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Original Article

CLOCK-BMAL1 regulates circadian oscillation of ventricular arrhythmias in failing hearts through β1 adrenergic receptor

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Abstract: The incidence of ventricular arrhythmias (VAs) in chronic heart failure (CHF) exhibits a notable circadian rhythm, for which the underlying mechanism has not yet been well defined. Thus, we aimed to investigate the role of cardiac core circadian genes on circadian VAs in CHF. First, a guinea pig CHF model was created by transaortic constriction. Circadian oscillation of core clock genes was evaluated by RT-PCR and was found to be unaltered in CHF ($P > 0.05$). Using programmed electrical stimulation in Langendorff-perfused failing hearts, we discovered that the CHF group exhibited increased VAs with greater incidence at CT3 compared to CT15 upon isoproterenol (ISO) stimulation. Circadian VAs was blunted by a β1-AR-selective blocker rather than a β2-AR-selective blocker. Circadian oscillation of β1-AR was retained in CHF ($P > 0.05$) and a 4-h phase delay between β1-AR and CLOCK-BMAL1 was recorded. Therefore, when CLOCK-BMAL1 was overexpressed using adenovirus infection, an induced overexpression of β1-AR also ensued, which resulted in prolonged action potential duration (APD) and enhanced arrhythmic response to ISO stimulation in cardiomyocytes ($P < 0.05$). Finally, chromatin immunoprecipitation and luciferase assays confirmed that CLOCK-BMAL1 binds to the enhancer of β1-AR gene and upregulates β1-AR expression. Therefore, in this study, we discovered that CLOCK-BMAL1 regulates the expression of β1-AR on a transcriptional level and subsequently modulates circadian VAs in CHF.

Keywords: Arrhythmia, chronic heart failure, β1 adrenergic receptor, circadian clock

Introduction

Sudden cardiac death (SCD) related to ventricular arrhythmias (VAs) is a major cause of mortality in chronic heart failure (CHF) patients [1, 2] and remains an unsolved problem worldwide. The incidence of SCD exhibits diurnal variation [3-10]. Its occurrence increases sharply in the morning and is regulated by the biological clock system.

Light stimulus is relayed through eyes to suprachiasmatic nucleus (central biological clock system, SCN), which subsequently synchronizes periphery biological clock system to external environment by the neuroendocrine system [11]. Brain and muscle Arnt-like protein-1 (BMAL1) is a critical transcription factor in the modulation of circadian rhythms [11-14]. BMAL1 forms heterodimers with circadian locomotor output cycles kaput (CLOCK), a transcription factor that activates E-box enhancers in the promoter region of circadian and circadian-controlled genes, including cryptochrome (CRY), period (PER), nuclear receptor subfamily 1, and nuclear receptor group D member 1/2 (REV-ERBα/β; NR1D1/2). As a result, PER and CRY reenter nucleus and repress CLOCK/BMAL1-mediated transcription. Another feedback loop comes into play when REV-ERBα/β represses BMAL1 transcription. These mechanisms regulate the transcription of clock-controlled genes, which play crucial roles in circadian cardiovascular physiology [10, 15].

The molecular mechanism for the morning peaks of VAs in CHF involves a morning spike of sympathetic activities [3, 5, 7, 14, 16], which

are regulated by the central circadian system. High levels of sympathetic activation lead to VAs when L-type Ca^{2+} channels are reactivated during early after-depolarization or when Ca^{2+} ions are spontaneously released by the sarcoplasmic reticulum during delayed after-depolarization [17-19]. Recent studies have suggested several reasons for the cardiac circadian system's contributions to the modulation of arrhythmogenesis. First, the QT interval [20, 21], ventricular effective refractory period [22], and the expression of ion channels [23-27] all exhibit diurnal variation. Second, cardiomyocyte-specific BMAL1 knockout (CBK) mice and cardiomyocyte clock mutant (CCM) mice both exhibit loss of rhythmic repolarizing ion channels or ionic currents [7, 23, 24, 26]. More importantly, sinus bradycardia has been recorded in CCM mice [28, 29], while CBK mice experienced a decreased heart rate, prolonged QRS duration, and increased episodes of VAs [24].

