Ischemia/Reperfusion Tolerance Is Time-of-Day–Dependent Mediation by the Cardiomyocyte Circadian Clock

David J. Durgan, Thomas Puliniilkunnal, Carolina Villegas-Montoya, Merissa E. Garvey, Nikolaos G. Frangogiannis, Lloyd H. Michael, Chi-Wing Chow, Jason R.B. Dyck, Martin E. Young

Rationale: Cardiovascular physiology and pathophysiology vary dramatically over the course of the day. For example, myocardial infarction onset occurs with greater incidence during the early morning hours in humans. However, whether myocardial infarction tolerance exhibits a time-of-day dependence is unknown.

Objective: To investigate whether time of day of an ischemic insult influences clinically relevant outcomes in mice.

Methods and Results: Wild-type mice were subjected to ischemia/reperfusion (I/R) (45 minutes of ischemia followed by 1 day or 1 month of reperfusion) at distinct times of the day, using the closed-chest left anterior descending coronary artery occlusion model. Following 1 day of reperfusion, hearts subjected to ischemia at the sleep-to-wake transition (zeitgeber time [ZT]12) resulted in 3.5-fold increases in infarct size compared to hearts subjected to ischemia at the wake-to-sleep transition (ZT0). Following 1 month of reperfusion, prior ischemic event at ZT12 versus ZT0 resulted in significantly greater infarct volume, fibrosis, and adverse remodeling, as well as greater depression of contractile function. Genetic ablation of the cardiomyocyte circadian clock (termed cardiomyocyte-specific circadian clock mutant [CCM] mice) attenuated/abolished time-of-day variations in I/R outcomes observed in wild-type hearts. Investigation of Akt and glycogen synthase kinase-3β in wild-type and CCM hearts identified these kinases as potential mechanistic ties between the cardiomyocyte circadian clock and I/R tolerance.

Conclusions: We expose a profound time-of-day dependence for I/R tolerance, which is mediated by the cardiomyocyte circadian clock. Further understanding of I/R tolerance rhythms will potentially provide novel insight regarding the etiology and treatment of ischemia-induced cardiac dysfunction. (Circ Res. 2010;106:546-550.)

Key Words: chronobiology ■ ischemia/reperfusion ■ myocardium

Numerous aspects of cardiovascular physiology and pathophysiology demonstrate circadian rhythms.1 In humans, heart rate, blood pressure, and cardiac output all increase in the early hours of the morning, as does the onset of adverse cardiac events, such as myocardial infarction.2,3 These rhythms have been attributed primarily to time-of-day oscillations in neurohumoral influences, such as sympathetic or autonomic stimulation.3,4 Although extracardiac factors undoubtedly play critical roles in modulation of cardiovascular function/dysfunction, increasing evidence suggests that intrinsic factors, such as cell-autonomous circadian clocks, likely contribute.1

Circadian clocks are transcriptionally based molecular mechanisms, composed of positive- and negative-feedback loops, with a free-running period of ≈24 hours.5 This mechanism allows the cell to anticipate alterations in environmental stimuli, through time-of-day–dependent modulation of cellular responsiveness to extrinsic factors.5 Circadian clocks have been identified/characterized in multiple cardiovascular-relevant cell types, including cardiomyocytes, vascular smooth muscle cells, and endothelial cells.6–8 Ubiquitous genetic ablation of circadian clock function markedly influences multiple cardiovascular parameters, including heart rate and blood pressure.9 We have recently used a CCM (cardiomyocyte-specific circadian clock mutant) mouse to reveal regulation of myocardial gene expression, β-adrenergic responsiveness, metabolism, heart rate, and cardiac power by this mechanism.10,11

Although circadian rhythms in myocardial infarction onset are well established, time-of-day oscillations in myocardial ischemia/reperfusion (I/R) tolerance have not been reported. Given that the cardiomyocyte circadian clock influences

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myocardial physiology, and that this mechanism becomes rapidly inactivated following an ischemic event, we hypothesized that the cardiomyocyte circadian clock modulates I/R tolerance in a time-of-day–dependent manner.

