Peripheral Circadian Clocks in the Vasculature

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Abstract—Living organisms have adapted to the daily rotation of the earth and regular changes in the light environment. Life forms anticipate environmental transitions, adapt their own physiology, and perform activities at behaviorally advantageous times during the day. This is achieved by means of endogenous circadian clocks that can be synchronized to the daily changes in external cues, most notably light and temperature. For many years it was thought that neurons of the suprachiasmatic nucleus (SCN) uniquely controlled circadian rhythmicity of peripheral tissues via neural and humoral signals. The cloning and characterization of mammalian clock genes revealed that they are expressed in a circadian manner throughout the body. It is now accepted that peripheral cells, including those of the cardiovascular system, contain a circadian clock similar to that in the SCN. Many aspects of cardiovascular physiology are subject to diurnal variation, and serious adverse cardiovascular events including myocardial infarction, sudden cardiac death, and stroke occur with a frequency conditioned by time of day. This has raised the possibility that biological responses under the control of the molecular clock might interact with environmental cues to influence the phenotype of human cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2007;27:1694-1705.)

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Circadian rhythms are daily cycles of physiology and behavior that are driven by an endogenous oscillator with a period of approximately (circa-) one day (dies). The most obvious circadian rhythm in humans is the cycle of sleep and wakefulness. These cycles are not simply consequences of light perception, but are generated by endogenous circadian clocks that can adapt the physiology of an organism to its needs in an anticipatory manner. Their expression continues (free-runs) when subjects are isolated from the light cycle, with the oscillator defining predicted day and night, organizing our behavior and physiology appropriately to adapt to the contrasting demands encountered throughout the 24-hour period.

Cardiovascular or hemodynamic parameters such as heart rate, blood pressure, endothelial function, and fibrinolytic activity exhibit variations consistent with circadian rhythm. Additionally, several types of acute pathological cardiac events exhibit diurnal patterns. The incidence of acute myocardial infarction, myocardial ischemia, cardiac arrest, ventricular tachycardia, post myocardial infarction, and sudden death in heart failure all vary according to the time of day. Social and commercial pressures such as shift work, which oppose the temporal circadian order, may be underlying factors contributing to the incidence of chronic illnesses such as cardiovascular disease and cancer. Understanding this molecular clock and its mechanisms may ultimately allow treatment of conditions where either the severity of the illness or therapeutic efficacy exhibit circadian rhythmicity.

Molecular Basis of Circadian Clocks
Circadian rhythms are regulated by three components: (1) the circadian pacemaker or “clock”, (2) an input mechanism which allows the clock to be reset by environmental stimuli, and (3) an output mechanism which regulates physiological and behavioral processes. In mammals, the master circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Circadian clocks are a series of self sustained transcriptional and translational feedback loops, which have a free running period of ≈24 hours. These clocks are intrinsic to the cell, persisting when tissues and cells are isolated or cultured in vitro. Core clock genes have been identified and characterized in drosophila and rodent models harboring naturally occurring, chemically induced, and targeted (knockout) mutations and through various comparative genomic approaches. Our current understanding of the clock architecture consists of 3 negative and 1 positive loop of transcription, translation, and posttranslational events. Heterodimers of basic helix-loop-helix/PER-arylhydrocarbon receptor nuclear translocator (ARNT)-SIM (bHLH/PAS) transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein-1 (BMAL1, also known as MOP3) drive transcription through E boxes located within the promoters of various target genes including period (Per1-3) and cryptochrome (Cry1-2) genes. When Per and Cry are translated, they heterodimerize, translocate to the nucleus, and repress Clock/Bmal1-mediated transcriptional activity at E boxes (Figure 1). Increases in Per protein levels are delayed 6 hours relative...
to their mRNA, and serine phosphorylation plays a critical role in regulating their ubiquitin mediated degradation.\textsuperscript{15} Inhibition of Clock/Bmal1 transcription by Per/Cry complexes ultimately decreases their own expression levels resulting in relief of inhibition, thus restarting the cycle. Fbxl3, a component of a specific ubiquitin ligase complex, was recently implicated in the proteasome mediated decay of Cry proteins.\textsuperscript{16–18} Additional interacting feedback loops involve bHLH domain containing transcription factors Dec1, Dec2,\textsuperscript{19} and Rev-Erb\textsubscript{a}.\textsuperscript{20} Both Dec1/2 and Rev-Erb\textsubscript{a} contain E boxes in their promoters and are thus transcriptionally regulated by Clock/Bmal1 heterodimers. On translation of the corresponding proteins, Rev-Erb\textsubscript{a} specifically represses Bmal1 transcription, whereas Dec1/2 are thought to impair Clock/Bmal1 transactivational capacity.\textsuperscript{19} Bmal1 expression is positively regulated by Per2 and by Ror\textsubscript{a} acting through RORE sequences in the Bmal1 promoter.\textsuperscript{13} The overall result of these transcriptional/translational feedback loops is oscillatory expression of clock genes and proteins with a period at or near 24 hours.