Previous studies have indicated the important role of cardiac circadian system on arrhythmogenesis in normal or transgenic animals [24, 28, 29]. However, they did not report the role of cardiac circadian genes on circadian VAs in CHF, particularly during sympathetic activation. It is generally recognized that the adverse effects of sympathetic activation on the heart are mediated by the adrenergic receptors (ARs). Moreover, extensive research has indicated that various subtypes, especially β1 and β2 ARs, are involved in the pathophysiology of CHF differently [30, 31]. Thus, we sought to investigate the role of cardiac circadian system on circadian VAs in CHF. As a result, we show that CLOCK-BMAL1 mediates circadian occurrence of VAs in failing hearts via β1-AR.

Materials and methods

Development of guinea pig CHF model and animal preparation

All experimental protocols were complied with the ethical standards outlined in the 1964 Declaration of Helsinki and its later amendments. This study was approved by the Nanjing Medical University Institutional Animal Care and Use Committee. Transaortic constriction (TAC) was carried out as described previously to create the CHF model of guinea pig [32, 33]. Male guinea pigs (250-280 g) were injected with atropine (0.1 mg/kg), and then were under anaesthetic using pentobarbital (30 mg/kg). After exposing the aorta along the third intercostal space, a nylon thread was used to constrict the vessel, and a needle was strapped to the aorta as a space holder. The needle was pulled out gently before suturing the incision. The control group (CON) underwent the same operation without aortic constriction.

A 12-12 h light-dark cycle for two weeks was performed to reset circadian rhythm of guinea pigs. Circadian Time (CT) was used because of the artificial light-dark cycle. After a two-week period, guinea pigs were maintained in constant darkness for 36 h (starting at the end of the light phase at CT12). Guinea pigs were then euthanized by stunning-induced areflexic coma in the dark (dim red light). Left ventricular (LV) tissues were collected every 4 h for 24 h.

Western blotting

Total protein were extracted with ice-cold lysis buffer, and the bicinchoninic acid method (Pierce, Rockford, IL) was used to measure protein concentrations. The immunoblotting procedure was performed as described previously [34] and specific primary antibodies for CLOCK (1:200, Abcam), BMAL1 (1:200, Abcam), β1-AR (1:1000, Abcam), β2-AR (1:1000, Abcam), and tubulin (1:1000, Cell Signaling Technology) were used. The protein bands were visualized with the ECL system (Bio-Rad Laboratories, Hercules, CA), and the Image J software (NIH) was used for the quantification of the bands.

Real-time PCR

Total RNA was obtained using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and transcribed to cDNA using an RT kit (Takara Biotech). The expression of CLOCK, BMAL1, PER1, NR1D2, ADRB1, and ADRB2 were analyzed by real-time quantitative polymerase chain reaction (RT-PCR) using SYBR green dye on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). β-actin was used as a reference for internal control. The primer sequences are summarized in Table S2. Relative gene expression was determined by the $2^{-\Delta\Delta ct}$ method.

Cardiomyocyte isolation, cell culture, and adenovirus infection

A Langendorff perfusion system was used to isolate cardiomyocytes of guinea pig as de-

