Circadian Control of Inflammatory Processes in Atherosclerosis and Its Complications

Sabine Steffens, Carla Winter, Maximilian J. Schloss, Andres Hidalgo, Christian Weber, Oliver Soehnlein

Abstract—Physiological cardiovascular functions show daily diurnal variations, which are synchronized by intrinsic molecular clocks and environment-driven cues. The clinical manifestation of cardiovascular disease also exhibits diurnal variation, with an increased incidence in the early morning. This coincides with circadian oscillations of circulating parameters, such as hormones and leukocyte counts. We are just at the beginning of understanding how circadian rhythms of immune functions are related to cardiovascular disease progression and outcome after an acute ischemic event. Here, we briefly summarize clinical data on oscillations of circulating inflammatory parameters, as well as experimental evidences for the role of circadian clocks in atherosclerosis, postmyocardial infarction inflammatory responses, and cardiac healing.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37:1022-1028. DOI: 10.1161/ATVBAHA.117.309374.)**

Key Words: atherosclerosis ■ cardiovascular disease ■ circadian rhythm ■ incidence ■ myocardial infarction

Circadian rhythms refer to endogenous 24-hour cycles driven by internal molecular clocks, which adjust behavior and physiological activities to environmental changes. This involves regulation of sleep–wake cycles, feeding, body temperature, blood pressure, heart rate, hormone secretion, metabolism (including lipid metabolism), and many other biological functions.[1,2] It is estimated that 10% to 20% of all mammalian genes are under circadian transcriptional control.[3,4] But according to murine transcriptome analysis, remarkable organ-specific differences in the number of circadian-regulated genes exist.[5]

A mounting body of evidence suggests that circulating counts of leukocytes involved in atherosclerosis show circadian oscillations.[6] At least some of the regulatory mechanisms underlying diurnal fluctuations of immune cell numbers have been recently identified in experimental mouse studies.[6] Leukocyte numbers oscillate in the murine blood with a peak during the inactive phase,[7] whereas leukocyte counts in tissues, such as bone marrow, skeletal muscle, or the heart, oscillate in antiphase to the blood, showing a peak at the beginning of the active phase.[7,8] Classical monocytes are one of the major immune cell types recruited into atherosclerotic lesions.[9] Their numbers oscillate in blood and tissue via transcriptional changes regulated by the clock transcription factor BMAL1 (brain and muscle ARNT [arylhydrocarbon receptor nuclear translocator]-like).[10] At the molecular level, monocyte-intrinsic BMAL1 was identified as a transcriptional repressor of chemokines involved in monocyte recruitment, such as CCL2 (CC-chemokine ligand-2).[10]

Atherosclerosis is a chronic inflammatory condition, which is initiated by endothelial dysfunction and upregulation of adhesion molecules.[11] This promotes the recruitment of leukocytes to inflamed endothelium and formation of atherosclerotic plaques. It is unknown whether arterial leukocyte recruitment to athero-prone vessels is affected by circadian rhythms and whether circadian expression profiles of endothelial adhesion molecules exist within arteries and are sustained during chronic inflammation in atherosclerosis.

Molecular Regulation of Core and Peripheral Clocks

Circadian rhythms are regulated by the suprachiasmatic nuclei (SCN) of the hypothalamus, as well as temperature, food intake, or behavior.[12] These circadian regulators control and synchronize peripheral clocks to ensure a synchronal rhythm in all existing clocks within an organism.[13] The SCN receive signals from the eye through the retino-hypothalamic tract, thereby, linking the central clock with the environment (Figure). Exposure to light modulates gene expression in adrenal glands and secretion of glucocorticoids driven by the SCN–sympathetic nervous system.[14] Hence, the SCN translates light signals into hormone release or sympathetic innervation to synchronize rhythms in peripheral clocks with the daily rotational cycle of the earth. In addition, individual cells throughout the body also contain autonomous peripheral clocks, which are capable to maintain circadian timing

Received on: February 1, 2017; final version accepted on: April 18, 2017.

