectively, these observations suggest that circadian clocks in numerous cells likely contribute towards increased adverse cardiovascular events in the early hours of the morning.

Although triggers in adverse ischemic events are primarily extra-cardiac in nature, and are not necessarily mediated by rhythms in cardiac metabolism/redox tone, the same may not be true for arrhythmias and sudden cardiac death. Furthermore, the tolerance of the myocardium to ischemic events also exhibits a time-of-day-dependent rhythm, to which metabolism and redox tone likely contribute. In humans, tolerance to a myocardial infarction appears to exhibit a biphasic pattern, with lowest tolerance in the early hours of the morning (similar to peak incidence) [128]. Rodent-based studies have confirmed these findings, revealing largest infarct sizes at the sleep-to-wake transition following ischemia-reperfusion (I/R) [129]. Furthermore, rhythms in I/R tolerance are mediated by the cardiomyocyte circadian clock, potentially in a Rev-erba dependent manner [129, 130]. ROS generation burst has been implicated as a significant contributor to reperfusion damage; greater H₂O₂-mediated damage of the heart during the active period therefore has the potential to contribute towards poorer reperfusion tolerance at this time [67]. Whether time-of-day-dependent rhythms in I/R-induced ROS generation occur has not been reported, but is a distinct possibility. With regards to cardiac metabolism, previous studies suggest that promotion of glucose oxidation (more specifically, a coupling between glucose oxidation and glycolysis) increases I/R tolerance [131]. Based on these observations, one could hypothesize that the rise in glucose oxidation may contribute towards increased I/R tolerance observed as the active period continues. Autophagy is known to be cardioprotective in nature, leading to the suggestion that decreased autophagy at the beginning of the active period may exacerbate I/R damage at this time. Evidence also exists that clock control of cardiac metabolism may play an important role in terms of hypertrophic remodeling. Indeed, greater hypertrophic growth of the heart (in response to isoproterenol) is observed at the beginning of the sleep phase (a time at which the cardiomyocyte circadian clock promotes protein synthesis) [33, 132], and pharmacological activation of Rev-erba attenuated pressure overload induced adverse remodeling [133].

Circadian rhythms are frequently perturbed during pathologic states. Metabolic and redox homeostasis are also often disrupted, and in many cases, have been casually linked with pathogenesis. This leads to speculation that misalignment of circadian rhythms might predispose and/or accentuate disease progression through disruption of cellular processes, including metabolism and/or redox status. Evidence exists in support of this concept. For

example, disruption of circadian rhythms through environmental (e.g., sleep/awake cycle manipulation or shift work in mice or humans, respectively) and/or genetic (e.g., targeted deletion or polymorphisms of clock genes in mice or humans, respectively) means increases cardiometabolic disease risk [134–140]. In terms of the heart, circadian clock gene oscillations are altered during obesity, diabetes mellitus, pressure overload-induced hypertrophy, hypertension, I/R, and aging [141–146]. For example, animal models of type 1 and type 2 diabetes exhibit a phase shift and (in the case of type 2 diabetes) attenuation in clock gene oscillations in the heart [142, 147], while hypertrophied and ischemic hearts both exhibit attenuated rhythms in clock genes [141, 144]. Moreover, genetic disruption of the circadian clock, either in a germline or cardiomyocyte-specific manner, results in contractile dysfunction of the heart [37, 140]. Despite these observations, little data is available regarding whether time-of-day-dependent oscillations in cardiac metabolism and/or redox tone are altered in pathologic states. However, some indirect evidence exists in support of this concept. For example, candidate gene approaches indicate that 24-hr rhythms in genes encoding for key metabolic genes are attenuated in the hypertrophied and ischemic heart [26, 74, 144]. Additional studies are required to address this important question. In Figure 2A we speculate how the physiological function of the clock can integrate metabolism and ROS signaling in the heart. In Figure 2B we suggest that loss of circadian control of Nrf2 transcriptional regulation depresses levels of cellular antioxidants (GSH and heme oxygenase-1 (HO-1)) during the sleep phase, resulting in increased susceptibility to the toxic effects of ROS generation at the onset of metabolism. Over time this would lead to increased protein oxidation and could then explain the increased severity of cardiac events in the early morning hours for human populations [22]. An important implication of these concepts is that consideration of the time of day administration of therapeutics designed to modulate the Nrf2/Keap1 system will be critical in order to achieve beneficial effects.