scribed previously [32]. In brief, heart was isolated from anesthetized Guinea pig and transferred to the Langendorff system. The heart was perfused with Tyrode's solution (in mmol/L): NaCl 143, KCl 5.4, NaH_2PO_4 0.25, HEPES 5.0, $MgCl_2$ 0.5, $CaCl_2$ 1.8, and D-glucose 5.6 (pH 7.4 adjusted with 10 M NaOH) for 2 min, and then was perfused with Ca^{2+}-free Tyrode's solution for 5 min. After that, the perfusion medium was changed to Ca^{2+}-free Tyrode's solution containing 1% bovine serum albumin and 0.3 mg/mL collagenase type II. When the heart was softened, cells were isolated by trituration and stored in KB solution (in mmol/L): KOH 85, KCl 30, KH_2PO_4 30, $MgSO_4$ 3, HEPES 10, EGTA 0.5, taurine 20, glucose 10, and L-glutamic acid 50 (pH 7.35 with KOH) at room temperature for 60 min. Myocytes were allowed to settle by gravity. The supernatant was then changed to KB solution containing higher concentration of Ca^{2+} (successively 300, 600, 900, and 1800 μmol/L). Finally, cardiomyocytes were resuspended in culture medium (Hyclone M199+Earle's salts and L-glutamine) containing 10% fetal bovine serum, and incubated at 37°C in a CO_2 incubator for 2 h, and then medium was replaced by M199 without serum.

The recombinant adenoviruses Ad CMV-CLOCK-IRES-RFP, Ad CMV-BMAL1-IRES-GFP, and Ad CMV-ShBMAL1-IRES-GFP were bought from Hanbio (Shanghai, China). The adenoviruses Ad CMV-IRES-GFP and Ad CMV-IRES-RFP were used as controls. The nucleotide sequence for siRNA was 5'-CCACCAACCCAUATACAGAAGCAAA-3' and scrambled siRNA was used for control experiments. Cardiomyocytes were infected with the virus at a multiplicity of infection of 30 (Figure S2). Infected cells were serum shocked (50%) for 2 h. All experiments on cultured cells were conducted after 48 h in culture.

Determination of arrhythmias by field stimulation in single ventricular myocytes

Cells were field-stimulated at 1 Hz and incubated with Tyrode's solution containing ISO and oxygen at 36°C for 5 min as described previously with minor modifications [35]. Extra contractions represented arrhythmic activity of myocytes and the percentage of arrhythmic cells was measured from a field of myocytes (1-8 cells) at the end of the 5-min superfusion. In a separate set of experiments, action potentials (AP) were recorded from single myocytes stimulated at 1 Hz and incubated with Tyrode's solution containing 100 nM ISO, where the electrode solution contained 150 mmol/L KCl and none of BAPTA. Early afterdepolarizations and delayed afterdepolarizations were recorded, which lead to extra systoles.

Cell electrophysiological recordings

Whole-cell patch-clamp currents of myocytes were measured by an Axopatch 200B (Axon, USA) amplifier. AP were evoked with whole-cell current-clamp mode by suprathreshold current pulse of 5 ms at a frequency of 1 Hz. AP was generated by the same amplifier and action potential duration (APD) was measured at 90% repolarization (APD90) and 50% repolarization (APD50). Pipettes resistance is about 2-4 MΩ.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were carried out using a Magna-ChIP G Tissue kit (17-20000, Millipore, USA). The cardiac tissues were embedded by OCT (Sakura, Japan) and sectioned at 300 μm. Samples 1 mm in thickness were collected into a labeled microfuge tube using a 1-mm microdissection punch. The samples were then superfused by formaldehyde (Sigma-Aldrich, St. Louis, MO, USA), and then were neutralized with glycine. The supernatant was then discarded, and the samples were collected in PBS and treated using ultrasonic lysis. The chromatins and magnetic beads were then immunoprecipitated with anti-BMAL1 (Abcam, USA) or normal rabbit IgG (Abcam) antibodies. The complexes were then washed stepwise with low salt buffer, high salt buffer, LiCl buffer and TE buffer. The complexes were eluted and incubated at 62°C for 2 h with shaking followed by 95°C for 10 min. DNA were recovered by spin columns. For ChIP samples, Real-time PCR was performed in an Agilent system (Agilent, USA) using SYBR green (Qiagen, German). The primer sequences are listed in Table S1. The data were analyzed using the fold enrichment method.