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This manuscript was sent to Robert A. Hegele, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

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Nonstandard Abbreviations and Acronyms	
Apoe	gene encoding apolipoprotein E
Arntl	gene encoding clock transcription factor BMAL1
BMAL	brain and muscle ARNT (arylhydrocarbon receptor nuclear translocator)-like
CCL	CC-chemokine ligand
CLOCK	circadian locomotor output cycles kaput
CRY	clock transcriptional repressor cryptochrome
HSPC	hematopoietic progenitors and stem cells
MI	myocardial infarction
PER	clock transcriptional repressor Period
REV-ERB	nuclear receptor reverse-ERB
ROR	receptor-related orphan receptors
SCN	suprachiasmatic nuclei

independent of the central clock.[6] Glucocorticoids have been identified as phase shifters by stimulating core clock transcriptional regulator Period (PER) expression in peripheral tissues because glucocorticoid receptors are only expressed in the periphery, but not the SCN.[15]

All peripheral clocks as well as the central clock are regulated by the core clock proteins BMAL1 and CLOCK (circadian locomotor output cycles kaput), which are expressed in a circadian manner (Figure). The core clock proteins form transcription–translation feedback loops that enable a cell-autonomous oscillation of clock-controlled genes.[16] The first feedback loop consists of BMAL1 and CLOCK. BMAL1 dimerizes with CLOCK in the cytoplasm before being translocated to the nucleus to induce E-box-dependent transcription that triggers the expression of the repressors PER and cryptochrome (CRY) and the nuclear receptors reverse ERB (REV-ERB) and retinoic acid receptor-related orphan receptor (ROR). The second loop consists of CRY and PER, which form a heterocomplex and interfere with the activity of BMAL1 and CLOCK, thereby representing a negative feedback loop. Besides these 2 core clock feedback loops, REV-ERB and retinoic acid ROR form a third loop by binding to ROR response elements within the *Arntl* promoter (encoding BMAL1).[17] ROR functions as a transcriptional activator by controlling the expression of *Arntl*, *Cry*, and *Per*, whereas REV-ERB represents a transcriptional repressor by interfering with ROR.[18] These 3 transcriptional–translational feedback loops form the molecular core clock and regulate their own oscillatory expression. Additionally, core clock proteins are also known to bind to promoters of many other genes, thereby, not only regulating their own expression, but also the rhythmic expression of other proteins, including markers of inflammation.[10,19] For example, BMAL1 recruits the histone complex polycomb repressive complex 2 to the E-box binding site in the promoter of *Ccl2*, thereby, regulating its gene expression under steady state.[10] Similar results were observed for *Ccl8*, *S100a8*, and *Tlr9*.[10,19]

Circadian Control of Human Cardiovascular and Metabolic Physiology

Circadian fluctuations of glucocorticoids, catecholamines, blood viscosity, and platelet reactivity have been well established in humans and correlate with plaque rupture and thrombus formation in the early morning hours.[1,20] Similar fluctuations have been found for heart rate and blood pressure.[21] An increase in heart rate and blood pressure during sleep–awake transition leads to an enhanced cardiac energy and oxygen demand, while coronary blood flow is decreased, bearing the risk of supply–demand mismatch.[1] Concomitantly, the frequency of acute cardiovascular events, such as myocardial infarction (MI), ischemic stroke, and arrhythmias, is much higher in the early morning hours.[1,22–26] A disruption of the circadian rhythm, which occurs in shift workers, increases the risk for an acute cardiovascular event.[27] In addition, human blood metabolites, like glucose, low-density lipoprotein, triglycerides, and the regulating hormone insulin oscillate throughout the course of a day.[28,29] Chronic circadian disruption leads to an increased susceptibility for metabolic disorders because of misalignment of rest/activity phases with metabolic processes.[2]

Moreover, circadian rhythms of white blood cells involved in atherosclerosis pathophysiology, for example, neutrophils, monocytes, and lymphocytes, have been reported in human blood, which all peak around midnight.[30] Circadian oscillations of blood parameters should be taken into account when evaluating blood parameters in a clinical setting. In addition, intervention and application of medication may have a time-dependent effect, which could offer novel approaches for preventing and treating atherosclerosis and its complications.