Initial output from the core clock manifests at the level of altered gene expression. Genes that are directly regulated by clock components but are not integral components of the clock are termed clock controlled or output genes. Oscillating protein expression of these clock output genes is then thought to occur, thus potentially impacting cell/tissue function in a rhythmic manner. Clock/Bmal1 heterodimers can bind to Ebox elements in numerous output genes including members of the PAR transcription factor family D-element binding protein (dbp), hepatic leukemia factor (hlf), and thyrotrophic embryonic factor (tef) as well as vasopressin, wee1, lactate dehydrogenase A, and prokineticin2. Expression of genes containing promoter elements RORE or D box elements known to be involved in the circadian clock could similarly be regulated in a rhythmic manner.\textsuperscript{21}

Circadian Clocks in the Periphery

As each of the genes comprising the core circadian clock was identified, their expression was detected outside the SCN.\textsuperscript{22} Core circadian clock genes are expressed in non-SCN neural tissues, as well as in peripheral tissues throughout the body where most exhibit an oscillating circadian pattern of expression. Indeed, even in culture, immortalized rat-1 fibroblasts and other cell lines show a circadian pattern of expression after treatment with serum.\textsuperscript{8,23} The best known exception to this rule is the testis, which shows constant rather than cyclic expression of circadian clock genes in multiple animal species.\textsuperscript{24} The central clock in the SCN is entrained by light signals transmitted along the retinohypothalamic tract, whereas peripheral oscillators are synchronized by various neurohumoral stimuli\textsuperscript{25} and food or metabolic cues\textsuperscript{26} (Figure 2). Metabolic signals from feeding (or perhaps more importantly starving) appear to be dominant cues for peripheral clocks. It is becoming increasingly apparent that daily me-
metabolism and the circadian cycle are intertwined. Circannual cycles in mammals (e.g., hibernation) are similarly known to influence and be influenced by metabolism. It is believed that the SCN can synchronize peripheral clocks through neurohumoral stimuli directly and indirectly (e.g., feeding behavior). In peripheral tissues such as the liver, kidney, and heart, circadian rhythms in RNA abundance are apparent for each of the Per genes, although the phase of oscillation is delayed 3 to 9 hours relative to the oscillation in the SCN. Oscillations in cultures of peripheral tissues were observed to dampen more rapidly than SCN cells in vitro, which sustain rhythms for weeks. However, sophisticated luciferase reporter methods have revealed that peripheral oscillators are capable of self-sustained oscillations for more than 20 cycles in isolation. Phase dispersion of luciferase rhythms were observed in tissues explanted from SCN lesioned mice. Hence, signals emanating from the SCN are now thought to synchronize, rather than sustain rhythms in peripheral oscillators. Circadian clock genes are expressed in many organs, and these peripheral oscillators may tailor circadian responses to tissue-specific alterations in physiological demands, such as might pertain during starving, feeding, or exercise.

One potential function of a peripheral clock is to regenerate a weak or dampened SCN signal, thus amplifying the oscillation of the signal in each tissue. Another is to coordinate locally clock controlled gene expression in each tissue generating an amplified and synchronized rhythm in response to asynchronous blood borne signals. Several microarray studies have helped elucidate that 5% to 10% of the transcriptome is under circadian control in the liver, the heart, and the aorta. Surprisingly, the complement of rhythmic genes between tissues differs substantially, with a very small percentage of clock controlled genes common to any 2 tissues. Transcripts found to be oscillating in more than 1 tissue tend to be core clock components or transcription factors (dbp and Rev Erbα). Recently, a previously unappreciated requirement for Per1, Per2, and Cry1 in sustaining cellular circadian rhythmicity was established. It appears that intercellular coupling in the SCN acts not only to synchronize component cellular oscillators but also for robustness against genetic perturbations.

Peripheral oscillators also differ from the SCN in that they display tissue-specific molecular components of the transcriptional feedback loop that sustain rhythmicity. Bmal2 (also
known as MOP9 or CLIF) expressed in endothelial cell cultures dimerizes with Clock to drive clock controlled genes (CCGs). Npas2 (also known as MOP4) is a paralogue of Clock. It is expressed in the forebrain and vasculature and can also dimerize with Bmal1 and drive gene expression through E box sequences. Transcriptional coactivators and histone acetyltransferases, p300/CBP PCAF, and ACTR associate with Npas2 and CLOCK to regulate positively clock gene expression. Temporal coactivator recruitment and chromatin remodeling on the relevant promoters permits the mammalian clock to orchestrate circadian gene expression. Indeed, a recent report suggests Clock has intrinsic histone acetyltransferase activity. Temporal coactivator recruitment and chromatin remodeling on the relevant promoters permits the mammalian clock to orchestrate circadian gene expression. However, the relative importance of Npas2 and Clock to the core loop in many tissues remains to be determined.