Luciferase assays

ADRB1 promoter was amplified from guinea pig genomic DNA and subcloned into the pGL3 basic luciferase reporter plasmid using XhoI

and MluI sites. Expression vectors of CLOCK and BMAL1 (500 ng) were co-transfected with ADRB1 luciferase reporter plasmids (100 ng) and control Renilla plasmid (10 ng) into HEK293 cells. Transfected cells were serum shocked (20%) for 2 h and incubated in 2% medium for 48 h before harvesting. Data were presented as relative luciferase activity calculated as the ratio of the luciferase activity to the activity of Renella.

Langendorff preparation, ECG recordings, and programmed electrical stimulation

Hearts were harvested from anaesthetized guinea pigs and loaded on the Langendorff system, and then were perfused with Tyrode's solution. Electrocardiogram (ECG) was recorded using an Animal BioAmp amplifier (Lab/8 s, AD Instruments). Programmed electrical stimulation (PES) was used to investigate the incidence of VAs. Heart preparations were stimulated with 2-ms rectangular pulses and a bipolar electrode at the LV apex was used to double the threshold. Continuous pacing started with a cycle length of 150 ms (S1-S1 interval) and a premature extrastimulus (S2) was delivered after every eight beats, while the interval (S1-S2) was progressively shortened by 5 ms down to the effective refractory period. The PES protocol was invoked until ventricular fibrillation [36] was induced or until the protocol was exhausted.

Statistical analysis

All data were expressed as the mean ± SEM. For the determination of rhythmic gene expression, data were analyzed using JTK_CYCLE analysis, where an established wave-fitting algorithm was used to analyze cosinar rhythms in RT-PCR for pertinent genes utilizing the R statistical package (version 2.12.1) [37]. A two-way ANOVA was used to determine the significant effects of two factors simultaneously. If a significant interaction was detected, Dunnett's post hoc comparison was performed. The statistical analysis was conducted using one-way ANOVA for multiple group comparisons. Frequency of ISO-induced arrhythmic myocytes and PES-induced arrhythmia was compared using chi-squared test. All analyses were performed using the SPSS software (Version 20.0, SPSS Inc., Chicago, IL, USA). The reported *P*-value was two-sided and a value < 0.05 was considered statistically significant.

Results

Circadian clock gene oscillations were maintained in CHF

To examine the role of cardiac circadian gene rhythms in the pathophysiology of cardiovascular disease, a guinea pig model of CHF was created by transaortic constriction. The animals successfully developed CHF (Table S1) as previously described [32, 33]. A circadian time course collection was then carried out with CON and CHF guinea pigs. Hearts were collected every 4 h over a 24-h period, RNA was extracted, and expressions of BMAL1, CLOCK, NR1D2, PER1, and DBP were measured by real-time PCR.

BMAL1 ($P < 0.05$; Period = 24, Phase = 16, Amplitude = 1.34), CLOCK ($P < 0.05$; Period = 24, Phase = 18, Amplitude = 0.29), NR1D2 ($P < 0.05$; Period = 24, Phase = 2, Amplitude = 0.33), PER1 ($P < 0.05$; Period = 24, Phase = 4, Amplitude = 0.24), and DBP ($P < 0.05$; Period = 24, Phase = 4, Amplitude = 0.25) all exhibited significant circadian rhythms. Circadian oscillations of these core clock genes (BMAL1: Period = 24, Phase = 16, Amplitude = 1.53; CLOCK: Period = 24, Phase = 18, Amplitude = 0.34; NR1D2: Period = 24, Phase = 2, Amplitude = 0.19; PER1: Period = 24, Phase = 4, Amplitude = 0.14; DBP: Period = 24, Phase = 4, Amplitude = 0.21) did not change significantly in CHF ($P > 0.05$; **Figure 1**). These results suggested that clock gene circadian oscillations were maintained in CHF, which might explain why VAs in CHF exhibit a circadian rhythm.

