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## Vascular Rhythms and Adaptation: Do Your Arteries Know What Time It Is?

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The notion that the clinical manifestations of cardiovascular disease do not occur randomly throughout the course of the day has been known for over 4 decades. Cohort studies documenting the time of chest pain onset demonstrated a morning predilection for the presentation of myocardial infarction (MI).<sup>1</sup> At the time, however, it was difficult to discern a morning rise in the incidence of MI from the simple realization of longstanding nocturnal symptoms upon awakening. With the advent of studies to limit myocardial infarct size, it became possible to accurately identify the MI onset by the time-dependent increase in circulating creatine kinase levels and these data confirmed a distribution roughly peaking in the hours between 6 a.m. and noon.<sup>2</sup> It soon became clear that other manifestations of cardiovascular disease, such as sudden cardiac death<sup>3</sup>, stroke,<sup>4</sup> and myocardial ischemia<sup>5</sup> all exhibited similar time-of-day dependence. If one corrects for individual variations in the time of sleep cessation, the preponderance of events occur within the first three hours after awakening.<sup>6</sup>

This clear clustering of cardiovascular events to a specific time of day prompted considerable investigation as to potential causes, with particular attention to phenomena that exhibit a preference to the morning hours. Arterial pressure, for instance, was noted to have a rhythmic pattern characterized by a nadir at 3 AM followed by a rise to peak pressures before noon. Since heart rate exhibits a similar pattern, one could suppose increased morning adrenergic tone might contribute to the morning clustering of cardiovascular disease events. Consistent with this notion, platelets are more susceptible to stimulation soon after awakening, when circulating catecholamine levels are higher than other periods of the day.<sup>7</sup> Several circulating factors such as cortisol and tissue-type plasminogen activator<sup>8</sup> exhibit rhythmic variation that could, in principle, contribute to the onset of cardiovascular disease.

In fact, most aspects of physiology vary on a rhythmic basis. While these rhythms could be generated as simple passive responses to an environment that changes over the day, many rhythms persist with a cycle length near 24 hours, even in constant environmental conditions. These endogenous rhythms are termed circadian rhythms, while rhythms observed in the presence of a rhythmic environment are termed daily or diurnal rhythms. In mammals, the most important environmental cue for synchronizing circadian rhythms to the 24-hour solar day is the daily light-dark cycle.<sup>9, 10</sup> When placed in constant darkness, rhythms of activity, sleep/wake, body temperature, food intake, hormone levels, blood pressure, heart rate, and myriad other endpoints persist.<sup>9,10</sup>

A central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus orchestrates this rhythmicity, maintaining internal temporal order so that, for example,

intestinal transporters that facilitate glucose uptake are expressed even before the animal eats,<sup>11</sup> improving efficiency by predicting recurring events rather than merely reacting to them. The SCN receive light input via the retina, and communicate timing information to other tissues by multiple redundant mechanisms.<sup>12</sup> (Figure 1A) These output pathways include hormonal rhythms (e.g., cortisol and melatonin), autonomic activity, and through regulation of the timing of feeding and activity, which in turn imposes additional cues on peripheral tissues.<sup>13</sup> Individual neurons within the SCN contain the biochemical machinery necessary to measure out 24 hours, and the population of ~ 10,000 SCN neurons communicate and dictate “SCN central time.” Remarkably, the capacity for rhythmicity is not restricted to the SCN, and indeed most organs and cell types express rhythms in gene expression, and continue to do so when isolated *in vitro*.<sup>13</sup> Thus, the SCN serve as a master pacemaker, a population of neuronal oscillators that coordinate a body full of oscillators. The light-dark cycle sets the timing of the SCN neuronal oscillations, and also reinforces and amplifies many physiological and behavioral rhythms. When an animal (or human) is placed in constant darkness, rhythms persist and they do so with the appropriate temporal order, but they are no longer coordinated to the solar day. In the absence of a functional circadian clock, rhythmicity is artificially imposed by the lighting cycle. Only when a circadian clock is studied in constant conditions can its true impact be seen.