Circadian Gene Expression in the Vasculature

The diurnal incidence of cardiovascular disease arises from a complex interplay among local oscillators in the heart, endothelium, and vascular smooth muscle, their endocrine interactions, and their regulation by SCN-dependent changes in autonomic tone, feeding, stress, and energetic demands. Dramatic oscillations in circadian clock components have been observed in vascular smooth muscle cells and in mouse aorta isolated at different times throughout the 24-hour period. Rhythmic accumulation of transcripts for almost all of the core clock components have been observed in murine vascular tissue. To date, aortic vascular smooth muscle cells are the most well characterized in vitro model of the vascular clock. Rhythms in Per1, Per2, Cry1, Cry2, Bmal1, Clock, Npas2, Rev Erb, dbp, and E4bp4 have been observed in smooth muscle cells in vitro and aortic tissue ex vivo. Oscillations in Per1/2, Cry1/2 mRNA levels in the mouse aorta peak during the early circadian night, whereas Bmal1, Npas2, and Rev Erb peak at the beginning of circadian day. Smooth muscle cell models show a similar phase relationship to that seen in vivo. Rhythms in clock gene mRNA expression have not yet been observed in vivo in vascular tissues outside the aorta; however, using tissues cultured from a Per1 luciferase transgenic rat, Davidson et al demonstrated rhythms in Per1 promoter activity in a wide variety of arteries and veins. The aorta has been the most widely studied vascular tissue in terms of gene expression in a circadian context, in part because of its accessibility and relative ease of isolation; however, it is quite possible that molecular clocks within venous and arterial vascular beds may express differing clusters of circadian output genes. Indeed, within any single vessel, endothelial, smooth muscle cell, and fibroblasts express widely differing transcriptomes.
circadian control may differ between these cell types. Oscillating clock or clock output gene expression has not, so far, been reported in isolated endothelial cells in vitro. Cultured NIH3T3 fibroblasts harbor cell-autonomous and self-sustained circadian oscillators with a relatively wide distribution of period length. However, they can be synchronized transiently by a short treatment with substances that activate a wide variety of signaling pathways. Single cell recordings and coculture experiments indicated that cultured fibroblasts do not influence each other's rhythms to any measurable degree. However, different tissue explants harvested from the same SCN-lesioned animal display circadian oscillations with different phases and period lengths, hence intercellular communication within an organ must be present but may be lost in culture. It is tempting to speculate that clocks within different cellular compartments within a blood vessel may communicate timing information via paracrine signaling.

Previously, the circadian pattern of gene expression in the thoracic aorta was examined using Affymetrix high density arrays. Circadian expression of about 8000 probesets was examined using U74A arrays. Three hundred seven genes exhibited a circadian pattern of oscillation in mouse aorta, including those intrinsic to the function of the molecular clock. In addition, many genes relevant to protein folding, protein degradation, glucose and lipid metabolism, adipocyte maturation, vascular integrity, and the response to injury demonstrated profound circadian oscillation. It is poorly understood how oscillating gene expression in vascular tissues might contribute to the temporal pattern of cardiovascular disease. Assessing cardiovascular parameters throughout the circadian day in transgenic mouse models with disrupted circadian clocks has provided initial information in this regard. However, forthcoming studies of mouse models with tissue restricted dysfunctional molecular clocks will allow a greater understanding of the relative contribution of both systemic cues and these peripheral oscillators to physiology and disease.

The identification of a vascular clock implicates local tissue-specific events as potential contributors to the temporal patterning of cardiovascular disease and, as such, may be amenable to local modulation. The circadian oscillatory mechanism throughout the tissues of the vasculature is potentially a target for tissue-specific therapy, independent of the SCN and other clock mechanisms. Factors which entrain timing or sustain rhythmicity within the peripheral vascular clock are potentially avenues of management.

