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REVIEW

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Old and New Roles and Evolving Complexities of Cardiovascular Clocks

Yanyan Xu, Wenhu Pi, and R. D. Rudic*

Department of Pharmacology & Toxicology, Augusta University, Augusta, GA

The cardiovascular (CV†) system has been established to be significantly influenced by the molecular components of circadian rhythm. Oscillations of circadian rhythm occur within the circulation to affect thrombosis and blood pressure and within CV tissues including arteries, heart, and kidney to control function. Physiologic and molecular oscillations of circadian rhythm have been well connected via global, tissue-specific, and transgenic reporter mouse models of key core clock signals such as *Bmal1*, *Period*, and *Clock,* which can produce both pathology and protection with their mutation. With different nuances of CV clock action continuing to emerge in studies of the cardiovascular system, new questions are raised in both new and old mouse model system observations that underscore the importance, complexity, and continued study of the circadian clock mechanism in cardiovascular disease.

THE CIRCADIAN CLOCK

Anticipation and the ability to respond to unexpected stresses is an important aspect of survival at the organismal and cellular level. In biology, the circadian clock acts like a temporal receptor, receiving information regarding physiologic timing and adapting accordingly. This 24-hour sensor has adapted and evolved as a characteristic of the 24-hour earth rotation, which on average provides us with an oscillating light pattern, of 12 hours of light and 12 hours of darkness. The mechanism by which this light information is relayed to the brain is through the melanopsin photopigment [1] contained in retinal ganglion cells [2] and as melanopsin is a non-visual photopigment, it can also relay this information in blindness [3,4] though enucleation (eye removal) abolishes this ability [5], as this opsin is also localized within the eyes. Within the cells of land organisms, a unique set of genes/proteins receive this temporal information via a cascade of signals that are modified by the environmental (zeitge-

*To whom all correspondence should be addressed: Dan Rudic, Augusta University, 1120 15th Street, Augusta, GA, 30912, CB3620; Tel:706 721-7649, Fax 706 721-2347, E-mail: rrudic@augusta.edu.

†Abbreviations: CV, cardiovascular; SCN, suprachiasmatic nucleus; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; eNOS, endothelial nitric oxide synthase; PAI-1, plasminogen activator inhibitor-1; VWF, von Willebrand Factor; HUVEC, human umbilical vein endothelial cells; PDGF, platelet derived growth factor; bHLH, basic helix loop helix; ATP1B1, ATPase Na+/K+ Transporting Subunit Beta 1; dec, deleted in esophageal cancer; eNAC, epithelial sodium channel; AngII, angiotensin II; ARB, angiotensin receptor blocker; AT1, angiotensin type 1 receptor; PVAT, perivascular adipose tissue; SMC, smooth muscle cell; TIMP, tissue inhibitor of metallopeptidase.

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bers), which include light, temperature, and food intake (eating). Circadian timing gives organisms the ability to anticipate and adapt to a change in the environment to maximize life at minimal metabolic cost. When we rest, the clock informs metabolic cues to change accordingly, and when we rise, metabolism adapts in anticipation of the demands that arise in activity. Conceptually, the clock regulates on the level of whole animal to organ to cell and the molecular components of the circadian clock are found in nearly all cells with 43 percent of all protein encoding genes exhibiting circadian rhythms [6]. The components of the circadian clock that localize within the brain to regulate locomotor rhythms, dubbed the central clock, are found in a particular pair of bilateral brain nuclei above the optic chiasm. This suprachiasmatic nucleus (SCN) is comprised of approximately 20,000 neurons in this region of the hypothalamus, to form a network that can synchronize the internal clock timing to the external stimuli, such as sunlight, via input from the retina and those melanopsin photopigments. The intensity, frequency, and timing of the external environment thus play an important role in controlling the clock [7]. For example, the time at which we eat or the time at which we receive light, or the frequency of meal or light bursts, and the caloric or illuminance (lux) strength of these stimuli impact the responsiveness of the circadian clocks. Within the cardiovascular system, changes in external rhythms can be sensed by the circadian clock and translated into changes in blood pressure, vascular or heart function. In addition, it may be that clock mechanisms in the cardiovascular system can also interact to influence locomotor activity [8] For example, disruption of *Clock* in the heart [8] decreased locomotor activity while disruption of *Bmal1* in the endothelium [9] increased activity, but in both of these studies, despite peripheral clock mutation influencing locomotor activity, overall locomotor rhythm was not changed. Further evidence suggestive of a CV clock to brain axis is recent data that showed that clock mutant mice undergoing left anterior descending coronary artery ligation exhibited changes in neuronal dendrite trees in the prefrontal cortex and hippocampus relative to wild type mice [10]. It is evident that circadian clocks can act in the periphery, can influence CV function by direct actions, but it seems they may also feed back and control aspect of central functions, which may again feed forward.

