HHS Public Access

Author manuscript

Chronobiol Int. Author manuscript; available in PMC 2022 September 01.

Published in final edited form as:

Chronobiol Int. 2021 September ; 38(9): 1262–1273. doi:10.1080/07420528.2021.1928160.

Targeting circadian PER2 as therapy in myocardial ischemia and reperfusion injury

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Abstract

The cycle of day and night dominates life on earth. Therefore, almost all living organisms adopted a molecular clock linked to the light-dark cycles. It is now well established that this molecular clock is crucial for human health and wellbeing. Disruption of the molecular clockwork directly results in a myriad of disorders, including cardiovascular diseases. Further, the onset of many cardiovascular diseases such as acute myocardial infarction exhibits a circadian periodicity with worse outcomes in the early morning hours. Based on these observations, the research community became interested in manipulating the molecular clock to treat cardiovascular diseases. In recent years, several exciting discoveries of pharmacological agents or molecular mechanisms targeting the molecular clockwork have paved the way for circadian medicine's arrival in cardiovascular diseases. The current review will outline the most recent circadian therapeutic advances related to the circadian rhythm protein Period2 (PER2) to treat myocardial ischemia and summarize future research in the respective field.

Keywords

Per2; circadian; intense light; amplitude enhancement; myocardial ischemia; anticoagulation; micro-RNA; adenosine; nobiletin; inflammation; SR9009; miR-21

Introduction

In the 1970s, research began to study the circadian system in Drosophila melanogaster. This led to discovering genes such as Period (Per) as critical molecular mechanisms of the

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circadian clockwork (Tei, Okamura et al., 1997). In the early '80s, a multicenter analysis reported a circadian pattern for acute myocardial ischemia (MI), with a higher occurrence of MIs at 9.00h compared to 21.00h (Muller, Stone et al., 1985). These studies indicated that light cycles a critical for the onset of MI. Interestingly, an increase of MI during the winter, where light periods are shorter, is a well-documented phenomenon in the US (Spencer, Goldberg et al., 1998). Regardless, since MI follows a circadian pattern, it has been suggested that circadian rhythm disruption contributes to cardiovascular disease (Staels, 2006). Other studies also reported an increased incidence of unstable angina, sudden death, stroke, ventricular arrhythmias, cardiogenic shock, aortic aneurysm rupture, stent thrombosis, and transient myocardial ischemia in the early morning hours (Braunwald, 2012). Clinical studies have found that myocardial ischemia is even significantly worse in the early morning when compared to other times of the day (Suarez-Barrientos, Lopez-Romero et al., 2011; Ibanez, Suarez-Barrientos et al., 2012; Reiter, Swingen et al., 2012). Since these initial observations have been made, research has significantly contributed to our understanding of the underlying molecular mechanisms and their potential for therapy.

Research from our group has mainly been focused on the circadian protein Period 2 (PER2) in cardioprotection. PER2 is a critical component of the circadian clockwork and is an essential regulator of the circadian amplitude. The importance of PER2 for circadian rhythmicity has been illustrated in mouse studies showing that the precise rhythmicity of PER2 is critical for driving cellular circadian oscillations (Hallows, Ptacek et al., 2013). *Per2*$^{-/-}$ mice have a significantly shorter night period, and *Per2* deficiency is associated with changes in daily locomotor activity and disturbance of the resting period (Nakamura, Takumi et al., 2013). As many of the core clock proteins, PER2 also plays a vital role in metabolic processes. *Per2*$^{-/-}$ mice have altered lipid metabolism, reduced adiposity, and the lack of PER2 affects gene expression related to lipid metabolism (Grimaldi, Bellet et al., 2010). This phenotype has been attributed to the interaction of PER2 with the nuclear receptors PPARγ, PPARα, and REV-ERBα, which regulate cellular metabolism in white adipose tissue and the liver (Schmutz, Ripperger et al., 2010) (Grimaldi, Bellet et al., 2010). Furthermore, PER2 was found to regulate gluconeogenesis and glycogen catabolism by working in concert with nuclear receptors (Panda, Antoch et al., 2002; Schmutz, Ripperger et al., 2010) (Storch, Lipan et al., 2002). Our initial investigations also found a necessity of PER2 in cardioprotection by regulating metabolic pathways. Studies from *in vivo* stable isotope glucose tracers during baseline, myocardial ischemia, or myocardial ischemia and reperfusion injury revealed the inability of *Per2*$^{-/-}$ mice to rely on glycolysis during ischemia. Additionally, *Per2*$^{-/-}$ mice had an increase in the flux of the TCA cycle during ischemia, whereas wildtype mice attenuated TCA cycle flux (Eckle, Hartmann et al., 2012). This article will discuss a variety of PER2 mechanisms for cardioprotection and highlight preclinical therapies targeting these pathways. Our review fully adheres to the standards of Chronobiology International (Portaluppi, Smolensky et al., 2010).