Circadian Gene Expression in the Heart
Oscillations in gene expression in the heart have been examined extensively in mouse models using both real-time polymerase chain reaction (PCR) and expression array analysis. Genes encoding both core clock components and CCGs, relevant to cardiac function, have demonstrated dramatic oscillations in heart tissue isolated at intervals throughout the circadian day. Included in these oscillating transcripts are genes relevant to carbohydrate utilization, mitochondrial function, and fatty acid metabolism. While numerous studies have provided insight into rhythmic heart gene expression, we are only now beginning to understand the impact of the molecular oscillator on cardiac physiology. The role of the circadian clock within the cardiomyocyte may allow for the anticipation of diurnal variations in workload, substrate availability, or the energy supply-to-demand ratio.

Circadian variation in cardiac function persists in isolated heart tissue, highlighting the cell-autonomous function of the cardiac clock. Indeed, rat hearts in vitro continue to display circadian variation in contractile function, sustained by a circadian variation in oxidative metabolism. Diurnal variations in oxidative stress tolerance and lipid peroxidation have also been observed in isolated perfused rat hearts. Similarly, myocytes isolated at different times of the day have displayed circadian variation in transient outward and steady state currents ex vivo. The observation that rhythmic cardiac function persists in the absence of external cues (i.e., ex vivo) highlights the importance of this autonomous clock within the heart in the regulation of rhythmic cardiac physiology. Other myocardial processes under circadian control likely include the responsiveness to sympathetic stimulation, electrical properties, calcium homeostasis, and antioxidant capacity.

Although the circadian clock within the heart drives cardiologic physiology, function of this clock can be disrupted under pathological conditions. In a model of experimentally induced cardiac hypertrophy, the core molecular oscillator continues to cycle, but the amplitude of oscillations in transcription factors such as dbp are blunted and the circadian cycle of metabolic gene expression is lost. Hence, the tissue would be less prepared to cope with routine increases in physiological demand, predisposing it to metabolic crisis. Streptozotocin-induced diabetes in the rat is another model of contractile dysfunction which alters clock gene expression in the heart; clock component oscillations show normal amplitude but are phase advanced by approximately 3 hours in this model. Spontaneously hypertensive rats show a marked increase in the amplitude of daily cardiac mRNA rhythms of components of the renin-angiotensin system. Thus, the impact of disease states on the cardiac circadian clock seems to be at the level of both circadian clock genes as well as clock controlled output genes relevant to tissue specific functions.

Environmental and Neurohumoral Influences on Cardiovascular Function
Chronobiologists have long struggled with the question of whether physiological processes such as the daily rhythms in cardiovascular parameters, energy metabolism, body temperature, and hormone release are gated simply by the behavioral sleep/wake and fasting/feeding rhythms or are subject to independent control by a circadian oscillator. Locomotor activity rhythms and circadian oscillations in circulating hormones (e.g., norepinephrine, epinephrine) add a layer of complexity when trying to decipher the relative contribution of the central (SCN) and peripheral oscillators to circadian cardiovascular physiology. We now have good evidence in many species that sleep and wakefulness are affected by the circadian clock, but we are only beginning to decipher to what extent rhythms in physiological functions are themselves directly influenced by the molecular clockwork or...
rather are merely reflective of behavioral rhythms. The existence of a circadian pattern of body temperature, blood pressure, endothelial function, fibrinolytic function, and circulating hormones has long been known. This section will focus on blood pressure, whereas endothelial function and the fibrinolytic system will be discussed in the final section.

Peak blood pressure levels in humans occur during the mid morning (at about 10:00 AM) then decrease progressively throughout the remainder of the day to reach a trough value the following morning around 3:00 AM. A slow but steady increase in blood pressure is then observed over the early morning hours before awakening, with an abrupt and steep increase at approximately 6:00 AM coincident with arousal and arising from overnight sleep. This morning blood pressure surge from low nighttime levels to higher daytime levels continues for 4 to 6 hours after awakening, with a secondary dip around 2 pm, thus variations in blood pressure in general tend to reflect the sleep activity cycle. It has been shown that the rhythms of blood pressure and heart rate are controlled by an endogenous circadian circulating system in which the SCN plays an important role in rats. However, it is unknown how the circadian information from the SCN is modulated/processed to regulate the 24-hour rhythm of blood pressure and heart rate. The amplitude of the diurnal variation in blood pressure is increased in patients with hypertension and the oscillation again coincides with the temporal variability in their incidence of acute vascular events, such as myocardial infarction, sudden cardiac death, and stroke.

A circadian pattern of blood pressure is maintained in hypertensive patients, although there is an upward shift to the blood pressure curve throughout the entire 24-hour period compared with normotensive subjects and the amplitude of the rhythm may be altered. The absence of a normal drop in systolic blood pressure (known as “nondippers”) from day to night has been found to be predictive of heart failure, stroke, and myocardial infarction, as well as sudden death in elderly patients with hypertension. Recently, Halberg and colleagues found that chronobiological analysis of human blood pressure recordings interpreted in the light of reference values specified by gender and age predicts actual and proxy outcomes when dipping fails.