To date, it is understood that the circadian clock itself is a sedentary mechanism, one that resides within cells, but it may control other endocrine/humoral/electrical signals that could circulate its message [11,12], either from the SCN to the vasculature, vice versa, or cell to cell. This local transcriptional/translational/post-translational/epigenetic self-regulated mechanism was first discovered in flies [13,14]. There, the *Drosophila* circadian clock is comprised of four factors: *clock* (*clk*), *cycle* (*cyc*), *period* (*per*), and *timeless*; in mammals Bmal1 and Cry are homologs of *clock* and *cyc*. Clk, Cyc (flies), BMAL1, and CLOCK (mammals) are the positive limb that drives transcription. Per, Tim (flies), PER, and CRY (mice) inhibit *clk*/*cycle* (flies) or *Bmal1*/*Clock* (mice). Also, in mammals there are three *Per* and two *Cry* gene isoforms. A protein-protein interaction between BMAL1 and CLOCK proteins facilitates binding to E-box cis-regulatory enhancer sequences in *Per* or *Cry* genes or other clock-controlled genes (that are not part of the circadian clock regulatory loop) to promote transcription. The protein-protein interaction or heterodimerization of BMAL1 and CLOCK is conferred by a contained protein domain (in both BMAL1 and CLOCK) called Period-ARNT-single-minded (PAS) domain. In addition, PAS domains are sensors to changes in redox and oxidant stress state. The formation of BMAL1/CLOCK heterodimers (also there are BMAL1/NPAS2 heterodimers in mammals), enables binding to the E-boxes (CACGTG) in the regulatory regions of the targeted genes, including the negative feedback components of the circadian clock loop *Per* and *Cry genes,* resulting in increased expression of PER (PER1, PER2, PER3) and CRY (CRY1 and CRY2) in cytoplasm. Thus, the *Per* and *Cry* RNA levels are increased while the *Bmal1* RNA levels fall because of the positive feedback [15,16].

CIRCADIAN CLOCKS IN BLOOD VESSELS

Similar to the way SCN can receive input, the peripheral clocks themselves can also be entrained or modified by different molecular, mechanical, or metabolic signals. Peripheral clocks are present and regulate transcription of thousands of genes in different tissues, affecting multiple physiological functions, including the function of the vascular system [17].

It is well known that the fluctuations of blood pressure and heart rate show circadian rhythm. In addition, other cardiovascular outcomes such as acute myocardial infarction, cerebral infarction, stroke, and sudden cardiac death also tend to present a peak onset frequency in the early morning [18]. The evidence is compelling that circadian rhythm dysfunction contributes to many aspects of cardiovascular disease [19,20] and is becoming more appreciated as an important factor in improving both therapeutics [21] and general well-being. Mechanistic studies have proved informative in identifying a clear role of the circadian clock loop and its targets [22-24] in CV function, though gaps in knowledge remain. Indeed, new data has emerged that is uncovering unexpected aspects and complexities of the circadian clock in cardiovascular function and signaling.