Circadian Control of Hematopoiesis

As with other processes in the organism, hematopoiesis displays circadian variations that accommodate to organismal demands and environmental inputs.[31] In adult mammals, hematopoiesis mainly occurs in the bone marrow through a series of events that balance self-renewal of hematopoietic progenitors and stem cells (HSPC), proliferation, differentiation, and mobilization of the mature (or immature) cells into the bloodstream.[32] At least 2 of these processes, proliferation and mobilization, are known to display circadian patterns. For example, in humans, the peak of proliferation of total bone marrow cells (measured as DNA synthesis) occurs at noon and troughs at around midnight, with similar daily patterns displayed by myeloid and erythroid progenitors.[33] This proliferative synchrony likely couples changes in other physiological parameters, such as the levels of cytoprotective metabolites or the release of mature cells into the circulation. Notably, these fluctuating patterns have clinical implications, for example, for the time of administration of antiproliferative drugs.[33]

The circadian nature of HSPC mobilization has been best studied in the mouse and compared with that of humans.[34,35] The timing of spontaneous or enforced mobilization adjusts to the active–rest cycles in each species, such that mice mobilize significantly better in the morning and humans in the evening in coincidence with their respective resting phases.[34] Mechanistic studies in the mouse demonstrated that circadian mobilization is regulated by adrenergic stimulation delivered by sympathetic nerves,[36] resulting in disruption of the capacity of stromal components of the hematopoietic niche to retain HSPC. Agonists for both β2 and β3 adrenergic receptors elicit these inhibitory functions by targeting osteoblastic and