Day to night differences in physical and mental activity are thought to be major determinants of blood pressure rhythmicity. Analysis of blood pressure rhythms in shift workers revealed an almost complete resynchronization within the first 24 hours of the shift rotation. This may reflect variation in sympathetic activity, consistent with the correlation between diurnal variation in plasma catecholamines and blood pressure and heart rate. Sympathetic activity appears to integrate the major driving factors of temporal variability in blood pressure, but evidence also indicates a role of the hypothalamic-pituitary-adrenal, hypothalamic-pituitary-thyroid, opioid, renin-angiotensin-aldosterone, and endothelial vasoregulatory systems, as well as other vasoactive peptides. Many hormones with established actions on the cardiovascular system such as arginine vasopressin (AVP), vasoactive intestinal peptide (VIP), melatonin, somatotropin, insulin, steroids, serotonin, CRF, corticotropin (ACTH), thyrotropin-releasing hormone (TRH), and endogenous opioids, show diurnal variabilities. Physical, mental, and pathologic stimuli, which may drive activation or inhibition of these neuroendocrine effectors of biologic rhythmicity, may also interfere with the temporal blood pressure structure. However, the time-dependent responsiveness of cardiovascular tissues to such stimuli may be just as important. Recently, the circadian blood pressure pattern was examined in Clock mice, and Npas2 mice which have disrupted circadian rhythmicity to varying extents. It was found that genes that subserve core functions in the molecular clock regulate differentially enzymes relevant to the synthesis and disposition of catecholamines. This resulted in alterations in blood pressure, plasma norepinephrine and epinephrine, their diurnal variation, and, surprisingly, their response to immobilization stress. It appears that the circadian clock may influence the vascular response to stress indirectly, by controlling the underlying rhythm of BP on which asynchronous cues are imposed but also directly by modulating pressor response, irrespective of timing. Both effects reflect the observed influence of the clock on sympathoadrenal function, which is activated in the integrated arousal response and many of its discrete elements, such as assumption of an upright posture, exercise, and emotional stress. Clock-dependent effects on blood pressure may also interact with diurnal variation of hemostatic variables, such as plasminogen activator inhibitor (PAI)-1, to determine the diurnal influence of cardiovascular events.

Neurohumoral Factors: Inputs to Peripheral Circadian Clocks?

Peripheral oscillators are incapable of receiving direct input from light; therefore, they rely on the master clock to provide time cues via entrainment signals. Although peripheral oscillators are capable of maintaining rhythmic output without the master clock (eg, single cells or single organ cultures), daily entrainment of peripheral oscillators avoids dampening or desynchronization of these rhythms. Recent studies using clock gene reporter systems (eg, Per2 fused to a luciferase reporter) demonstrated that oscillations could be sustained for numerous cycles in cultured peripheral tissues. Tissues from SCN-lesioned mice similarly displayed rhythms when placed in culture, but significant desynchronization was observed both within and among SCN lesioned animals. Thus, the hierarchical organization of the circadian system places the SCN in a role for coordinating and synchronizing the phase of peripheral oscillations.