Summary

It is increasing apparent that cardiomyocyte processes are temporally partitioned, in a manner that is critical for maintenance of normal cardiac function. Such processes includes metabolic fluxes and redox tone, thus impacting the magnitude of anabolic/catabolic pathways and ROS signaling in a time-of-day-dependent manner; this temporal partitioning appears to be governed by a complex interplay between intrinsic (*i.e.*, cardiomyocyte circadian clock) and extrinsic (*i.e.*, neurohumoral factors) (Figure 3). Common cardiometabolic disease states (e.g., obesity, diabetes) disrupt this interplay, leading to misalignment of cardiac processes, which likely increases susceptibility of the heart to pathologic remodeling and subsequent contractile dysfunction. Recent pharmacologic strategies modulating distinct circadian clock components (*e.g.*, Rev-erba) have yielded promising beneficial outcomes in murine models of imbalance metabolism and redox tone. Continued consideration and study of chronobiology may lead to new fundamental insights regarding the molecular mechanisms underlying the role of aberrant circadian function in the etiology of cardiac dysfunction, ultimately identifying new approaches for disease treatment/prevention.

Acknowledgements

This work was supported by the National Heart, Lung, and Blood Institute (HL106199, HL074259, HL123574, and HL122975), and an UAB AMC21 reload multi-investigator grant (VDU, MEY).

Abbreviations:

NOS	nitric oxide synthase
NO	Nitric Oxide
NOX	NADPH oxidase
LC3	Microtubule associated protein light chain 3
ROS	reactive oxygen species
NRF2	nuclear factor (erythroid-derived 2)-like 2
KEAP1	kelch-like ECH-associated protein 1
PPP	pentose phosphate pathway

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Figure1: Time-of-day-dependent fluctuations in biventricular weight of the mouse heart.

A: Assimilated data from McGinnis et al and Brewer et al shows time-of-day-dependent fluctuations in biventricular weight (mg). **B:** Murine biventricular weight is 6.9% higher at the awake-to-sleep (dark-to-light; average of ventricles collected at ZT21, ZT24/0, and ZT3; n=28) transition compared to the sleep-to-awake (light-to-dark; average of ventricles collected at ZT9, ZT12, and ZT15; n=26) transition.

Figure 2: Hypothetical mechanism for circadian control of redox tone in the heart, and increased susceptibility to cardiac dysfunction when disrupted.

A: Based on the current literature (as discussed in the text) we propose that decreased metabolic activity during sleep results in a higher resting mitochondrial membrane potential ($\Delta\Psi_m$). This condition is known to produce more ROS (superoxide/hydrogen peroxide), which we then propose controls redox signaling. In addition, through the modification of specific thiol groups on Keap1, Nrf2 is released to translocate to the nucleus. It can then initiate the transcriptional regulation of antioxidant enzymes which control levels of antioxidants such as glutathione (GSH) and HO-1, such that at the onset of increased metabolic demand, ROS are efficiently scavenged and do not damage contractile or metabolic proteins. Intrinsic circadian clocks likely optimize this system, through temporal regulation of both antioxidant and metabolic enzymes. This hypothesis suggests that the peak antioxidant levels in the circadian cycle will be at the sleep-to-awake transition. **B:** In pathological settings, increased oxidative stress, coupled with dysfunctional circadian clocks, suppress circadian control of the Keap1/Nrf2 system, leading to decreased levels of intracellular antioxidants. Oxidative protein modification and cardiac dysfunction then accumulates over time.

Figure 3. The interplay between intrinsic (i.e., circadian clock) and neurohumoral factors in circadian control of both metabolism and redox tone.

In this scheme some of the key mechanisms influencing temporal control of cardiac metabolism and redox tone are summarized in the cardiomyocyte. Dashed lines depict metabolic pathways. Italics depicts examples of clock regulated genes. In this model, circadian-dependent transcriptional regulation in the nucleus (represented by the clock at an arbitrary time of day) controls the protein levels of key metabolic proteins (examples shown here are *Pik3r1*, phosphoinositide-3-kinase regulator subunit 1; *Hk2*, hexokinase 2 and *Glut 4* Glut4, glucose transporter isoform 4; and *Dgat2*, diacylglycerol O-acyltransferase 2). This leads to changes in protein expression and substrate uptake/utilization, which in turn affects both mitochondria function and the hexose monophosphate pathway (HMP). A further level of regulation is provided by the impact of neurohumoral factors on glucose and fatty acid metabolism, as described in the text. The net effect is to integrate ATP (adenosine triphosphate) demand with metabolism, therefore meeting fluctuations in metabolic/energetic demands during the light/day cycle. The interface with redox signaling occurs at a number of levels, some of which involve the transcription factor Nrf2 (nuclear factor (erythroid-derived 2)-like 2). Increased levels of Nrf2 in the nucleus controls levels of glutathione (GSH) in the cell and the redox tone. Higher levels of GSH attenuate ROS (reactive oxygen species) signaling by changing the steady state levels of ROS signaling molecules such as H_2O_2 , which can be generated by the mitochondria.