The mechanisms by which individual mammalian cells can measure time in ~24-hour increments has been revealed over the last 12 years (Figure 1B). The core of the circadian clock mechanism is a molecular feedback loop that produces and destroys key proteins in an oscillating manner with a cycle length of 24 hours. At the heart of this mechanism are three basic helix-loop-helix transcription factors, CLOCK, BMAL1 and NPAS2.<sup>10,14</sup> These proteins pair up to form heterodimers (CLOCK:BMAL1 or NPAS2:BMAL1) which are transcriptional activators. Among the targets of these activator complexes are the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. The protein products of these genes (specifically PER1, PER2, CRY1 and CRY2), form complexes that inhibit the transcriptional activity of the activator complex, thus closing the negative feedback loop (Figure 1B). Posttranslational modification and degradation of the negative regulators prevent the feedback loop from closing too soon, resulting in a molecular feedback loop oscillating with a period of 24 hours.

It is important to keep in mind that in parallel to this rhythmicity of the so-called “clock genes” (gene families necessary for the core molecular loop), other genes are being rhythmically activated and inhibited. These genes include clock-controlled genes, which respond directly to the waxing and waning of the activator and repressor complexes, as well as other transcription factors. These transcription factors (both activators and inhibitors) generate second-order rhythms of expression in other genes, and can also modify the time of expression of genes affected by the activator complex. The overall impact of this molecular oscillation is stunning: microarray studies estimate that as much as 5–10% of the genome is rhythmically expressed (reviewed in ref<sup>15</sup>) However, the *specific* 5–10% of genes rhythmically expressed actually varies by tissue.

Studies with knockout mice show that disruption of genes within the core clock mechanism leads to loss of circadian rhythmicity.<sup>10</sup> The only single gene which leads to loss of behavioral and cellular rhythmicity when disrupted is *Bmal1*.<sup>16</sup> Other genes within the circadian clockwork have at least partial redundancy with other members of their gene family, such that disruption of more than one gene is necessary to produce an arrhythmic phenotype. This functional redundancy is the case for the *Per* genes (*Per1* and *Per2*), *Cry* genes (*Cry1* and *Cry2*), and also for *Clock* and the closely related gene *Npas2*.<sup>14</sup> However, a mutation of the *Clock* gene has been identified that leads to production of a mutant protein lacking the residues encoded by exon 19.<sup>17</sup> The CLOCK protein lacking exon 19 is devoid of transcriptional activity, and apparently disrupts rhythms by sequestering BMAL1 in non-productive

complexes. Mice homozygous for this mutant allele of *Clock* actually have a more severe phenotype than *Clock* knockout mice, losing rhythmicity within several cycles in constant darkness.<sup>14, 17</sup>

In this issue of *Circulation*, Anea and colleagues investigate chronic vascular adaptation as a function of the molecular components of the circadian clock.<sup>18</sup> Normally, chronic reductions in flow are associated with intimal thickening and inward remodeling of the lumen to reflect the lower flow demands.<sup>19</sup> However, in mice with disrupted circadian rhythmicity due to deletion of the essential clock element *Bmal1*,<sup>16</sup> intimal thickening is accentuated and there is no inward remodeling indicating a failure to adapt to changing flow.<sup>18</sup> In older mice, this defect was accentuated and associated with a predisposition to thrombus formation in the ligated artery. These pathologic arterial responses were unequivocally reproduced in another animal model, *Clock* mutant mice under arrhythmic conditions, clearly establishing that circadian rhythms at the molecular level have important implications for vascular homeostasis.

The approach by Anea and coworkers involved studying mice with alterations in two important components of the molecular circadian mechanism, CLOCK and BMAL1.<sup>18</sup> As noted above, these genes encode transcription factors, so one interpretation of their study is that these mutants have a vascular phenotype due to loss of the transcription factors and the resultant alterations in gene expression, but not necessarily due to a loss of *rhythmic* gene expression. The authors use a clever approach to address this issue. The circadian defect in *Clock* mutant mice is manifest under constant (dark) conditions, but can be “rescued” via housing under normal light-dark cycles. Since the remodeling defect in *Clock* mutant mice was not evident with light-dark cycling, it is clear that *rhythmic* gene expression is a key element of normal vascular adaptation. This latter point has important implications in that inherent circadian defects may be prevented from producing pathology if one maintains a rhythmic environment.