Intense light elicited PER2 in cardioprotection

The appearance of sunlight and oxygen on our planet was without question the most drastic change to our environment (Zerkle, Poulton et al., 2017; Bartman & Eckle, 2019). Therefore almost all organisms on earth have developed so-called light- and oxygen- sensing

mechanisms. It does not come as a surprise that light sensing and oxygen sensing pathways are mechanistically linked (Hogenesch, Gu et al., 1998). The transcription factor hypoxia-inducible factor 1α (HIF1A), which allows the cell to adapt to low oxygen levels (Semenza, 2011) and the light-regulated circadian core protein Period2 [PER2, (Liu, Shen et al., 2012)] are both parts of a PAS-domain containing protein superfamily. The PAS-domain, which was first described in the drosophila proteins PER, ARNT, and SIM, allows both proteins to sense oxygen or light (Hogenesch, Gu et al., 1998; Taylor & Zhulin, 1999). Indeed, cardiac *Hif1a* mRNA cycles in a circadian manner (Eckle, Hartmann et al., 2012), and hypoxia elicited HIF1A alters circadian gene transcription (Adamovich, Ladeuix et al., 2017; Wu, Tang et al., 2017). Through evolution, this relationship between light and oxygen-sensing pathways has been highly conserved. As such, this relationship strongly supports a role for light elicited circadian rhythm proteins as therapeutic targets to treat disease states of low oxygen availability, such as MI.

Our group exposed mice to intense light (10,000 LUX) and demonstrated that intense light therapy significantly reduced troponin I levels and infarct sizes following MI (Eckle, Hartmann et al., 2012). Subsequent analysis found a robust induction of cardiac PER2, where infarct sizes correlated reciprocally with cardiac PER2 levels. Further investigations revealed that *Per2*$^{-/-}$ mice were deficient in generating lactate during myocardial ischemia. The inability to generate lactate was associated with a deficiency to upregulate HIF1A-dependent glycolytic pathways. The importance of glycolysis and lactate production for the ischemic heart is illustrated by experiments inhibiting lactate production. In less than five minutes, the heart undergoes contracture-rigor, which usually occurs after 60-90 minutes of ischemia if anaerobic glycolysis is intact (Jennings & Reimer, 1991). In general, anaerobic glycolysis is a critical mechanism to adapt to ischemia and low oxygen conditions (Jennings & Reimer, 1991; Myrmel, McCully et al., 1994; Lopaschuk & Jaswal, 2010). A switch from a more "energy-efficient" state using fatty acids to a more "oxygen-efficient" usage of glucose is crucial to allow the heart to function under ischemic or hypoxic conditions (Neubauer, 2007; Aragones, Fraisl et al., 2009; Lopaschuk, Ussher et al., 2010; Lopaschuk & Jaswal, 2012). Our studies further demonstrated that light-elicited PER2 induced transcript levels of numerous HIF1A regulated glycolytic enzymes in hearts from wildtype mice. The abolished induction of glycolytic enzymes in *Per2*$^{-/-}$ mice resulted in the depletion of energy storages and increased myocardial cell death during ischemia (Eckle, Hartmann et al., 2012). Together, these findings revealed that intense light stabilizes cardiac PER2, enhancing oxygen-efficient glycolysis and protecting the heart from ischemia (Fig.1). Intense light is an easy and cost-effective therapy, and hopefully, future studies will show its effectiveness in cardiovascular disease such as MI.

Adenosine, a critical regulator of intense light elicited PER2 in cardioprotection

Interestingly, the discovery of light elicited PER2 was initially based on studies investigating adenosine signaling in cardioprotection. Extracellular adenosine signaling is an essential cellular adaptive mechanism (Ohta & Sitkovsky, 2001; Sitkovsky, Lukashev et al., 2004; Thiel, Chouker et al., 2005; Fredholm, 2007). In the extracellular space, adenosine