**Methods**

Wild-type (WT) and CCM mice were housed under controlled conditions in a 12-hour light/12-hour dark cycle (lights on at zeitgeber time [ZT]0, lights off at ZT12). The closed-chest left anterior descending coronary artery occlusion model of I/R was used (45 minutes ischemia); sham animals were subjected to identical surgical procedures, without coronary occlusion. Following 1 day of reperfusion, TTC (2,3,5-triphenyltetrazolium chloride) staining was performed for measurement of infarct size; area at risk was determined by Evan’s Blue staining. Echocardiographic (M-mode) assessment of cardiac function and histological analyses were performed following 1 month of reperfusion. Hearts from a separate set of mice not having undergone surgical intervention were isolated at distinct times of the day for gene and protein expression analyses. An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

Consistent with loss of cardiomyocyte circadian clock function, diurnal oscillations in clock (*bmal1*) (Figure 1A) and clock output (*dbp, e4bp4* [E4 promoter binding-protein 4]) (Figure 1B and 1C) gene expression were significantly attenuated in CCM hearts.

Following 1 day of reperfusion, WT hearts subjected to I/R at ZT12 exhibited an infarct size 3.5-fold greater than at ZT0 (*P*<0.05; Figure 2A; Online Figure I, A). ZT12 infarct size was ∼21%, consistent with previous studies using the murine closed-chest model. Area at risk did not vary between groups (Online Figure I, B). Following identification of the peak and trough in infarct size (ie, ZT12 and ZT0, respectively), these 2 time points were used for long-term studies (ie, 1-month reperfusion).

To investigate whether observed diurnal variations in infarct size following 1 day reperfusion were associated with differences in long-term remodeling, hearts were studied following 1 month of reperfusion. As predicted, infarct volume and fibrosis increased in WT hearts following I/R at both ZT0 and ZT12 (relative to respective shams; Figure 2B and 2C; Online Figure I, A). However, these parameters were significantly greater when the ischemic insult was performed at ZT12 versus ZT0. Similarly, histological analysis revealed increased left ventricular end diastolic diameter (LVEDD) (a marker of adverse remodeling) only for WT hearts subjected to I/R at ZT12 (Figure 3A). LVEDD values were reiterated by echocardiographic assessment of contractile function, whereas all I/R groups exhibited decreased ejection fraction and fractional shortening compared to their sham counterparts, WT hearts that underwent I/R at ZT12 exhibited the greatest depression of contractile function (Figure 3B and 3C).

Time-of-day–dependent oscillations in I/R tolerance in vivo could be secondary to extrinsic (eg, neurohumoral factors) and/or intrinsic (eg, circadian clocks) influences. To investigate the role of the cardiomyocyte circadian clock, CCM mice were used. Consistent with temporal suspension of CCM hearts at the dark-to-light phase transition (ie, ZT0; Figure 1 and Bray et al⁷), infarct size in CCM hearts (following 1 day reperfusion) was

### Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Akt</td>
<td>v-akt murine thymoma viral oncogene</td>
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<tr>
<td>CCM</td>
<td>cardiomyocyte-specific circadian clock mutant</td>
</tr>
<tr>
<td>E4BP4</td>
<td>E4 promoter binding-protein 4</td>
</tr>
<tr>
<td>GSK</td>
<td>glycogen synthase kinase</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
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<tr>
<td>LVEDD</td>
<td>left ventricular end diastolic diameter</td>
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<tr>
<td>P</td>
<td>phosphorylated</td>
</tr>
<tr>
<td>p70S6K</td>
<td>ribosomal protein S6 kinase, 70 kDa</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>ZT</td>
<td>zeitgeber time</td>
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### Figure 1

**A** *bmal1* mRNA. **B** *dbp* mRNA. **C** *e4bp4* mRNA diurnal variations in WT and CCM hearts. Data are shown as means±SEM for 5 to 6 hearts per group.
similar to that observed for WT hearts at ZT0, independent of the time of day (Figure 2A). Absence of time-of-day dependence in infarct size for CCM hearts was accompanied by attenuation/abolition of diurnal variations in infarct volume, fibrosis, remodeling, and contractile dysfunction following 1 month of reperfusion (Figures 2 and 3).

Previously published studies in extracardiac tissues suggest that glycogen synthase kinase (GSK)-3β, whose phosphorylation status oscillates over the course of the day in liver, is an integral circadian clock component. Given the known central roles of Akt (v-akt murine thymoma viral oncogene) and GSK-3β in modulating I/R-induced myocardial damage, we investigated Akt and GSK-3β in WT and CCM hearts. Figure 4A and 4B shows that the phosphorylation of Akt and GSK-3β oscillate in WT hearts...
The purpose of the present study was to determine whether I/R tolerance varies over the course of the day and, if so, to investigate the potential mediation by the cardiomyocyte circadian clock. Here, we expose dramatic time-of-day–dependent variations in I/R tolerance, which are lost following genetic ablation of the cardiomyocyte circadian clock. Initial interrogation of possible molecular links between the cardiomyocyte circadian clock and myocardial I/R tolerance suggest that Akt and/or GSK-3β are potential mediators.