The daily cycle of feeding and starvation, indirectly controlled by the SCN via activity rhythms, appears to be a dominant entrainment signal for peripheral clocks. When food is available ad libitum, food intake for mice is normally consolidated to the dark phase, corresponding to the activity phase for this nocturnal species. However, during a restricted feeding regime, where food access is restricted to the light phase, circadian gene expression in the periphery, but importantly, not in the SCN, is inverted by 12 hours. Thus food intake, which may present as a synchronous or asynchronous cue, is capable of entraining peripheral oscillators. Although restricted feeding appears to be a dominant entrainment signal for many tissues, the rate at which these various
oscillators have the capacity to alter phase can vary. There are many candidate hormones that may act in a more subtle way to fine tune peripheral rhythms. Npas2, a component of the molecular oscillator, heterodimerizes with retinoic acid receptors, and ligation of these receptors by retinoic acid phase shifts Per2 rhythms in mouse aorta and heart.53 Others have shown that the glucocorticoid analogue, dexamethasone, phase shifts peripheral clocks in fibroblasts, liver, heart, and kidney, without influencing the SCN.76,91 Glucocorticoid hormones have also been demonstrated to inhibit the uncoupling of central and peripheral oscillators that occurs during restricted feeding.52 Peripheral oscillators in the cardiovascular system are particularly well placed to receive entrainment signals from circulating hormones and sympathetic innervation. Adrenergic signaling can influence Per1 expression in cultured cells and tissues slices in culture93 and can initiate cycling in clock gene expression in cardiomyocytes,53 however the relative impact of adrenergic signaling on peripheral oscillators in the heart and vasculature in vivo remains to be determined. Immobilization stress in mice will induce a substantial physiologically relevant rise in plasma catecholamines and glucocorticoids. However, in a study by Yamamoto et al, acute stress elevated Per1 mRNA expression in mouse peripheral organs but behavioral rhythms and molecular rhythms of clock genes in the periphery were unaffected.94 Angiotensin II plays a central role in the regulation of systemic blood pressure and fluid homeostasis through its multiple effects on the vasculature, adrenal glands, kidneys, and brain. These pleiotropic actions are mediated by specific receptors (AT1 and AT2).95,96 In rats and mice, high concentrations of these receptors are found in the brain, including within the SCN. However, the significance of this latter observation is currently unknown. Components of the renin-angiotensin system exhibit considerable diurnal variations and therefore potentially influence blood pressure circadian rhythms.97–100 Nonaka et al have shown that angiotensin II induces significant oscillations in bmal1, per2, and dbp, in VSMCs. Overexpression of the renin2 gene in the rat is associated with a phase delayed or inverted circadian rhythm of blood pressure, attenuated circadian and photic induction of c-fos gene in SCN neurons, and attenuated phase shifting of behavioral and cardiovascular rhythms in response to light.101–103 Studies in AT2 receptor knockout mice revealed disrupted circadian rhythms in blood pressure and heart rate compared with wild type mice.104 These findings raise the possibility that angiotensin II may contribute to regulation of cardiovascular circadian function including integration of the SCN with the peripheral clock in the vasculature.

An elegant study by Guo et al used parabiosis between intact and SCN-lesioned mice to show that nonneuronal signals, either hormonal or behavioral, are capable of synchronizing some peripheral tissues but not others.54 Moreover, the rhythmic expression of Per2 mRNA105 and cell-adhesion molecules106 in circulating peripheral mononuclear leukocytes, which have no neuronal connections, provides further evidence for the existence of circulating phase shifters or inducers of peripheral clocks in vivo. Peripheral oscillations seem to be coordinated both by major entrainment cues as well as local phase shifting signals. It is likely that integration of diverse signals resulting from food ingestion, hormones (possibly SCN driven), or energy homeostasis may contribute to the entrainment of peripheral clocks and that these clocks may be entrained by the SCN through diverse mechanisms.

**Circadian Rhythms and Metabolism**

It has now been well established, by using restricted feeding paradigms, that food/metabolic signals can have a profound influence on the peripheral clock timing at the molecular level. Although this phenomenon is not yet fully understood, it serves to highlight the coregulation of circadian rhythms and metabolism. The SCN controls the phase of peripheral tissues mainly by imposing a rest/activity cycle, which in turn determines a daily feeding cycle. In addition, many neuroendocrine and metabolic systems are subject to strong circadian control. Metabolic genes have featured extensively as rhythmic transcripts in many tissues examined.34–36,107 One such examination of the mouse aorta56 showed circadian variations in genes of relevance to lipid metabolism, energy balance, adipocyte maturation, the maintenance of vascular integrity, and the vascular response to injury. Twenty-two genes relevant to glycolysis, gluconeogenesis, fatty acid synthesis and degradation, triglyceride mobilization and storage, and cholesterol biosynthesis exhibited pathway specific coordination subject to circadian variation. Indeed, several pathways have been identified that might link circadian networks with both gluconeogenic and lipogenic pathways. Important metabolic nuclear hormone receptors and transcription factors including SREBP 1a, SREBP 1c, RORα, Rev Erbα, THR, PPARα show significant circadian expression in numerous tissues. SREBP 1a and 1c regulate hepatic lipogenesis,108 RORα has been shown to regulate lipid flux, lipogenesis, and lipid storage in skeletal muscle,109 increased Rev Erbα and THR expression is observed during adipogenesis,110 whereas PPARα is involved in the regulation of numerous metabolic processes.111 Regulation of clock genes by the redox state of nicotinamide adenine dinucleotide cofactors (NAD and NADP) has been demonstrated in a human neuroblastoma cell culture system.112 The reduced forms of these cofactors NADH and NADPH, strongly enhance DNA binding activity of the Clock:Bmal1 and Clock:Npas2 heterodimers. In contrast, the oxidized form of these redox factors inhibits the DNA binding by these heterodimers. The regulation by NADPH in vitro implies that oscillations in metabolic flux participate in feedback loops with the clock genes. In particular, glycolytic flux results in cytosolic NADH production and depends on shuttle mechanisms for NADH transport into the mitochondria. A study by Young et al reported diurnal variations in myocardial metabolic flux and contractile function.58 Contractile performance, carbohydrate oxidation, and oxygen consumption in isolated working rat hearts were greatest in the middle of the night, with little variation in fatty acid oxidation. In addition, they observed circadian rhythmicity in a variety of genes involved in carbohydrate and fatty acid metabolism. In the context of the NAD/NADH enhancing Clock:Bmal1 binding to Ebox elements, the variations in carbohydrate flux demonstrated by Young et al in the intact
heart could represent an interacting feedback mechanism for the clock. It is therefore a possibility that abnormalities in metabolic flux and NADH generation could disrupt or reset the peripheral clock in the heart.