VAs exhibited circadian rhythm in failing hearts and circadian arrhythmic response to ISO stimulation was mediated by β1-AR

To investigate whether circadian oscillations of arrhythmic response to ISO stimulation in failing hearts occur and which β-AR mediates circadian VAs in CHF, ECGs of Langendorff-perfused hearts with ISO (β-AR nonselective agonist; concentration, 100 nmol), ISO + CGP-20712A (β1-AR selective antagonist, CGP; concentration, 300 nmol), and ISO + ICI118551 (β2-AR selective antagonist, ICI; concentration,

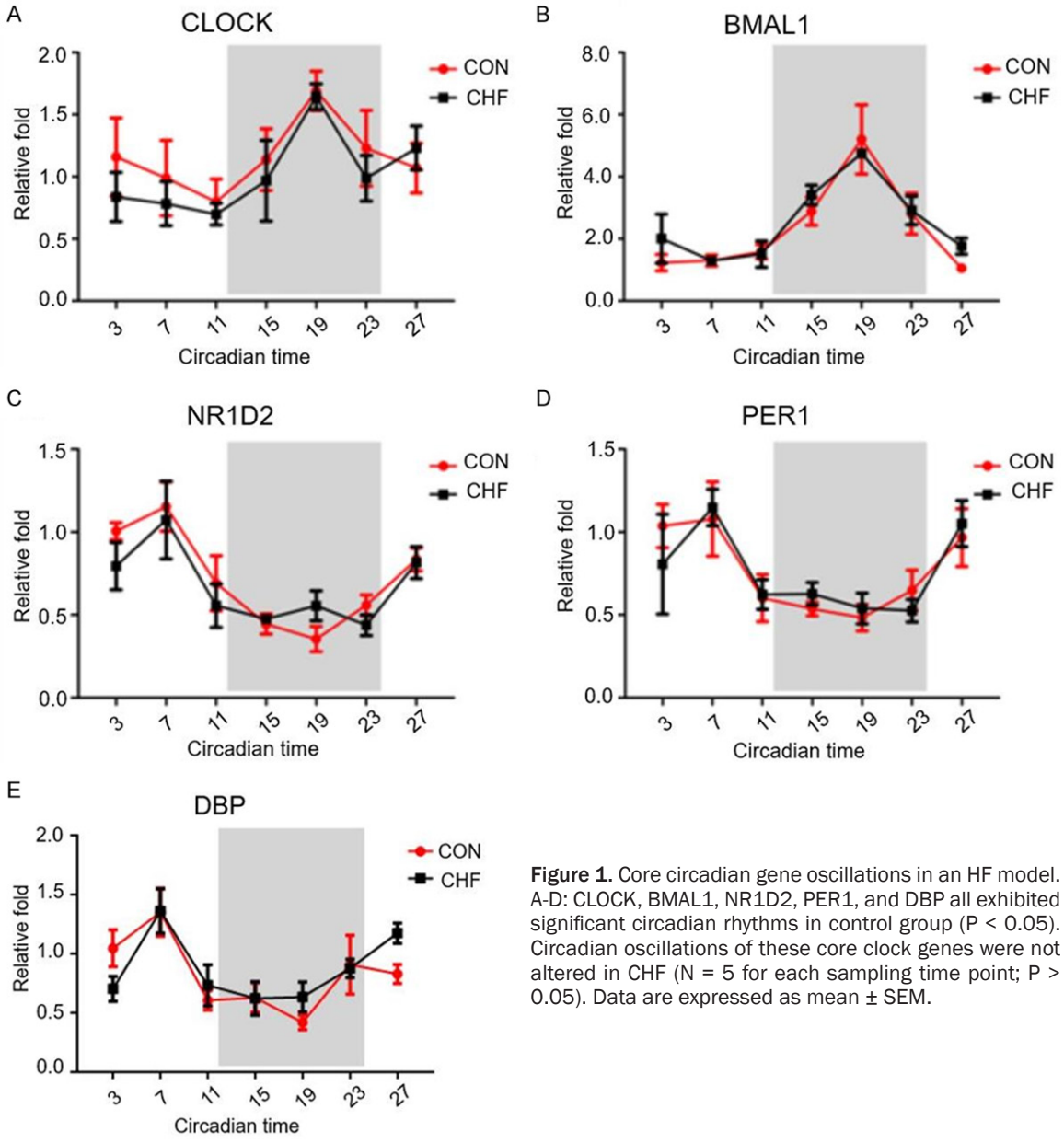

Figure 1. Core circadian gene oscillations in an HF model. A-D: CLOCK, BMAL1, NR1D2, PER1, and DBP all exhibited significant circadian rhythms in control group ($P < 0.05$). Circadian oscillations of these core clock genes were not altered in CHF (N = 5 for each sampling time point; $P > 0.05$). Data are expressed as mean ± SEM.

1 μmol) at CT3 and CT15 were recorded and VAs were induced by PES.

During ISO infusion, circadian variations in response to β-AR activation translated to a greater incidence of VAs at CT3 (resting period) compared to CT15 (active period) ($P < 0.05$, CHF-CT3: CHF-CT15 = 100%: 60%; **Figure 2**). However, during ISO + CGP infusion, VA circadian variations were blunted by β1-AR selective blocker ($P > 0.05$, CHF-CT3: CHF-CT15 = 26.7%: 26.7%; **Figure 2**). Furthermore, during ISO + ICI