Diurnal variations in the onset of myocardial infarction are well established.3 Humans are at greatest risk for myocardial infarction in the early hours of the morning, because of a combination of increased shear stress, sympathetic tone, and prothrombolytic factors at this time.1,3 However, whether the myocardium exhibits diurnal variations in I/R tolerance has not been reported. The brain exhibits a time-of-day dependence in ischemic tolerance.17 Rats subjected to an ischemic episode at different times of the day exhibit greatest infarct sizes at ZT14, similar to our observations in the mouse heart (greatest infarct size at ZT12; Figure 2). ZT12 corresponds to the sleep-to-wake transition in the nocturnal rodent. As such, diurnal oscillations in the stimulus (ie, ischemia) and responsiveness (ie, infarct development) are in phase.

Circadian dyssynchronization is classically associated with cardiovascular morbidity and mortality. In humans, shift work significantly increases risk for cardiovascular disease development.18 Similarly, subjecting cardiomyopathic hamsters to light/dark cycle manipulations augments early mortality.19 Genetic modulation of circadian clock timing, resulting in subtle circadian dyssynchronization accelerates cardiac and renal disease, which is rescued by light/dark cycle–mediated circadian resynchronization.20 In the present study, genetic ablation of the cardiomyocyte circadian clock results in cardioprotection. The apparent discrepancy is likely attributable to the observation that CCM hearts are temporally suspended at the wake-to-sleep (dark-to-light) phase transition (Figure 1),11 a time of the day at which WT hearts exhibit greatest I/R tolerance. CCM mice are therefore distinct from models wherein circadian dyssynchronization occurs following classic shift work paradigms.

In summary, the present study reveals profound time-of-day dependence in myocardial I/R tolerance, mediated by the cardiomyocyte circadian clock. Rhythms in I/R-induced myocardial damage/dysfunction are in phase with established rhythms of myocardial infarction onset. The implications of

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**Figure 4.** Akt (A) and GSK-3β (B) phosphoprotein diurnal variations in WT and CCM hearts (normalized to Ran-GTPase). Data are shown as means±SEM for 5 to 6 hearts per group. C and D illustrate relationships between P-Akt and P-GSK3β levels with infarct size (TTC assessment).
these observations range from bench (eg, time of day consideration for experiments) to bedside (eg, chronotherapy).

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Disclosures
None.

References

Novelty and Significance

What is Known?
- The onset of a myocardial infarction exhibits a marked time-of-day dependence in humans, peaking in the early hours of the morning (ie, on awakening).
- Factors external to the heart (eg, prothrombotic factors) correlate with time-of-day dependence in human myocardial infarction onset.
- A molecular mechanism known as the circadian clock has been identified and characterized within human and rodent hearts.

What New Information Does This Article Contribute?
- A normal mouse heart exhibits a dramatic time-of-day dependence in I/R tolerance.
- Worst I/R tolerance is observed on awakening for the rodent (ie, poorest I/R tolerance is at the time of day for which myocardial infarction incidence is greatest).
- The circadian clock within the cardiomyocyte mediates time-of-day dependence in cardiac I/R tolerance.

The onset of a myocardial infarction exhibits a marked time-of-day dependence in humans, peaking in the early hours of the morning (ie, on awakening). However, what is less clear is whether the time of day at which an ischemic event occurs influences the severity of the insult. We, therefore, investigated whether a normal mouse heart exhibits a time-of-day dependence in ischemia/reperfusion (I/R) tolerance. We report that a 3.5-fold difference in the extent of damage (ie, infarct size) is observed, depending on the time of day at which the ischemic event occurs. Poorest tolerance to I/R is observed at the sleep-to-wake transition. Disruption of the molecular clock specifically within the cardiomyocytes of mice completely abolishes time-of-day oscillations in I/R tolerance. Our studies therefore clearly show, for the first time, that the heart exhibits a time-of-day dependence in I/R tolerance, which is mediated by the cardiomyocyte circadian clock. Poorest tolerance to an ischemic event is at a time of day at which myocardial infarctions occur with greatest incidence in humans. Modulation of the cardiomyocyte circadian clock may therefore prove to be a prudent candidate to target for improving I/R tolerance.
Short Communication: Ischemia/Reperfusion Tolerance Is Time-of-Day–Dependent: Mediation by the Cardiomyocyte Circadian Clock