originates from 5'-adenosine monophosphate (AMP) via ecto-5'-nucleotidase (CD73) mediated phosphohydrolysis. Adenosine then signals through four well-described adenosine receptors, the ADORA1, ADORA2A, ADORA2B, or ADORA3 (Fredholm, 2007; Hasko, Linden et al., 2008). During hypoxia or ischemia, extracellular adenosine production and signaling increase significantly (Eckle, Krahn et al., 2007; Eckle, Grenz et al., 2008). Enhanced activation of adenosine receptors can then mediate endothelial barrier protective or anti-inflammatory effects (Eltzschig, Thompson et al., 2004; Eckle, Faigle et al., 2008; Rosenberger, Schwab et al., 2009). These adenosine signaling effects are ultimately organ protective during ischemia (Lappas, Day et al., 2006; Hart, Much et al., 2008) (Yang, Day et al., 2005; Linden, 2006). On the other side, genetic ablation of ADORA2B signaling abolishes ischemic-preconditioning elicited cardioprotection (Kohler, Eckle et al., 2007; Eckle, Kohler et al., 2008; Eltzschig, 2009). Interestingly, ischemic preconditioning, where short non-lethal ischemic periods precede a more extended ischemic period, has been described as the most potent cardioprotective mechanism discovered at the bench. Regardless, these studies implicate adenosine signaling events as a central mechanism in cardioprotection.

After our group had found a dominant role for ADORA2B signaling in cardioprotection, we performed a microarray study from ischemic-preconditioned murine hearts with genetic ablation of ADORA2B receptors to gain better insight into ADORA2B signaling events. These studies revealed a critical role for the circadian rhythm protein PER2 in mediating adenosine-elicited cardio adaptive responses during ischemia. Interestingly, Period1 (PER1) was the second top gene regulated by ADORA2b signaling in our array. Follow-up studies in *Per1*$^{-/-}$ mice also found larger infarct sizes when compared to wildtype controls which, however, was not significant (Eckle, Hartmann et al., 2012). This observation supports findings that PER1 and PER2 have distinct roles in the mouse clock mechanism (Zheng, Albrecht et al., 2001).

Consistent with previous studies implicating the molecular network of circadian rhythm proteins in the regulation of cellular metabolism (Rudic, McNamara et al., 2004; Turek, Joshu et al., 2005), we observed a critical role of PER2 in mediating a metabolic switch during myocardial ischemia towards more oxygen efficient glycolysis.

Not surprisingly, studies exploring liver metabolism under light-dark cycles and constant darkness identified adenosine as a circulating circadian signaling molecule (Zhang, Kaasik et al., 2006). These studies implicated adenosine signaling as a mechanism for synchronizing the central and the peripheral clock. Indeed, our group found that intense light increased cardiac adenosine levels under normoxia (Fig.1). Further, *Adora2b* deficiency in mice resulted in abolished light-cardioprotection. Therefore, we have proposed that adenosine signals the 'cardioprotective' effect of light from the brain to the heart. However, our studies were restricted to the use of whole-body knockout mice. Future studies in mice with a brain-specific deletion of adenosine signaling will be necessary to fully understand the role of adenosine in peripheral clock synchronization. Regardless, one drug class that can reset the circadian system and induce the circadian protein PER2 are cAMP enhancers (Ripperger & Albrecht, 2012), as cAMP is the core component of PER2 regulation (Wang & Zhou, 2010). Forskolin, an adenylyl cyclase activator, is the prototype of such a drug

(Wang & Zhou, 2010). Besides, our laboratory studies found that the ADORA2B receptor is a potent inducer of cardiac PER2 by enhancing cAMP signaling pathways (Eckle, Hartmann et al., 2012). Moreover, *Per2* deficiency completely abolished the specific ADORAB2 agonist BAY 60-6583 mediated protection from myocardial ischemia (Eckle, Hartmann et al., 2012). Together, these studies highlight the use of ADORA2B agonists to activate circadian mediated cardioprotection (Fig. 2).

Circadian PER2 amplitude enhancement in cardioprotection

In studies on intense light in cardioprotection, we initially used short light exposure times (Eckle, Hartmann et al., 2012). After studies investigating numerous light treatment protocols, we discovered that housing mice under *intense* instead of *ambient* light conditions (14h light:10h darkness, 10,000 LUX, full-spectrum with UV filter) robustly reduced infarct sizes in a PER2 dependent manner. As a mechanism, we found enhancement of the PER2 amplitude (He, Nohara et al., 2016; Wang, van Spyk et al., 2017) which was abolished in blind mice and initiated HIF1A-mediated transcription before an ischemic event [Fig.1, (Bartman, Oyama et al., 2017; Oyama, Bartman et al., 2019)].

Circadian amplitude enhancement has been implicated as a protective mechanism in different settings (Hatori, Vollmers et al., 2012; He, Nohara et al., 2016) and is currently under intense investigation (Gloston, Yoo et al., 2017; Wang, van Spyk et al., 2017; Gile, Scott et al., 2018). Therefore, we performed an unbiased, wide genome array to achieve mechanistic insight, profiling intense light-dependent gene expression changes in the non-ischemic heart. This array uncovered Angiopoietin-like 4 (ANGPTL4) as the gene with the most robust upregulation via light elicited PER2.