The two pathologic responses observed by Anea and colleagues with circadian disruption, loss of vascular adaptation and predisposition to thrombus,<sup>18</sup> are reminiscent of the defects seen in patients with vascular disease that exhibit endothelial dysfunction.<sup>20</sup> Indeed, animals with dysfunctional circadian rhythms exhibited severe defects in endothelium-dependent arterial relaxation that were normalized by the restoration of rhythmicity with normal light-dark cycles. The molecular mechanism responsible for abnormal endothelial function appeared due to an inability to normally phosphorylate and activate eNOS, the enzyme responsible for endothelium-dependent NO production and, in large part, vascular homeostasis.<sup>21</sup>

The findings of Anea and colleagues clearly indicate that rhythmic gene expression, via the circadian clock, has important implications for cardiovascular signaling. Their findings that phosphoinositide dependent kinase 1 (PDK1) expression was impaired leading to defective phosphorylation of the serine/threonine kinase Akt have broad implications in the vascular wall and beyond. Previous studies have demonstrated that Akt is needed for a host of vascular responses including nitric oxide (NO) bioactivity, endothelial migration, vascular remodeling, and the regulation of inflammation (reviewed in <sup>22</sup>) Experimental animals with defective Akt signaling also exhibit a profound predilection to atherosclerosis and coronary thrombosis.<sup>23</sup> Beyond the vasculature, Akt is known to be important for cardiac protection in the face of ischemic injury<sup>24</sup> and the regulation of left ventricular hypertrophy and the progression to heart failure.<sup>25</sup> Thus, disruption of circadian rhythms has the potential to causally impair a number of homeostatic responses in both the vasculature and the heart.

There is also reason to believe that impaired rhythmic gene expression, and the resultant defects in eNOS action will have implications beyond the vasculature. Mice lacking eNOS exhibit features of the metabolic syndrome including hypertension, hyperlipidemia, and insulin resistance.<sup>26</sup> It would follow, therefore, that defective eNOS signaling observed by Anea and

colleagues<sup>18</sup> could explain the obesity and insulin resistance seen in some models of impaired circadian rhythmicity.<sup>27</sup> However, it is likely the relation between the circadian clock and metabolism is more complex. For example, Obesity and insulin resistance develop in *Clock* mutant mice develop obesity and insulin resistance under normal light:dark cycling,<sup>27</sup> conditions not associated with abnormal eNOS signaling in the present study. The *Bmal1* null mouse actually shows increased insulin sensitivity and hypotension, clearly opposite to predicted consequences of impaired eNOS activation. No doubt more information is needed to understand the complete relation between the circadian clock components and metabolism.

So how might disruption of circadian rhythms alter the expression of cardiovascular disease? One hint comes from work in golden hamsters heterozygous for the mutation *tau*, that causes a shift to a 22 hour circadian period and results in fragmentation of diurnal activity in 24 hour light-dark cycles. The fragmented activity results in premature death characterized by a dilated cardiomyopathy, interstitial cardiac fibrosis, and progressive renal impairment.<sup>28</sup> If the animals were kept in a 22 hour light-dark cycle (concordant with their genotype), there was no fragmentation of activity and no evidence of cardiomyopathy or renal disease were evident. These data are consistent with observations in aged mice that phase advancing (shifting) the light cycle (as in jet lag) increases mortality.<sup>29</sup> Thus, shifting your biological clock, particularly towards less sleep, is a stressful phenomenon that has adverse cardiovascular consequences.