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Supplemental Methods

**Animals.** Male WT and CCM (FVB/N background) littermate mice were housed under controlled conditions in a 12-hr light/12-hr dark cycle at the Children’s Nutrition Research Center (Baylor College of Medicine), and were provided standard rodent chow and water *ad libitum*. All studies were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

**Closed-chest ischemia/reperfusion procedure.** In an attempt to avoid confounding influences of excessive immune response associated with the classic open-chest model of I/R, we utilized the previously described closed-chest model. At 11-13 weeks of age, male WT and CCM mice underwent surgical implantation of an occluding device around the left anterior descending coronary artery. Ten minutes prior to the initiation of surgery, mice were administered Buprenorphine (1mg/Kg; IP). Mice were anesthetized using isoflurane (5% at 2L/min oxygen). Mice were shaved from below the chin to the bottom of the sternum and placed in a supine position with paws taped to a heated electrocardiogram (ECG) board. Mice were ventilated throughout the procedure with isoflurane (1%; stroke volume of 250μl at a rate of 175 strokes/minute). Starting just below the clavicle, an incision was made along the left side of the sternum, 3 ribs in length. The incision was then held open using a retractor, and the pericardium was gently dissected. A Prolene 8-0 suture was passed under the LAD ~1 mm from the tip of the left auricle. The ends of the suture were thread through a 0.5mm length PE-10 tubing (occluding device) before being exteriorized through each side of the chest wall and tucked under the skin. The chest was then closed using Prolene 6-0 suture (3 individual stitches). Cefazolin was applied topically to the chest wall incision before closing the skin using Prolene 6-0 suture (running stitch). Mice were allowed to recover for 8-10 days.

On the day of the experiment, mice pre-implanted with the occluding device were anesthetized using isoflurane (5% at 2L/min oxygen) and secured to a heated ECG board. The skin incision was opened and the ends of the suture were taped to lead weights placed on either side of the animal. The lead weights were moved away from the animal until S-T segment elevation was visible on the ECG, indicating successful occlusion of the LAD. Sham animals underwent identical procedures without occlusion of the LAD. The incision area was moistened with Cefazolin and covered with gauze. After 45 minutes of occlusion, the sutures were cut near the chest wall and the skin was re-closed using Prolene 6-0 suture. Mice were allowed to recover for either 24 hours (infarct size measurement by TTC stain) or one month (histological analysis and echocardiography).

**Assessment of infarct size by TTC staining.** Following 24 hours of reperfusion mice were anesthetized with pentobarbital (40mg/Kg; IP) and an incision made along the midline of the neck, exposing the right jugular vein. Using a 30 gauge needle 1-2ml of cold cardioplegic solution was infused into the heart via the right jugular, until the heart stopped. Immediately after the heart stopped beating it was removed and placed in a beaker of cold cardioplegic solution. The heart was then moved to gauze covering crushed cardioplegic ice, keeping the heart cold and moist. Next the aorta was cannulated using a 23 gauge blunt-end needle and secured loosely with 7-0 silk suture. Blood was then flushed from the heart and coronaries with ~3ml of cold cardioplegic solution. Using Prolene 8-0 suture the LAD was tied off at the original site of snare implantation. A 1% Evan’s Blue solution was infused through the aortic cannulae to verify occlusion of the LAD. Excess Evan’s Blue solution was flushed out with ~2ml of cardioplegic solution. Next the heart was filled with melted 1% agarose and placed on crushed cardioplegic ice in the freezer for 15 minutes. The heart was then cut into 1 mm sections starting just above the
LAD suture, yielding 4-5 sections per heart. Sections were then sandwiched between filter paper and incubated in 1% TTC solution for 15 minutes. Sections were immediately transferred to Z-fix aqueous zinc buffered formalin and stored at 4°C. Infarct size was assessed using ImagePro software.

**Perfusion fixation.** Following one month of reperfusion, mice were anesthetized with pentobarbital (40mg/Kg; IP), and an incision made along the midline of the neck, exposing the right jugular vein. Using a 30 gauge needle 1-2ml of cold cardioplegic solution was infused into the heart via the right jugular, until the heart stopped. Immediately after the heart stopped beating it was removed and placed in a beaker of cold cardioplegic solution. The heart was then moved to gauze covering crushed cardioplegic ice, keeping the heart cold and moist. The aorta was then cannulated using a 23 gauge blunt-end needle and secured loosely with 7-0 silk suture. Blood was then flushed from the heart and coronaries with ~3ml of cold cardioplegic solution. The blunt-end needle was then passed through the semilunar valve into the left ventricle. Next, a small hole was cut in the left atrium, allowing for insertion of a 16cm length piece of PE-50 tubing. The tubing was passed through the bicuspid valve and into the left ventricle. The heart was next submerged in Z-fix aqueous buffered zinc formalin and perfused for 10 minutes, at a constant pressure of ~16cm H2O.