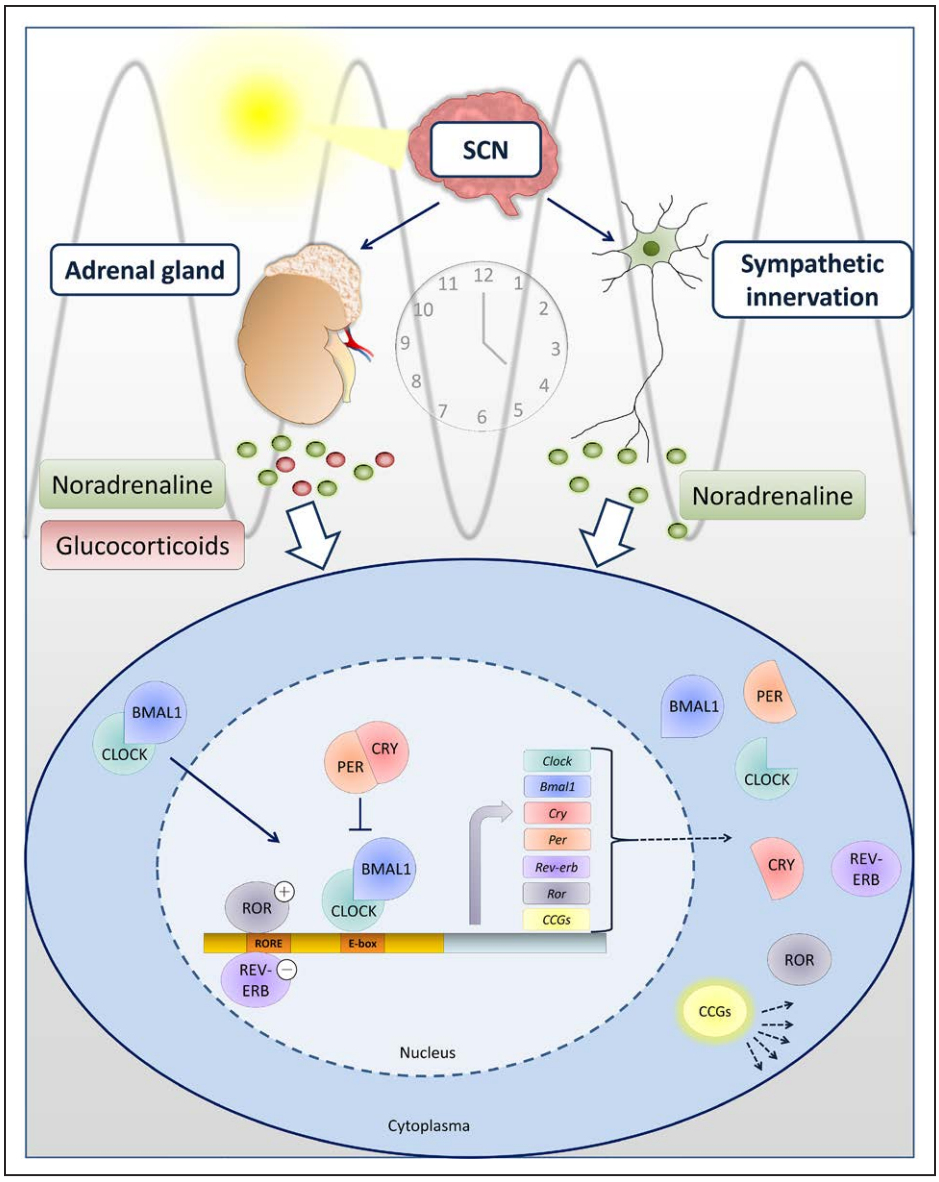

Figure. The circadian clock and its regulatory mechanisms. Light is one main entrainment factor of the core clock that synchronizes intrinsic circadian oscillations within an organism to overlap with the daily rotational cycle of the earth. The suprachiasmatic nuclei (SCN) translate the light signal into hormone release by the adrenal gland and innervation of sympathetic nerves that leads to noradrenaline release. This process regulates peripheral clocks to ensure the same rhythm in each cell within an organism. In cells, 3 transcriptional–translational feedback loops control the circadian oscillatory expression of the main core clock proteins BMAL1 (brain and muscle ARNT [arylhydrocarbon receptor nuclear translocator]-like) and CLOCK (circadian locomotor output cycles kaput). The 2 core clock proteins BMAL1 and CLOCK regulate their own expression and the expression of *Cry*, *Per*, *Rev-erb*, and *Ror* by binding to E-box sequences in the promoter region. CRY and PER interfere with the activity of the heterodimer BMAL1/CLOCK, forming a negative feedback loop. Additionally, the transcriptional inhibitor REV-ERB and the transcriptional activator ROR bind to ROR response elements (ROREs), thereby repressing or activating *Arntl* expression (encoding BMAL1). Because clock proteins induce the expression of clock-controlled genes (CCGs), they are able to induce circadian patterns in many genes. CRY indicates clock transcriptional repressor cryptochrome; PER, clock transcriptional repressor Period; REV-ERB, nuclear receptor reverse-ERB; and ROR, receptor-related orphan receptors.

mesenchymal cells, respectively.[36,37] Interestingly, these β3 adrenergic receptor–dependent circadian signals that regulate hematopoietic niches are also elicited under situations of inflammatory stress or mobilization induced by granulocyte-colony stimulating factor and favor massive release of immune and progenitor cells into peripheral tissues.[37,38] Circadian HSPC trafficking is additionally orchestrated by innate immune cells through periodic inhibition of hematopoietic niches, as shown for neutrophils that clear daily into the bone marrow.[39] This process, which requires uptake of incoming neutrophils by resident bone marrow macrophages and activation of cholesterol-sensing transcription factors (liver X receptors α and β), reveals temporal synchronization of HSPC and their myeloid descendants.[40]

Although these data suggest that circadian regulation of hematopoietic organs occurs primarily through extrinsic mechanisms, that is, via sympathetic or immune control of the stromal niche compartment, there is also evidence for cell-intrinsic, clock-controlled regulation of hematopoiesis. Expression of CXCL12 (CXC chemokine ligand-12), a key

regulator of hematopoietic activity, displays circadian patterns that fade in the absence of the clock gene *Arntl*,[35] and CXCR4 (CXC chemokine receptor-4) expression on murine HSPC is also regulated by BMAL1.[34] These complex pathways, which are regulated in a circadian manner and converge in the bone marrow, may be important to ensure a rapid but tightly controlled supply of blood and immune elements on demand.