infusion, circadian variations in response to β-AR activation maintained the same level compared to ISO infusion ($P < 0.05$, CHF-CT3: CHF-CT15 = 80%: 40%; **Figure 2**). Together, these data supported the hypothesis that circadian variation of VAs is mediated by β1-AR.

β1-AR exhibited circadian oscillations in CHF that were linked to clock genes

To investigate which genes in the β1-AR pathway exhibit circadian oscillations, a high-resolution CircaDB microarray dataset for the heart (http://bioinf.itmat.upenn.edu/circa/) was used for bioinformatics analysis, which examined gene expression every 2 h for 48 h in a combi-

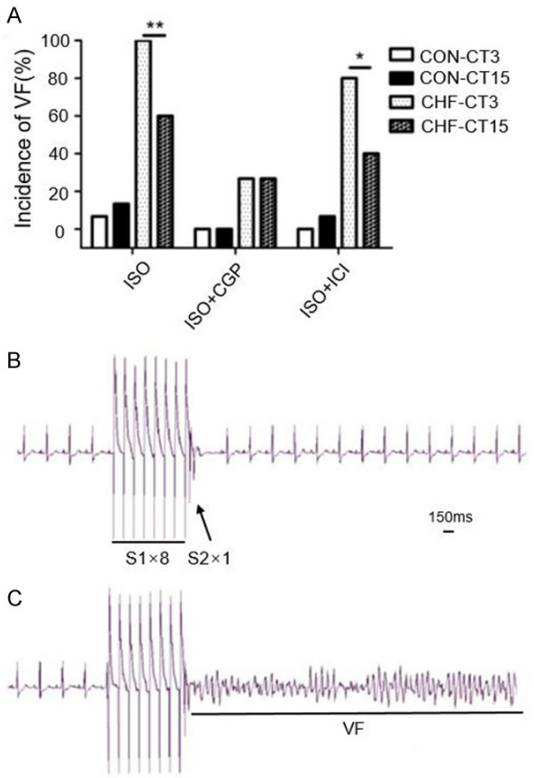

Figure 2. Circadian variation in ventricular arrhythmias (VAs) is blunted by selective β1-adrenoreceptor antagonist. A: CHF group exhibited greater incidence of VAs. Circadian variations in response to ISO stimulation in failing hearts translated to greater incidence of VAs at CT3 than CT15, whereas circadian VAs were blunted by β1-AR-selective blocker (CGP). In addition, circadian VAs were not altered by β2-AR-selective blocker (ICI). B: Protocol for programmed electrical stimulation (PES); Premature extrastimulus (S2) was delivered after eight basic stimuli (S1). S1-S1 interval was 150 ms. C: Example of VF induced by PES.VF was induced by S1–S2 (8:1) programmed stimulation. N = 15 for each group. *P < 0.05, **P < 0.01.