**Semi-quantitative histology and morphometry.** Following perfusion fixation, the entire heart was cross-sectioned from base to apex at 250μm intervals. The first ten 5μm sections were obtained for each 250μm interval. H&E staining was performed in order to assess infarct volume as well as LV dimensions, using ImagePro software. Picosirius Red staining of collagen fibers was used to assess fibrosis in the peri-infarct region, using ImageJ software.

**Echocardiography.** Using a Vevo 770 ultrasound machine equipped with a 30 MHz transducer (Visualsonics,Toronto, Canada), in vivo cardiac function and morphology were assessed at the Baylor College of Medicine Mouse Phenotyping Core. Mice were anesthetized in an induction chamber using 2.5% isoflurane and then transferred to a heated ECG platform for heart rate monitoring during the imaging procedure. The body temperature was monitored via rectal probe and was maintained at 37°C. While imaging, anesthesia was maintained via a nosecone with 1% isoflurane. Standard B-mode (2D) and M-mode images were taken in the short axis position for each animal. Using this method, ejection fraction should be considered as an estimate.

**Quantitative RT-PCR.** Gene expression analysis was performed by quantitative RT-PCR. Gene-specific Taqman assays were designed from mouse sequences available in GenBank. Primer and probe sequences have been published previously. Standard RNA was made for all assays by the T7 polymerase method (Ambion, Austin, Texas), using total RNA isolated from mouse hearts; the use of standard RNA allows absolute quantification of gene expression. The correlation between the Ct (the number of PCR cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over at least a 5-log range of RNA for all assays (data not shown). Quantitative RT-PCR data are represented as mRNA molecules per ng total RNA.

**Western blotting.** Akt, P-Akt, GSK-3β, P-GSK-3β, p70S6K, P-p70S6K, and RAN-GTPase proteins were detected in heart homogenates, using standard Western Blotting techniques. Myocardial proteins were separated by electrophoresis on a 10% gel, transferred to PVDF membranes, and probed with specific antibodies. Detection was achieved through secondary probing, followed by enhanced chemiluminescence.
Statistical analysis. Two- and three-way analysis of variance (ANOVA) was conducted to investigate the main effects of group (e.g. genotype, surgery) and time, using Stata version 8.0 (Stata Corp., San Antonio, TX). A full model including second-order interactions was conducted for each experiment. Significant differences were determined using Type III sums of squares. The null hypothesis of no model effects was rejected at $p < 0.05$. Bonferroni post-hoc analyses were performed for pair-wise comparisons.
Online Figure Legends

**Online Figure I.** Representative images for TTC staining and H&E staining (Panel A). Arrows delineate the edges and middle of the infarct region for H&E stained hearts. Area-at-risk for WT versus CCM hearts, over the course of the day (Panel B). Data are shown as means ± SEM for 6 hearts per group.

**Online Figure II.** AKT (A), GSK-3β (B), and Ran-GTPase (C) total protein levels in WT and CCM hearts. Protein levels (A-B) are normalized to Ran-GTPase. Data are shown as means ± SEM for 4-6 hearts per group.

**Online Figure III.** GSK-3β mRNA expression levels in WT and CCM hearts. Data are shown as means ± SEM for 5-6 hearts per group.

**Online Figure IV.** p70S6K phosphoprotein levels in WT and CCM hearts (A). Protein levels are normalized to Ran-GTPase. Panel B illustrates the relationship between P-p70S6K levels and infarct size (TCC assessment). Panel C shows total p70S6K levels in WT and CCM hearts. Data are shown as means ± SEM for 4-6 hearts per group.
Online Figure I A-B
Online Figure II A-C
Online Figure III

Zeitgeber Time (hours)

gsk-3β mRNA per ng Total mRNA

WT

CCM
p<0.05 for genotype main effect

Zeitgeber Time (hours)

P-p70S6K/Ran-GTPase (fold change from trough)

A)

B)

R2 = 0.1585

Infarct wt./AAR wt. (g)

P-p70S6K/Ran-GTPase

C)

Total p70S6K/Ran-GTPase (fold change from trough)

Zeitgeber Time (hours)