Role of Circadian Clocks in Atherosclerosis

Within the last few years, the concept emerged that circadian rhythms might be involved in the pathogenesis of atherosclerosis. A transcriptome analysis in mouse aortas identified around 330 genes expressed with circadian rhythmicity.[41] Human carotid plaque–derived vascular smooth muscle cells exhibit lower amplitudes of mRNA expression levels of core clock genes, such as *Arntl* and *Clock*, compared with normal carotid smooth muscle cells from same donors. It is, therefore, tempting to suggest that a mismatch of circadian gene expression patterns in atherosclerotic vessels with central clocks might play a role in plaque stability.[42] Apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice fed with chow or Western-type diet for 4 weeks exhibit an altered cardiac expression profile of diurnal regulated clock genes compared with wild-type mice.[43] In addition, apoptosis-related genes *c-Myc* and *p53*, which are also expressed in a circadian manner in wild-type hearts, lose their rhythmic expression pattern in *Apoe*-deficient mice. These findings deserve further confirmation with higher sample numbers to validate their relevance. Moreover, it would be interesting to assess potential effects of hypercholesterolemia on circadian expression patterns in atherosclerotic lesion–prone aortas and predilection sites of plaque formation and how this may be linked to plaque stability.

An experimental study addressed the potential causal implication of core clock protein CRY1 in atherosclerosis. Interestingly, blood analysis of patients with coronary artery disease revealed lower *Cry1* mRNA levels compared with healthy controls.[44] Overexpression of CRY1 reduced the expression of inflammatory markers, plasma lipid levels, and plaque development in *Apoe*$^{-/-}$ mice.[44] Conversely, lentiviral-mediated knockdown of REV-ERB in hematopoietic cells resulted in enhanced lesion formation,[45] while pharmacological activation of REV-ERB reduced atherosclerotic plaque development.[46] Overexpression or pharmacological REV-ERB activation also inhibited M1 macrophage polarization in vitro, while inducing the expression of M2 markers.[45,46] However, whether all these effects on atherosclerosis are directly linked to regulation of circadian rhythms remain unclear because circadian cycles were not assessed in these studies.

In this context, recent data comparing the effects of conventional and inducible core clock gene deficiency *Arntl* suggest that BMAL1 might also regulate many targets independent of circadian rhythms.[47] While constitutive lack of *Arntl* resulted in increased atherosclerotic lesions, less plaque area was observed in inducible *Arntl* knockout mice.[47] It should be taken into consideration that in full knockout mice, *Arntl* is already absent during embryogenesis. Expression of core clock genes (including *Arntl*) is detectable in the SCN from embryonic stage E19, but their expression does not exhibit circadian oscillations.[48] Instead, rhythmic expression of core clock genes in the SCN only develops after birth.[48] Thus, BMAL1 might exert important nonclock-related functions during embryogenesis, which may explain the profound effects on many physiological parameters in conventional, but not inducible *Arntl* knockout mice (including possible effects on the immune system, which might affect atherosclerosis).[47] In adult organisms, both conventional and inducible *Arntl* deficiency lead to a blunted circadian rhythm.[47] However, only a minority of genes expressed in a circadian manner are direct targets of core clock transcription factors.[4] Circadian regulation of the majority of genes is tissue specific, and most clusters of diurnally oscillating genes affect relevant organ functions, in particular, the rate-limiting steps of key pathways. Consequently, *Arntl* deficiency likely has many indirect effects on various downstream pathways, which highlights the importance of additional experiments to validate circadian expression patterns of downstream targets. In line with the observation that circadian regulation is highly tissue specific,[4] central and peripheral clocks in tissues or cells might play differential pathophysiological roles in atherosclerosis.[49]