In studies using Clock mutant and Bmal1−/− mice, a profound role for the clock in the recovery from insulin induced hypoglycemia and, specifically, in gluconeogenesis was observed. A high-fat diet amplified the diurnal variation in glucose tolerance and insulin sensitivity in a manner dependent on the molecular clock. Turek et al reported abnormalities in adipogenesis and metabolism in Clock mutant mice, including a subtle decrease in energy expenditure, hyperphagia exaggerated in response to a high-fat diet, and dysregulation of neuropeptides (Ghrelin, Cart, and Orexin) that regulate energy metabolism. However, on an ICR background, Clock mutant mice develop lipid malabsorption and hence do not develop obesity. The Clock mutant mouse, which was a triumph of forward genetics, is a dominant mutation which results in deletion of a putative activation domain and arrhythmicity in constant darkness. A recently generated Clock−/− mouse retains rhythmic activity, but the question of whether compensation by Npas2 occurs remains to be definitively addressed. Studies of metabolic phenotypes in Bmal1−/− mice are tempered by progressive arthropyathy and an advanced aging phenotype. No doubt emerging mouse models with tissue specific dysfunctional clocks will allow a greater understanding of the contribution of the molecular clock in distinct tissues to energy balance and metabolic homeostasis. How peripheral clocks in many tissues including the cardiovascular system impact the incidence of cardiovascular disease and metabolic syndrome remains a fertile area of investigation.

Circadian Rhythms and the Cell Cycle
Like many other biological processes relevant to the cardiovascular system, the cell cycle displays circadian rhythmicity. Key components of the cell cycle, including cyclin A, cyclin D1, Cdc2, c-myc, and Wee1 have been shown to be under transcriptional control by the molecular clock. More direct evidence for molecular clock regulation of the cell cycle in vivo stems from the work by Matsuo et al. Progression of the cell cycle through the G2/M transition, but not S-phase progression, is gated by the molecular clock to certain times of the day in a partial hepatectomy model of liver regeneration. This circadian gating involves the clock-controlled gene Wee1, which inactivates the CDC2-cyclin B complex required for G2/M transition. Interestingly, Wee1 shows a significant circadian expression pattern in vascular smooth muscle cells synchronized in vitro by the serum shock model (Reilly D, FitzGerald GA, unpublished observations, 2007). However, it has yet to be determined whether the vascular clock can influence the timing of cell proliferation in vascular cells, which may have implications for angiogenesis and vessel remodeling.

Mouse models of molecular clock dysfunction have provided further evidence for clock control of the cell cycle. Liver regeneration after partial hepatectomy is retarded in cryptochrome-deficient mice, whereas mutation of CLOCK was recently shown to inhibit the proliferation of mouse fibroblasts in vitro. Aberrant cell division in vivo has been observed in Per2 mutant mice, which have an increased susceptibility to tumor development on challenge with γ-irradiation. Although these data provide evidence for a role of the molecular clock in regulating the cell cycle, a functional oscillator is not absolutely required for cell division, as several mouse models of molecular clock dysfunction are viable. Nevertheless, the impact of the molecular clock on cell division is evident. Future work will likely shed light on the relative importance of the molecular clock on proliferative diseases of varying etiology, including cancer, atherosclerosis, arthritis, and others. Circadian time may also influence the outcome of coronary interventions such as angioplasty, where restenosis reflects a dysplastic response to injury.

Recent data have also revealed a link between the molecular clock and aging. Mice deficient in BMAL1 display an early aging phenotype with shortened life spans, sarcopenia, cataracts, and other signs of early aging. Similarly, Per1/Per2 double mutant mice age prematurely, as characterized by a drop in fertility and litter size, loss of soft tissues, and kyphosis. Cellular senescence, a marker of cellular aging, is evident in human vascular tissues and may contribute to the development of vascular disease. Interestingly, Kunieda et al showed that cellular senescence impairs circadian rhythmicity in cultured human aortic smooth muscle cells. In vivo, mouse embryo fibroblasts (MEFs) were capable of entrainment on implantation into mice, whereas senescent MEFs were impaired in this ability. Thus, an interaction between the circadian and cell cycles appears to influence aging at both the cellular and organismal level.