ANGPTL4 is upregulated in cardiac endothelial cells via HIF1A (Inoue, Kohro et al., 2014) and maintains vascular integrity during reperfusion injury following MI (Galaup, Gomez et al., 2012). Endothelial disruption is a recognized critical event in MI (Singhal, Symons et al., 2010), leading to functional abnormality, cellular edema, and apoptosis (Sezer, van Royen et al., 2018). Despite this knowledge, clinically applicable strategies to protect the endothelial barrier have not been established yet.

Our studies demonstrated that intense light-elicited cardioprotection or improved endothelial barrier function was abolished entirely in endothelial-specific *Per2*$^{-/-}$ mice (Oyama, Bartman et al., 2019). Subsequent studies found light induction of ANGPTL4 to be PER2 dependent and revealed light elicited increases of HIF1A binding to the promoter region of *Angptl4*. These data indicate that intense light stimulated amplitude amplification of endothelial PER2 boosts vascular integrity via induction of HIF1A-ANGPTL4, which is cardioprotective.

Recent studies found troponin values to peak in patients undergoing aortic valve replacement during the early morning hours. They, therefore, confirmed the diurnal nature of myocardial ischemia (Montaigne, Marechal et al., 2018). While we cannot change the circadian pattern of myocardial injury, the administration of intense light therapy, e.g., before high-risk surgery to enhance the circadian amplitude, might provide robust

cardioprotection through an entire day. Data from intense light elicited circadian amplitude enhancement demonstrated increased cardiac PER2 peak and trough levels associated with cardioprotection during a complete circadian cycle [Fig. 1, (Eckle, Hartmann et al., 2012; Oyama, Bartman et al., 2019)]. Therefore, this strategy could potentially decrease troponin levels in the morning and evening times. Future studies will be necessary to understand the clinical impact of intense light therapy or circadian PER2 amplitude enhancement in cardioprotection.

An anti-inflammatory role for PER2 in heart ischemia

The circadian clock is critical for innate and adaptive immunity (Gibbs, Blaikley et al., 2012; Narasimamurthy, Hatori et al., 2012; Silver, Arjona et al., 2012). This has been well established since the initial discovery that the circadian clock controls Toll-like receptor-9 mediated inflammation. Interestingly, numerous studies have shown that toll-like receptors and innate immunity are essential during myocardial ischemia and reperfusion injury (Eckle & Eltzschig, 2011; Eltzschig & Eckle, 2011; Timmers, Pasterkamp et al., 2012). Also, the circadian clock controls TNFα , which plays a significant role in innate immunity (Petrzilka, Taraborrelli et al., 2009). Together, these findings strongly suggest that the circadian clock impacts the inflammatory response during cardiac ischemia and reperfusion injury. In general, studies on the circadian role in inflammation during myocardial ischemia are rare. In our investigations, a microarray screen discovered that *Per2*$^{-/-}$ mice initiated a proinflammatory program following myocardial ischemia and reperfusion injury (Bonney, Kominsky et al., 2013). Subsequent studies found a strong upregulation of TNFα and IL6 in *Per2*$^{-/-}$ mice during reperfusion injury, confirming the microarray result. Data on PER2 regulating inflammation in conjunction with findings that PER2 controls metabolism favor the idea that metabolism and inflammation are interconnected (Baker, Hayden et al., 2011). Indeed, deficiency of circadian rhythm proteins in mice results in a metabolic syndrome (Turek, Joshu et al., 2005; Staels, 2006; Gomez-Abellan, Hernandez-Morante et al., 2008; Bonney, Hughes et al., 2013). Further, patients with metabolic syndrome have higher inflammatory markers with an increased risk of developing cardiovascular disease (Haffner, 2006). Therefore, understanding the systems linking circadian rhythmicity to cardiac cell metabolism and cardiac cell inflammation could provide novel insights into ischemic heart disease and future circadian-based therapeutic approaches.