There is indeed evidence for BMAL1-dependent peripheral circadian clocks in immune cells, which regulate the expression of inflammatory markers, such as CCL2.[10] Studies in mouse models of atherosclerosis indicate the importance of the CCL2–CCR2 axis in early lesion development. Genetic deficiency of *Ccl2* or its corresponding receptor *Ccr2* leads to a reduction in lesion development with lower lesional monocyte/macrophage content.[50,51] The implication of the Ccl2–Ccr2 axis in atherogenesis seems to be rather as a result of effects on mobilization and homeostasis of classical monocytes under steady state rather than their recruitment.[52] Moreover, deficiency of circadian clock gene PER2 promotes aortic endothelial dysfunction,[53] which is the initial stage of lesion development. During transition from resting to active phase, endothelium-dependent vasorelaxation was increased in isolated aortic rings of wild-type mice, confirming a circadian regulation of endothelial function.[53] Deficiency of PER2 was associated with decreased production of vasodilator nitric oxide, which might also promote enhanced leukocyte–endothelium adhesion.[54] Of note, dysregulation of nitric oxide synthesis did not occur at the level of endothelial nitric oxide synthase expression, but possibly by an alteration of its enzyme activity.[53] In support of circadian regulation of leukocyte recruitment, leukocytes show diurnal oscillations between blood and tissues under steady state and in response to an acute inflammatory stimulus.[7,10] These oscillations are regulated by rhythmic expression of chemokines and endothelial adhesion molecules, thereby, mediating circadian leukocyte trafficking into tissues.[7,10] In support of a role for circadian leukocyte recruitment in atherosclerosis, myeloid-specific deletion of *Arntl* on *Apoe*$^{-/-}$ background was recently shown to accelerate arterial monocyte recruitment, M1 macrophage polarization, and atherosclerotic plaque development.[49] Further evidence for pathophysiological implications of tissue-specific clocks was provided in an experimental model of transplant arteriosclerosis. While aortic transplants from *Arntl*$^{-/-}$ mice into wild-type mice developed robust arteriosclerosis, the reverse experiment failed to induce pathological signs in wild-type grafts transplanted into *Arntl*$^{-/-}$ mice.[55] Moreover, the core

clock transcriptional regulators CLOCK and BMAL1 have been implicated in the transcriptional regulation of prothrombotic mediators von Willebrand factor, fibrinogen, and PAI-1 (plasminogen activation inhibitor-1), and constitutive *Arntl* deficiency in mice leads to accelerated arterial thrombus formation.[56] This may suggest a causal implication of BMAL1 in the onset of an acute ischemic cardiovascular event.

In addition to in vivo data, ex vivo experiments assessing cell-intrinsic clocks in isolated macrophages from spleen, lymph node, and peritoneum identified that ≈8% of the macrophage transcriptome is under circadian control.[57] These macrophage-intrinsic clocks are not regulated by systemic glucocorticoid levels. In cell lines, cellular endogenous autonomous clocks function independent of the SCN. In a murine smooth muscle cell line, several genes with circadian patterns were identified, including the tissue inhibitor of metalloproteinase 1 and 3, collagen 3a1, transgelin (also known as sm22a), and calponin.[58] This may have pathophysiological relevance because all these genes are related to atherosclerotic plaque stability. Proinflammatory stimuli are able to disrupt circadian rhythms in isolated cells and contribute to disease onset by changing their cellular phenotype and behavior, which highlights the importance of cell-intrinsic clocks.

To conclude, mounting evidence suggests that circadian rhythmicity plays an important role in atherosclerosis by influencing atherosclerotic plaque development. In addition to the central molecular clock, many peripheral clocks exist, which might affect inflammatory processes underlying atherosclerosis. Cell-intrinsic molecular clocks in leukocytes, endothelial cells, macrophages, and smooth muscle cells have been identified in the past and have been linked to inflammatory processes underlying atherosclerotic lesion development. Because previously published studies are mostly based on global clock gene deficiency in atherosclerosis-prone mice, the question remains which role these peripheral clocks play in the pathogenesis of atherosclerosis. Moreover, phenotypes observed in global deficiency models might be, at least in part, a consequence of noncircadian effects during embryogenesis, as discussed earlier. Further in vivo studies based on inducible and cell-specific circadian clock deficiency and possibly in vitro experiments with isolated cells or vessels are, therefore, essential. Of particular clinical relevance will be to better understand how unbalanced circadian rhythms may promote atherosclerosis.