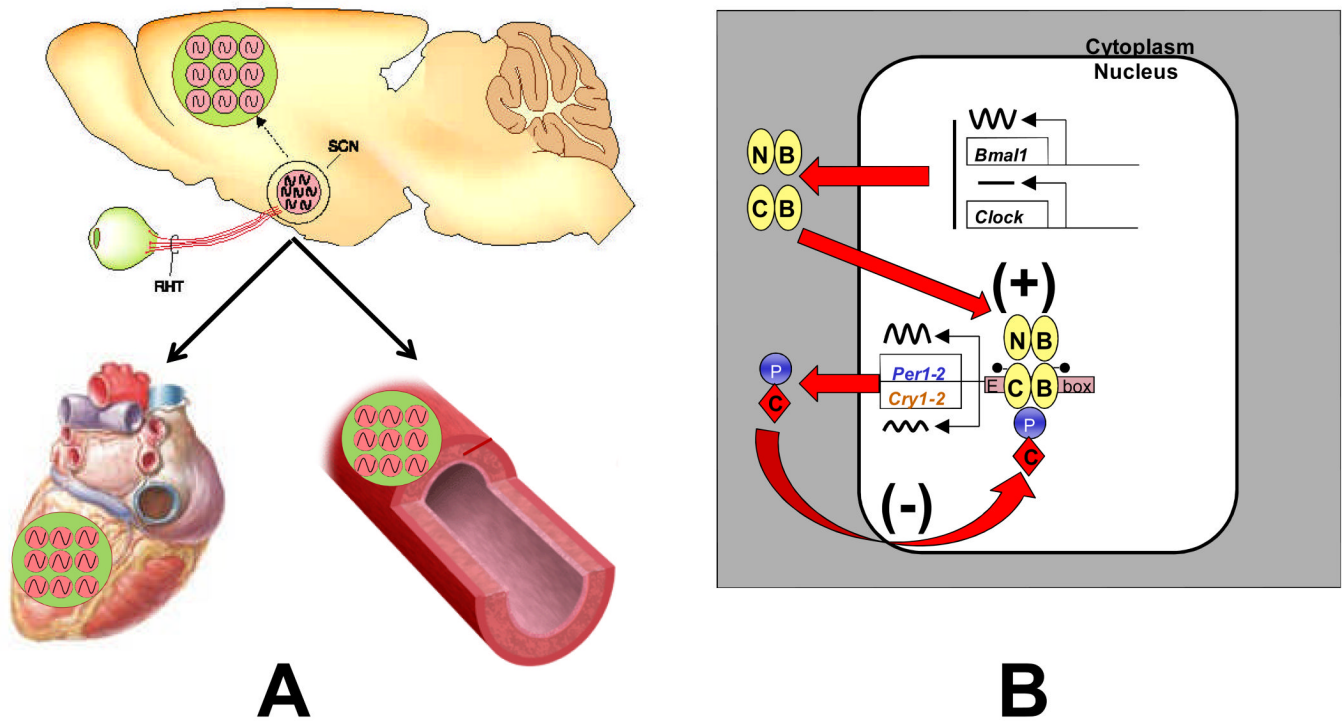
In summary, the work of Anea and colleagues<sup>18</sup> has linked disruption of the rhythmic circadian gene expression to impaired vascular homeostasis manifest as endothelial dysfunction and impaired vascular remodeling, largely due to disruption of normal signaling through PDK1, Akt, and eNOS. These findings highlight the role of rhythmic gene expression in the control of vascular homeostasis and will surely spark a search for the precise molecular link(s) that tie together rhythmicity to PDK1/Akt signaling.

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**Figure 1. Circadian clock mechanisms**

(A) Cells in the suprachiasmatic nuclei (SCN) of the hypothalamus maintain an internal 24h pacemaker via rhythmic expression/degradation of a small collection of proteins. The SCN are synchronized by light via a special population of retinal ganglion cells which comprise the retino-hypothalamic tract (RHT, red). The SCN communicate timing information to tissues by multiple redundant mechanisms. Tissues also exhibit the capacity for rhythmicity, usually coordinated by the SCN. (B) The molecular clock mechanism includes the transcription factors CLOCK (C, yellow oval), BMAL1 (B) and NPAS2 (N) that form heterodimers to activate the *Period* and *Cryptochrome* genes by binding to E-box elements in their promoters. The gene products, PER (P) and CRY (C, red diamond) form complexes that inhibit the activity of the transcriptional activator complex, resulting in a negative feedback loop. Posttranslational modification and degradation of the PER and CRY proteins (not shown) prevents the feedback loop from closing sooner than a period of 24 hours. Many other genes are rhythmically expressed due to rhythmic E-box-mediated transcription, or by the rhythmic activity of other transcriptional activators and inhibitors which are themselves rhythmically expressed.