The circadian microRNA MiR-21 in cardioprotection

In general, numerous circadian microRNAs are essential players in cardioprotection from ischemia. However, studies on cardiac circadian microRNAs are rare (Noyan, El-Mounayri et al., 2015). Profiling of PER2 dependent microRNAs in cardiac ischemia indicated a critical role for miR-21 (Bartman, Oyama et al., 2017). *In vitro* studies revealed that PER2 dependent miR-21 regulates glycolysis during cellular stress (Eckle, Hartmann et al., 2012). Similar to studies in *Per2*$^{-/-}$ mice, *miR-21*$^{-/-}$ abrogated intense light-elicited cardioprotection (Eckle, Hartmann et al., 2012). Further, we found increased cardiac miR-21 levels in wildtype mice following intense light exposure. As mechanistic studies on light exposure in humans are scarce, we also used intense light in healthy human volunteers. Here, intense light exposure for 30 minutes over five days increased miR-21 or PER2

dependent phosphofructokinase activity in plasma samples (Eckle, Hartmann et al., 2012; Bartman, Oyama et al., 2017). Interestingly analyses of human metabolic changes upon intense light therapy are otherwise not existent. This seems surprising considering that the effectiveness of intense or bright light therapy in humans is well accepted, and intense light therapy is e.g., used to treat seasonal affective disorders (Terman, Terman et al., 1990; Terman, Terman et al., 2001). Besides, light therapy has been shown to reduce delirium (Yang, Choi et al., 2012) or improve human subjects' sleep (Phipps-Nelson, Redman et al., 2003; Eckle, 2015). Regardless, our findings support light as a promising strategy to activate PER2 elicited cardioprotection (Eckle, Hartmann et al., 2012) and to increase the robustness of the circadian system in humans (Wright, McHill et al., 2013; Ritchie, 2015). More detailed studies on intense light therapy in humans will help us to dissect these mechanisms further.

The circadian amplitude enhancer nobiletin as therapy of MI

Based on studies uncovering a crucial role of PER2 in cardioprotection, our group evaluated pharmacological approaches to mimic light-elicited cardioprotection. Recent studies identified several circadian amplitude-enhancing small molecules in a high throughput screen (Chen, Yoo et al., 2012). One of the natural compounds identified was the flavonoid nobiletin, which robustly enhanced the amplitude of PER2 (He, Nohara et al., 2016). In these studies, nobiletin was found to enhance the amplitude of PER2 via Retinoic Acid Receptor-Related Orphan Receptor Alpha (RORα) (He, Nohara et al., 2016). Interestingly, RORα upregulates the above-mentioned PER2-regulated ANGPTL4, which improved cardiac repair and function following myocardial ischemia in recent studies (Cho, Kang et al., 2019).

Since the discovery of nobiletin, animal studies have shown that nobiletin protects from a metabolic syndrome (He, Nohara et al., 2016), from midazolam induced delirium (Gile, Scott et al., 2018), or from ischemia and reperfusion injury (Oyama, Bartman et al., 2018; Dusabimana, Kim et al., 2019; Zhang, Jiang et al., 2019; Güvenç, Cellat et al., 2020). Different mechanisms such as PI3K/AKT, SIRT-1/FOXO3a, or iNOS-eNOS were suggested. While most of these studies did not report the time of day when nobiletin was administered, it is not surprising that all the pathways investigated are PER2 regulated (Yang, He et al., 2012; Bhatwadekar, Yan et al., 2013; Wang, Zhao et al., 2016). Since our group demonstrated that intense light-elicited amplitude enhancement requires PER2, nobiletin mediated circadian amplitude enhancement and cardioprotection would be expected to require PER2. To further test the PER2-specificity of nobiletin, we treated wildtype or *Per2*$^{-/-}$ mice with nobiletin before myocardial ischemia. Here we found that nobiletin significantly reduced infarct sizes which was entirely abolished in *Per2*$^{-/-}$ mice (Oyama, Bartman et al., 2018). These studies revealed for the first time that the circadian amplitude enhancer nobiletin requires PER2 to mediate cardioprotection. Nobiletin could therefore represent a promising cardioprotective therapy in a clinical setting, such as critical care units (Brainard, Gobel et al., 2015b), where circadian disruption of PER2 is expected.

The circadian amplitude enhancer SR9009 in cardiac remodeling

Myocardial ischemia and reperfusion injury increase cardiac inflammation in patients. Inflammation can then increase infarct sizes and ultimately leads to heart failure (Eltzschig & Eckle, 2011; Ibáñez, Heusch et al., 2015). As pointed out earlier, the circadian clockwork is an essential regulator of inflammatory processes. Nevertheless, the circadian role during heart ischemia-induced inflammation is not fully explored yet. An exquisite study by Dr. Martino and her research team recently discovered a critical part of REV-ERB alpha in myocardial ischemia and reperfusion injury (Reitz, Alibhai et al., 2019). REV-ERB alpha is a vital regulator of the circadian clockwork and mediates circadian regulation of innate immunity (Gibbs, Blaikley et al., 2012). REV-ERB alpha agonists were initially developed to treat metabolic disorders and reduced obesity, and improved hyperglycemia in diet-induced obese mice (Solt, Wang et al., 2012). If a REV-ERB alpha-agonist could also treat myocardial ischemia and reperfusion injury associated inflammation was unknown until recently. Dr. Martino's research team demonstrated that short-term targeting of the circadian core regulator REV-ERB alpha with the synthetic agonist SR9009 (Solt, Wang et al., 2012) improved cardiac repair following myocardial ischemia and reperfusion injury in mice. One single administration of SR9009 following myocardial ischemia and reperfusion injury dampened cardiac inflammation via inhibition of the NLRP3 inflammasome and thereby decreased the recruitment of inflammatory cells. The anti-inflammatory effects of SR9009 resulted in less myocardial scar tissue and improved myocardial function, measured by the left ventricle's ejection fraction. Mechanistically, SR9009 targeted the inflammasome in fibroblasts which was the cause of less scar formation.