The circadian clock is found in all layers of the vas-

culature: the endothelium, the media, and the adventitia. The endothelium of the vasculature is unique as it is a single circumferential layer of cells, and as such oscillating endothelial-specific circadian signals might be under-represented or even missed in profiling studies of whole aorta, given that the more massive smooth muscle and adventitial multilayers are more prominent. But indeed, the clock is located in endothelial cells [25]. Endothelial cells, which line the blood vessels, function as a sensor to flow hemodynamics to regulate blood exchange between vessels and different tissues, thus maintaining tissue oxygen supply and normal end-organ function. Damage from different blood borne insults such as activated macrophages, turbulent blood flow forces, hyperglycemia, and hyperlipidemia, most likely are first experienced by endothelial cells of the arteries wall which may cause the endothelial cell to become inflamed, de-differentiated, and dysfunctional. Target effects include altered regulation of adhesion molecules (such as ICAM-1 and VCAM-1) expression, imbalanced eNOS production, increased superoxide and coagulation cascade components (such as plasminogen activator inhibitor PAI-1, fibrinogen and von Willebrand factor VWF) production [26], and enhanced inflammatory responses. Circadian clocks are involved in all these processes. It has been shown that circadian clock dysfunction translates into endothelial dysfunction, shown in *Bmal1* knockouts, Clockmut mice and *Per2* mutant mice [27-29]. The endothelial dysfunction is corroborated by deficits in key aspects of endothelial signaling, as the loss of core circadian clock causes a reduced phosphorylation form of *eNOS* and *AKT* in aorta, and also in the long-term impairs vascular remodeling, to cause vessel stiffness [30-32]. More specific evidence implicating the endothelial clock was shown demonstrating that endothelial disruption of *Bmal1* worsens microvascular injury in retinal capillaries and large arteries [33].

Aside from these former studies, more recent work also suggested that the clock plays an important role in the permeability barrier of the vasculature, a role that involves the endothelium and pericytes. In the brain circulatory system, *Bmal1* has been shown to regulate pericytes in the mouse blood-brain barrier. Disruption of the *Bmal1* clock component in mice via nestin-targeting to brain pericytes (which are in close apposition to the endothelium) caused an increase in blood-brain barrier permeability, that was mediated through pericyte dysfunction as evidenced by decreased PDGF-bb production [34]. A circadian input into permeability and the blood-brain barrier has also been shown in *Drosophila.* In flies, the blood-brain barrier is more restrictive at night, a barrier oscillation that is mediated by a fly-specific junction molecule called innexin [35] (invertebrate analog of connexins) [36], similar to the restrictive barrier timing of mice in the periphery [37]. In human endothelial cells, the sleep hormone melatonin has been shown to modulate other growth factors to regulate permeability. In HUVEC cells, it was shown that melatonin could reduce the increased vascular permeability caused by VEGF and EGF, by altering VE-cadherin phosphorylation and *Akt* [38]. Indeed, melatonin is a key output of the clock, and may be an important signal that also entrains the clock, to control cardiovascular function and blood pressure in addition to endothelial permeability [39,40].

BLOOD PRESSURE CLOCKS

The circadian clock is known to influence blood pressure. This has been shown from global *Bmal1* disruption [41], tamoxifen induced disruption of *Bmal1* [42], smooth muscle cell disruption of *Bmal1* [43], *Cry* [44], and *Per* disruption in mice [45]. Given the importance of the heart and cardiac output in blood pressure regulation, it should also be stated numerous studies have demonstrated the key importance of the heart and more specifically the cardiomyocyte clock in heart metabolism and function [46-55]. Recent work now implicates *Dec* (*deleted in esophageal cancer*) transcription factors in blood pressure regulation. Like the positive limb of the core clock, *Dec1* and *Dec2* are bHLH transcription factors and also play a role in controlling circadian rhythms [56], albeit less well studied. Recent data has revealed that *Dec1* KO mice exhibited decreased blood pressure, and mechanistically it was found that *Dec1* suppressed expression of *ATP1B1* which encodes the beta subunit Na^+/K^+-ATPase. This effect was through a heterodimeric partnership between Dec and Clock that bound the *ATP1B1* promoter to inhibit ATP1B1 transcription. While Dec1/Clock was a repressor, the Bmal1/Clock heterodimer was an activator of *ATP1B1* promoter activity. Such ascribed alterations in the dynamics of circadian clock heterodimerization with Dec1 that occur in the kidney likely also contribute to the altered *ATP1B1* expression observed in the heart and vasculature in *Dec1* KO mice [57]. Other work demonstrated the importance of other transporters/channels such as eNAC in blood pressure regulation [58] and alterations in clock oscillations in the hypertensive, high salt-challenged kidney [59]. There is also evidence that the renin-angiotensin pathway also contributes to circadian clock mediated blood pressure regulation [60], although the site of action (central or peripheral receptors) of AngII signaling in circadian blood pressure regulation has not been fully elucidated. One recent study approached this by administering angiotensin receptor blockers infused either in the brain or in the periphery in a hypertensive strain of mice at different times of day. In these studies, the ARBs exhibited greater effects to lower blood pressure when given at night in the BPH/2J mice, and this hypotensive effect was compara-