Chronobiological Implications for Cardiovascular Therapy
Several lines of evidence suggest that an understanding of the chronobiological implications for cardiovascular therapy may prove fruitful. Low-dose aspirin appears to reduce blood pressure, but only if administered in the evening. The mechanism is unknown. Controlled-onset extended-release (COER) verapamil, a calcium channel blocker, administered in the evening to blunt the morning surge in blood pressure, has shown clinical improvement over morning doses of other antihypertensive drugs. 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase, the target of the statin drugs, such as propranolol, nifedipine, and enalapril, is
apparent.\textsuperscript{134} Both transporters and P450 isozymes are among the most oscillatory of transcripts.

**Contribution of the Circadian Clock to the Temporal Incidence of Cardiovascular Disease**

There is a well observed temporal incidence in adverse cardiovascular (CV) events, including transient myocardial ischemia,\textsuperscript{135} myocardial infarction,\textsuperscript{136} sudden cardiac death,\textsuperscript{137} and stroke,\textsuperscript{138–140} which occur most frequently in the early morning hours just after awakening, but also display a secondary more subtle peak in the late afternoon. Skeptics of the circadian argument for the temporal incidence of cardiovascular disease raise the objection that the classification of morning events might be artifactual or biased. In particular, unwitnessed events, such as sudden cardiac death occurring during sleep might be falsely attributed to the morning hours, when the deceased patient is discovered. In addition, the patient might sleep through the onset of a heart attack, wake up at 8 AM and report pain. The increased morning onset of cardiovascular events may also be the result of exogenous factors, such as assumption of upright posture and initiation of daily activities, all of which activate the sympathoadrenal system and other aspects of the stress response. Although these arguments may be valid, they do not account for the consistently observed temporal incidence of adverse events.

Several factors involved in the development of cardiovascular disease are temporally modulated. Blood pressure, endothelial function, vascular tone, lipid metabolism, platelet and leukocyte reactivity, and fibrinolysis vary with the time of day. Moreover, core molecular oscillators have been identified in the heart\textsuperscript{59} and vascular tissue,\textsuperscript{36} encompassing both vascular smooth muscle and endothelial compartments, and recent evidence has pointed toward a role of molecular oscillators in regulating cardiovascular physiology.\textsuperscript{48,141} The endothelium secretes low levels of tPA along with the platelet inhibitors, prostacyclin (PGI\(_2\)), and nitric oxide (NO),\textsuperscript{142,143} which are also responsible for regulating vascular tone\textsuperscript{144} and blood pressure.\textsuperscript{145} An early morning surge in blood pressure is accompanied by a decline in endothelial function, as assessed by flow-mediated vasodilation\textsuperscript{146–148}, both phenomena coincide with the clinically observed morning peak incidence in thrombotic events.\textsuperscript{149} Per2 mutant mice display decreased endothelium-dependent vasodilation in response to acetylcholine,\textsuperscript{141} further implicating the molecular clock in the regulation of endothelial-dependent vascular function.

The tendency of platelets to aggregate, which can promote thrombogenesis, has been suggested to show a diurnal pattern in humans, however aggregometry studies are conflicting and potentially confounded by artifact. Other mediators of the hemostatic system display diurnal variation, including coagulation factors (II, VII, X, and tissue factor pathway inhibitor [TFPI]).\textsuperscript{150–152} The morning onset of myocardial infarction may partly result from circadian variation of fibrinolytic activity. Fibrinogen, the circulating precursor of fibrin (a clot stabilizing protein), displays circadian variation in humans,\textsuperscript{153} with a peak in the early morning, whereas the expression of the fibrinogen gene in mouse liver displays 2 peaks, the first of which seems to be regulated by the molecular clock.\textsuperscript{154} PAI-1 is the main inhibitor of the plasminogen activators, and thus fibrinolytic activity. PAI-1 activity shows a circadian oscillation peaking in the early morning, and evidence has indicated a role of the molecular clock components CLOCK, BMAL1, and CLIF (also known as MOP9 or BMAL2) in driving E-box–mediated PAI-1 transcription subject to inhibition by PER/CRY complexes.\textsuperscript{155} Thus, numerous mediators of cardiovascular pathology display circadian variation, and evidence for molecular clock regulation of these mediators is beginning to unfold. The physiological importance of control by the circadian clock remains a fertile area of investigation.

Tempora mutantur et mutamur in illis (The times change and we change with them.) John Owen.

**Disclosures**

None.

**References**


Vascular Clocks and Cardiovascular Disease

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Peripheral Circadian Clocks in the Vasculature
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