Interestingly, SR9009 is a potent PER2 amplitude enhancer which is illustrated by studies on synthetic REV-ERB agonists regulating circadian behavior and metabolism (Solt, Wang et al., 2012). Our investigations uncovered that amplitude enhancement of PER2 is cardioprotective and that PER2 exerts anti-inflammatory effects during myocardial ischemia and reperfusion injury (Bonney, Kominsky et al., 2013; Oyama, Bartman et al., 2019). Based on these observations, future studies will have to evaluate the role of PER2 for an anti-inflammatory role of SR9009 in remodeling from myocardial ischemia.

In summary, these studies showed for the first time that REV-ERB alpha is critical for cardiac repair following ischemia and highlight that targeting the circadian clockwork is a powerful strategy to reduce myocardial ischemia and reperfusion injury. As these studies demonstrated improved cardiac function, one can postulate that this therapy would prevent the otherwise natural progression to heart failure following severe MI. Hopefully, this study will stimulate clinicians to initiate clinical trials using REV-ERB alpha agonists to further explore their efficacy and mechanisms in treating ischemia-associated inflammatory responses.

Light as circadian rhythm targeting therapy in humans

As intense or bright light is used as therapy [10,000 LUX] to treat seasonal mood disorders in humans (Yorguner Kupeli, Bulut et al., 2017), we evaluated light therapy's effects on circadian gene expression and associated metabolic pathways. Human healthy human

volunteers were exposed to intense light for 30 min from 8.30h to 9.00h on five consecutive days. Following light therapy, we collected blood samples at 9.00h each day. These studies revealed that intense light therapy increased PER2 protein levels in human buccal or plasma samples in the morning (9.00h) or even 12 h after light treatment in the evening (21.00h). Our data further suggested that intense light enhanced the circadian amplitude in different human tissue samples simultaneously. We also tested the effects of intense light therapy on plasma melatonin levels (Lewy, Wehr et al., 1980). We found that intense light significantly suppressed melatonin levels. In contrast, standard room light could not reduce substantially plasma melatonin levels required for the circadian clock to function.

Subsequent analyses revealed that intense light therapy also decreased plasma triglycerides. Plasma triglycerides have been suggested as a clinical marker for insulin sensitivity and efficient glucose metabolism (Ginsberg, Zhang et al., 2005). Therefore, this finding indicated that intense light might increase insulin sensitivity and thereby glucose metabolism. To get further mechanistic insight, we performed a targeted metabolomics screen from these human plasma samples. Here, we found mainly metabolic pathways such as glycolysis or the TCA cycle affected by intense light therapy (Oyama, Bartman et al., 2019).

As sleep deprivation decreases insulin sensitivity and results in a diabetic phenotype in humans (Depner, Stothard et al., 2014), we next analyzed the impact of bright light therapy on healthy human subjects' sleep behavior. Using actigraphy analyses (Lee & Suen, 2017), we demonstrated that one week of intense light therapy reduced wake-up episodes after sleep onset, resulting in improved sleep efficiency. We further found that intense light therapy increased daily activities. Combined analyses of improved sleep and increased day-activity revealed that intense light increased the circadian amplitude in healthy human subjects. These human studies are in support of our findings in mice. In fact, they suggest that intense light therapy can increase the circadian amplitude and target similar PER2 dependent metabolic pathways in humans as seen in our murine studies. Future translational studies in patients will be necessary to evaluate intense light for the treatment of diseases where low oxygen levels are the culprit, such as myocardial ischemia.