ble when ARBs were administered either via intracerebroventricular or subcutaneous routes of delivery, with the authors concluding that central angiotensin II receptor type I inhibition was not contributing to the hypertension in this strain of mice [61]. The db/db obese mouse has also been a valuable model to examine circadian rhythm in blood pressure in conditions of a genetically intact clock. In the obese and diabetic db/db mouse, blood pressure dipping is impaired [62], and recent studies identify numerous disruptions in peripheral clock oscillations. In these studies, the db/db mouse was crossed to the reporter PerLuc mice to generate an obese non-dipping BP mice with this circadian luciferase readout; the results showed that there were impaired rhythms in liver, kidney, and submandibular glands, but without interrupting SCN Per rhythms [63].

Some interesting complexities remain with regard to blood pressure and the circadian clock. Like *Bmal1*-KO mice [64], the *Dec*-KO [57] and *Per1*-KO mice [65] were also shown to have a lower blood pressure than WT mice, though *Clock* mutant mice in the Dec1 studies were shown to have a higher blood pressure than their WT counterparts [57]. That blood pressure is lower with circadian clock gene mutation is counter-intuitive. However, this has now been shown in multiple models. Most recently, disruption of *Bmal1* in perivascular adipose tissue (PVAT) caused a super-dipper phenotype, via a lower blood pressure dip during the rest period, and mechanistically by a *Bmal1* induced regulation of angiotensinogen specific to the PVAT [66]. Thus, circadian clock gene mutation can cause endothelial dysfunction [25,28], accelerated thrombosis [67,68], and vascular pathology [33,69-73] despite modest lowering of blood pressure. Studies in SMC *Bmal1*-KO mice may have shed some insight on this quandary. SMC Bmal1-KO mice were shown to exhibit lower blood pressure like the global knockout of Bmal1. The investigators parsed systolic and diastolic blood pressure data and found that the increment of diastolic pressure reduction was greater than that of systolic blood pressure resulting in an increase in the derived pulse pressure [43]. Thus, it may be that increased pulse pressure (which can be a predictor of vessel stiffening, and has been shown to occur in Bmal1-KO and Per-KO mice [31]) could explain the susceptibility to vascular disease in face of lowered blood pressure in the circadian clock knockout models. There are other mechanisms also likely at play in this paradox. Formation of Dec/Clock heterodimers to influence the core clock mechanism may effect kidney-controlled blood pressure and vascular function controlled remodeling differently. Moreover, there could be additional tissue specific bHLH's that can modify signaling and CV responses. Additionally, there are bi-directional signals like Akt that have exhibited complex up or down regulation depending on tissue and circadian clock mutation, with data showing Akt is upregulated by Clock mutation [50], downregulated by *Bmal1* mutation [27,74,75], and upregulated by *Per* mutation, while Akt also can act to feedback and regulate Bmal1 [76] and Clock phosphorylation [77], which could condition their ability to transactivate target genes. Indeed, more evidence is emerging regarding the good and bad of broken clocks.

Can a Broken Clock be a Good Thing?