nation JTK-CYCLE analysis [38]. As a result, β1-AR gene expression exhibited circadian oscillations. In addition, to confirm circadian oscillations of β-ARs in CHF, a circadian time course collection was carried out using CON and CHF guinea pigs. Hearts were collected every 4 h over a 24-h period, RNA was extracted, and β-AR expression was determined using real-time PCR.

The results suggested that β1-AR exhibited significant circadian rhythms (P < 0.05, Period = 24, Phase = 20, Amplitude = 0.18). Circadian oscillations of β1-AR (Period = 24, Phase = 20, Amplitude = 0.26) were maintained in CHF (P > 0.05), while the mean expression of β1-AR was significantly attenuated in guinea pigs with CHF (n = 5, P < 0.01). One the other hand, β2-AR did not follow a circadian rhythm (P > 0.05; **Figure 3**). The β1-AR phase was delayed 4 h compared to CLOCK and BMAL1 phases in mRNA levels. To further verify the hypothesis that β1-AR is a CLOCK-BMAL1-regulated gene, hearts were collected every 4 h over a 24 h period, protein was extracted, and β-AR and CLOCK-BMAL1 expressions were measured by western blotting.

BMAL1 (P < 0.05; Period = 24, Phase = 0, Amplitude = 0.22), CLOCK (P < 0.05; Period = 24, Phase = 23, Amplitude = 0.17), and β1-AR (P < 0.05; Period = 24, Phase = 3, Amplitude = 0.12) all exhibited significant circadian rhythms. Circadian oscillations of core clock genes (BMAL1 - Period = 24, Phase = 23, Amplitude = 0.22; CLOCK - Period = 24, Phase = 21, Amplitude = 0.10) and β1-AR (Period = 24, Phase = 3, Amplitude = 0.10) were maintained in CHF (P > 0.05). However, the mean expression of β1-AR was attenuated in guinea pigs with CHF (n = 5, P < 0.01). Again, β2-AR did not follow a circadian rhythm (P > 0.05) and the β1-AR phase was delayed 4-6 h compared to the CLOCK and BMAL1 phases in protein levels (**Figure 4**). A 4-6 h phase delay was discovered between mRNA expression and protein abundance, in accord with the phase delay anticipated for core clock mechanism-regulated proteins [39]. The results indicated that β1-AR exhibited circadian oscillations in CHF and that it might be regulated by CLOCK and BMAL1.

CLOCK-BMAL1 induces arrhythmic cardiomyocyte response to ISO stimulation

To confirm that CLOCK-BMAL1 is involved in arrhythmic response to ISO stimulation of cardiomyocytes, ISO-induced arrhythmic activity of cardiomyocytes was examined after CLOCK overexpression, BMAL1 overexpression, CLOCK-BMAL1 overexpression, and in control groups. ISO-induced arrhythmia was investigated in field-stimulated cardiomyocytes when exposure to different concentrations of ISO (10, 50, and 100 nmol/L).