Intense light as anticoagulant therapy in humans

Acute coronary thrombosis can result in nonfatal myocardial infarction (Takada, Saito et al., 2003). This process is well defined in patients with heart failure or with coronary artery disease. Circadian mechanisms regulating thrombosis have been reported but are limited due to the lack of tissue-specific studies (Zheng, Larkin et al., 1999; Tracey, Pan et al., 2012). We recently observed that tissue-specific deletion of PER2 in the megakaryocyte lineage resulted in increased platelet aggregation and increased myocardial damage. Further, we found that intense light therapy inhibited procoagulant pathways and reduced the clot rate in healthy human subjects.

Several studies on how circadian proteins influence coagulation have been published. Unfortunately, most studies have used whole body knockout mouse models to evaluate the circadian clock in hemostasis. A study using whole-body *Per2* knockout mice found

that *Per2*$^{-/-}$ null mice had reduced platelet counts, and platelets were compromised to aggregate (Zhao, Zhang et al., 2011). In another study, it was found that there was a diurnal rhythm in the expression of thrombopoietin in wildtype mice, and *Clock* mutant mice showed disrupted thrombopoietin expression (Tracey, Pan et al., 2012). In contrast to the study using whole body *Per2*$^{-/-}$ mice, however, *Clock* mutant mice showed an increase in thrombopoietin, a significant increase in megakaryocyte numbers and significantly higher platelet counts. Unfortunately, no analysis of the platelet function was performed. Another study using whole-body *Bmal1*−/− mice found enhanced platelet aggregation upon ADP stimulation (Somanath, Podrez et al., 2011). In our studies using mice with a *Per2* deletion in the megakaryocyte lineage, we did not see changes in platelet counts but found increased platelet aggregation.

In general, these contradictory data indicate that different clock proteins control various functions in various tissues. Further, these data show that results from whole-body null mice affecting circadian core clock proteins are challenging to interpret and underscore the need for further research aimed at tissue-specific regulation of circadian mechanisms.

As discussed throughout this review, the importance of light as a regulator of the circadian system has been well described (Bonney, Hughes et al., 2013; Brainard, Gobel et al., 2015a; Brainard, Gobel et al., 2015b; Bartman, Oyama et al., 2017; Oyama, Bartman et al., 2019). Interestingly, studies on platelet turnover found that megakaryopoiesis is regulated by light signals which emerge from the suprachiasmatic nuclei, the master oscillator of circadian rhythms (Hartley, Sheward et al., 2009). Our studies have shown that light increases PER2 levels in peripheral tissues in mice and humans (Oyama, Bartman et al., 2019). Based on these observations, we evaluated intense light as a therapy in healthy human subjects to possibly affect coagulation. Here a protein array revealed that intense light creates an anti-thrombotic signature in plasma samples. Further, the clot rate, which is platelet dependent, was significantly reduced after five days of intense light therapy.

Conclusions

Discovering circadian rhythm mechanisms in myocardial ischemia and identifying novel therapeutic targets has revealed numerous options for future translational studies in humans. Intense light to enhance the circadian amplitude of PER2 and related pathways seems feasible and cost-efficient. However, this therapy might not become a reality in a clinical setting until appropriate high-intensity light sources are available in all hospitals. Pharmacological approaches such as nobiletin, BAY60-6583, or SR9009 to target PER2 seem promising alternatives (Fig. 2). While some data exist on intense light therapy in humans, there are no data on drugs targeting circadian pathways in patients. As evidence emerges, this research will hopefully stimulate clinicians to investigate circadian medicine such as PER2 mediated cardioprotection in a clinical setting. In this endeavor, the time-of-day administration of drugs, targeting PER2 pathways, will be critical to watch.

Source of financial support for the work:

National Heart, Lung, and Blood Institute (NIH-NHLBI) 5R01HL122472 Grant to T.E.; American Heart Association (AHA) Postdoctoral Fellowship 19POST34380105 to YO.