More evidence has emerged that the absence of a functional clock may not always induce a bad outcome for CV health (in mice). Disruption of *Bmal1* in vascular smooth muscle cells was protective in one study assessing experimentally induced aortic aneurysm. In those studies aneurysm induced by either an aldosterone infusion combined with a high salt diet or AngII infusion combined with hypercholesterolemia model robustly caused aneurysm in wild type mice, but *Bmal1*-KO mice did not develop robust aneurysms, potentially through an increase in TIMP-4 to suppress MMP activation and elastin breaks [78]. In a different set of studies using another model of cardiovascular insult, induced hypertension via high salt and mineralocorticoids, induced hypertension in wild type mice, but blood pressure in mice with Per1 disruption was actually lower [65]. These studies were done in mice where clock genes were disrupted prenatally, but what about clock disruption postnatally?. Recently, studies in which Bmal1 was disrupted in adult mice (tamoxifen-induced Bmal1 disruption) also demonstrated that mice with broken clocks are protected from jet-lag. In these studies, induced Bmal1 disruption in 3-month-old mice, facilitated adaptation to a range of light cycle perturbation models, resulting in improved adaptation with regard to central locomotor activity and peripheral metabolic homeostasis [79]. These observations in clock knockout mice suggest that lacking an oscillator may be protective by rendering the mice impervious to environmental disturbances of circadian rhythm. There may be additional complexities that involve differences between clock disruption or dysfunction pre-natally versus post-natally. In utero, clock genes are thought to not oscillate in the fetus [80,81]. That said, there are still "non-clock" fetal rhythms persisting in the fetus coordinated through the mother [82,83] (perhaps reminiscent of the way single cells pass clock time to daughter cells [84]). Work by Yang *et al*. identified phenotypic disparities between pre-natal and post-natal clock gene disruption in mice challenging "rhythm" roles in the progression of some pathologies [42]. In these studies, some phenotypes previously reported in global embryonic Bmal1 knockout mice were not recapitulated by inducible disruption of Bmal1 at 3 months (tamoxifen treatment in 3 month-old floxed Bmal1 mice) leading to Fitzgerald and colleagues to propose a circadian modification of the Barker hy-

pothesis [42,85], the latter hypothesis being that the fetal environment can impact future adult disease including hypertension [86]. That compensatory signaling occurs in knockout mice has long been a consideration and a challenge in identifying direct causation in genetic mouse models perhaps reflecting a molecular flow chart whose primary order is sustenance and life [87]. In the current context of clocks, it may be that embryonic Bmal1 disruption can: one, cause *injury* due to the inability to anticipate time appropriately to experimentally-induced circadian stresses; two, (the opposite) cause *protection* due to the ambivalence (via loss of a time sensor) to experimentally-induced circadian stresses; or three, cause injury or protection that is induced by compensatory changes *in utero* that persist through life and are independent of circadian rhythmicity. It is also interesting that the embryo and its clock, though not oscillating, can still be sensitive to circadian disturbances experienced maternally, sensitivities that become more evident with advancing age. In this recent work, a pregnant mother (gestational mouse) exposed to circadian stress produced offspring that developed heart and bone disease at 10 months of age, while the livers of younger 4-week old pups that were so-exposed while *in utero* exhibited a correlative change in epigenetic memory at a CV-related microRNA cluster that was hypermethylated [88].These data demonstrated that pregnant mice exposed to experimental jet lag ("a fly East, lose sleep model") have offspring (aged to 10 months) that exhibit increased cardiac disease as quantified by left ventricular hypertrophy. Despite this peripheral clock defect in the cardiovascular system, these offspring mice born and developing in conditions of prenatal stress were better able to adapt their central clock circadian timing in locomotor rhythms to jet-lag as adults (or advances), while (as expected) "a fly West, gain extra hours" was less hurtful to health and life as has also been shown in the mortality of aged mice [89].

CONCLUSION

Generally speaking, circadian rhythm disruption is likely not good for human or mouse health. That said, the current discussion underscores the complexity of clocks; there are circadian clock effects that appear to occur even independent of oscillation; there are clock perturbations in the fetus that can influence biology and disease into adulthood; a mutated genetic clock can be in certain models of disease protective and in other models of disease injurious. Another possibility is that these complexities reflect some divergence of the mouse models from the human condition. Perhaps subtle lowering of blood pressure in a mouse is not such a significant influence to disease in a mouse, or another possibility is that blood pressure and coincident endothelial dysfunction are disconnected in the mouse models or even disconnected altogether. Many of the disparities are observed in models of mice where the clock is genetically mutated. And, while there are human mutations in clocks that may relate to the mouse models [90], non-genomic dysfunctions of circadian clocks and rhythms that arise in the timing of drug dosing, social jet lag, shift work, and sleep disorders are important to be studied in both the genetic mouse models of mutation and in conditions of sleep deprivation and light cycle alteration that may yield more insight into the complexities of the circadian clock in cardiovascular disease. Given these complexities there are certain things that are well established:

- Circadian clocks oscillate after birth.
- Circadian clocks regulate downstream signals, (whether those target signals oscillate or not).
- Circadian clocks can sense changes in timing.

Given the significance of temporal routines (or lack thereof in human life), the science of clock signaling remains a very important and complicated biology to understand, but one that is certain to provide new clues into cardiovascular health.

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