Role of Circadian Clocks in MI Damage

In addition to the increased prevalence of MI in the morning, it has been suggested that the severity of an acute cardiovascular event is affected by the time-of-day of ischemia onset. In support of this hypothesis, time-of-day-dependent differences in plasma levels of creatine kinase used for diagnosis of MI have been reported.[59–61] There is certainly a relatively high variability in such clinical parameters, given that plasma levels of cardiac enzymes are affected by the delay of measurement after MI onset and possibly individual differences in time of rest and activity. To better assess direct causal relationships between circadian rhythmicity and infarct size, mouse models of MI have been used. Durgan et al[62] have first demonstrated that the infarct size after cardiac ischemia/reperfusion is strongly affected by diurnal variations. The authors reported significantly larger infarct size, fibrosis, and adverse remodeling after ischemia onset at the sleep-to-wake transition period. In support of a possible role for circadian clocks in acute MI damage, genetic disruption of clock gene *Per2* leads to a reduced infarct size in mice.[63] The time-of-day of ischemia onset also has major consequences on the cardiac healing response after MI.[8,62] Neutrophils play a crucial role in this process because their production and retention in the bone marrow is time-of-day dependent.[39] Moreover, circulating neutrophils at the beginning of the active phase have higher capacity to migrate into the myocardium because of upregulated CXCR2 expression.[8] It is likely that circadian oscillations of classical monocytes in the blood and tissues[10] also contribute to the time-of-day-dependent consequences of MI outcome. Finally, disruption of circadian rhythms promotes unfavorable healing response after experimental MI in mice.[64]

Conclusions

Mounting evidence suggests a link between circadian rhythms and cardiovascular disease. The time-of-day when an acute cardiovascular event occurs has profound effects on the severity of ischemic tissue injury and the subsequent inflammatory and healing response. Circadian rhythms in vascular and immune cells might contribute to atherosclerotic lesion development. Disturbances of the normal activity and resting phase (eg, by sleep deprivation or shift work) induce a misalignment between physical activity and intrinsic clocks, with adverse effects on cardiovascular parameters, healing responses, and remodeling. Of particular clinical relevance will be, therefore, to better understand how unbalanced circadian rhythms may promote atherosclerosis and outcome after an acute cardiovascular event. To date, available experimental studies investigating the link between atherosclerosis and circadian rhythms are mostly based on full knockout mouse models of molecular clock transcription factor disruption. However, this might also affect the expression of genes that are controlled by these transcription factors in a circadian-independent manner. Further investigations to confirm causal relationships between circadian rhythmicity and the onset and progression of atherosclerosis are, therefore, needed to draw firm conclusions. Moreover, the validation of clinical correlations between the time of day of acute MI onset and clinical outcome in large cohorts would be of interest. A better understanding of the link between circadian rhythms and cardiovascular pathologies might help developing more targeted and personalized therapeutic strategies for cardiovascular disease patients.

Acknowledgments

Servier Medical Art was used for the Graphical Abstract.

Sources of Funding

This work was funded in part by the Deutsche Forschungsgemeinschaft (STE-1053/3-1 and STE-1053/5-1 to S. Steffens; SO876/6-1, SO876/11-1 and SFB1123 TP A6 and B5 to O. Soehnlein, SFB1123 TP A1 to C. Weber and SFB914 TP B8 to O. Soehnlein and C.W.), the German Centre for Cardiovascular Research (DZHK MHA VD1.2 to O. Soehnlein and C. Weber, and doctoral fellowship to M.J. Schloss), the European Research Council (ERC AdG 692511 to C. Weber), the Netherlands Organisation for Scientific Research (VIDI project

91712303 to O. Soehnlein), the FoeFoLe (Foerderung von Forschung und Lehre) program of the Ludwig-Maximilians-University Munich (to S. Steffens and M.J. Schloss); the Ministerio of Economy, Industry and Competitiveness (MINECO) grant SAF2015-65607R, and Fundació La Marató TV3 (120/C/2015-20153032) to A. Hidalgo.

Disclosures

None.

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Highlights

- Circadian rhythms refer to endogenous 24-hour cycles driven by internal molecular clocks.
- Clinical manifestations of cardiovascular disease coincide with circadian oscillations of circulating parameters, such as hormones and blood leukocyte numbers.
- This review summarizes emerging experimental evidence how circadian rhythms of immune functions affect cardiovascular disease and outcome after an acute ischemic event.
- It is of clinical interest to investigate how misalignments between physical activity or environmental factors and intrinsic clocks might affect cardiovascular disease manifestations
- A better knowledge of the link between circadian rhythms and cardiovascular pathologies might help developing more targeted and personalized therapeutic interventions.

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2017;37:1022-1028; originally published online April 27, 2017;
doi: 10.1161/ATVBAHA.117.309374
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636