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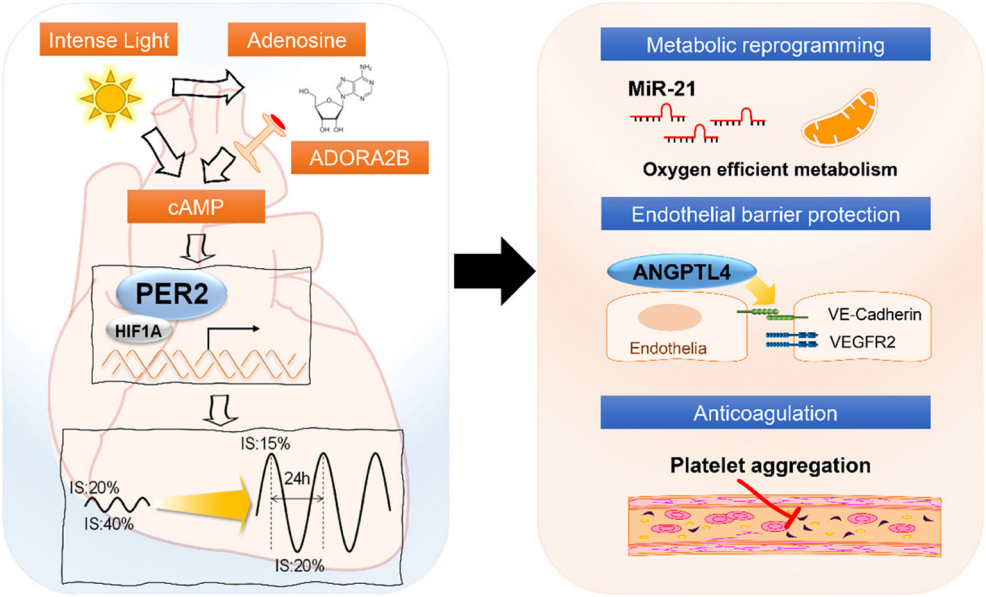

Figure 1. Circadian Mechanisms in myocardial ischemia.

Left panel: intense light or adenosine signaling via the adenosine A2B receptor (ADORA2B) increases cAMP in the heart, resulting in increased PER2 protein levels. Intense light can also increase adenosine and cAMP by itself. PER2 binds to HIF1A and initiates the transcription of cardioprotective genes. Intense light increases the amplitude of PER2, which results in cardioprotection throughout a complete circadian cycle (IS=infarct size). **Right panel**: Amplitude enhancement of PER2 results in improved oxygen utilization via various mechanisms (e.g., MiR-21), improves the endothelial barrier function via ANGPTL4 mediated regulation of tight junctions, and can also inhibit thrombus formation.

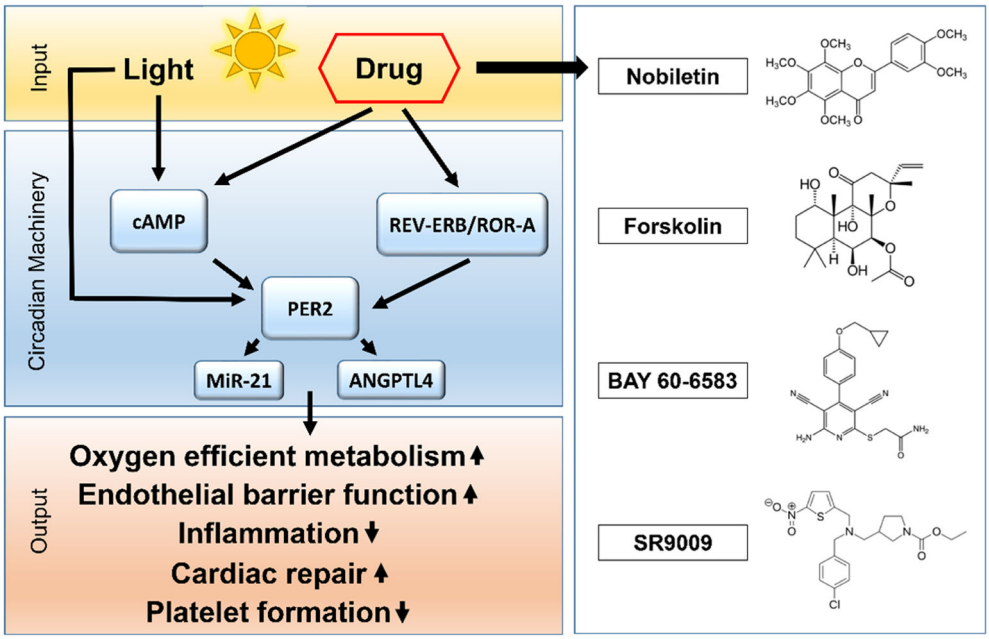

Figure 2. Targeting the circadian clock as therapy in myocardial ischemia and reperfusion injury.

Left panel: Intense light, cAMP, or circadian amplitude enhancers increase PER2, resulting in PER2 elicited cardioprotection. **Right panel**: Nobiletin, a natural flavonoid, enhances the PER2 amplitude via ROR-A. Forskolin activates adenylyl cyclase and increases cAMP. Forskolin is the traditional enhancer of circadian rhythms as cAMP is the core regulator of PER2. BAY 60-6583, a specific adenosine A2B receptor agonist which enhances cAMP, increases PER2 and mimics cardiac ischemic preconditioning. SR9009 is a REV-ERB agonist which decreases obesity by reducing fat mass and improves dyslipidemia and hyperglycemia. SR9009 also increases the amplitude of PER2.