Overexpression of CLOCK-BMAL1 enhanced arrhythmic activity of cardiomyocytes following simulation with 50 and 100 nmol/L of ISO (P < 0.05; **Figure 5A**). In addition, action potentials from single myocytes were recorded in the

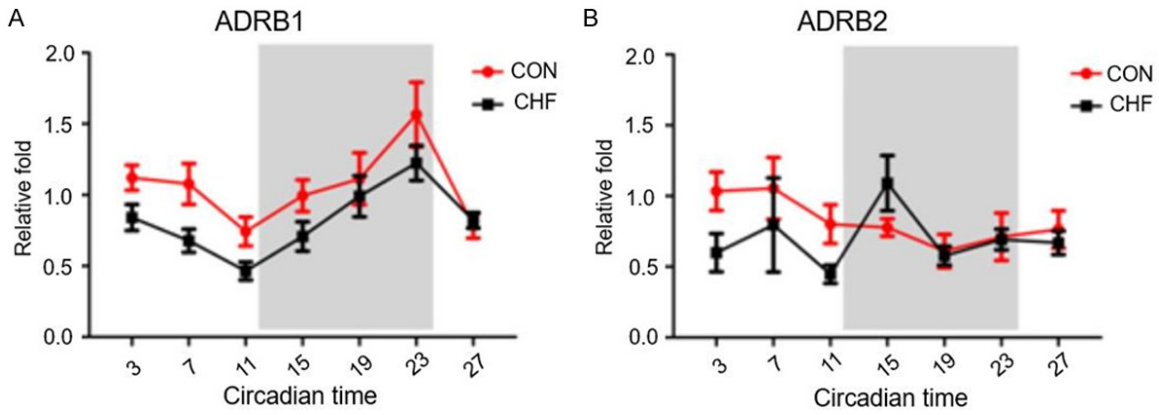

Figure 3. Circadian oscillations for β-ARs mRNA level in CHF model. A: β1-AR exhibited significant circadian rhythms in control group ($P < 0.05$). Circadian oscillations of β1-AR were maintained in CHF model ($P > 0.05$). However, the mean expression of β1-AR was significantly attenuated in guinea pigs with CHF ($N = 5$ for each sampling time point, $P < 0.01$). B: β2-AR did not follow a circadian rhythm ($N = 5$ for each sampling time point, $P > 0.05$). Data are expressed as mean ± SEM.

same experiments and demonstrated that co-infection of CLOCK-BMAL1 prolonged cardiomyocyte ADP and induced extrasystole as well as early and delayed afterdepolarization (**Figure 5B**).

CLOCK-BMAL1 regulates action potential duration of cardiomyocytes via β1-AR

To assess which β-AR mediated the prolonged APD in CLOCK-BMAL1 over-expression group, cardiomyocytes with added ISO and highly selective β1 (CGP-20712A) or β2 (ICI-118,551) antagonists were compared to ISO-alone groups.

Co-infection of CLOCK-BMAL1 prolonged myocyte APD in the ISO-alone group ($n = 10$, $P < 0.05$; **Figure 6A**). The β1-blocker (CGP-20712A) abolished the ISO-effect ($n = 10$, $P > 0.05$; **Figure 6D**), while β2-blocker (ICI-118,551) failed to alter the ISO effect ($n = 10$, $P < 0.05$; **Figure 6G**), confirming that it is biased towards β1-adrenergic activation.

CLOCK-BMAL1 enhances expression of β1-AR transcriptionally

To further support the notion that CLOCK-BMAL1 enhances β1-AR expression transcriptionally, CLOCK and/or BMAL1 infections were used in myocytes (Figure S1) and β1-AR and CLOCK-BMAL1 expressions were measured using western blotting. As expected, co-infection of CLOCK-BMAL1 enhanced β1-AR expression (**Figure 7**).

Furthermore, to determine β1-AR (gene name: ADRB1) circadian modulation, three putative E-box sequences in the ADRB1 promoter region located at -741 bp, -806 bp, and -1005 bp were examined using a bioinformatics search (**Figures 8A** and S2). To examine whether the putative E-box binds to BMAL1, precipitation was performed using anti-BMAL1 antibodies followed by RT-PCR. As a result, BMAL1 was precipitated by the E-box located at -741 bp and -806 bp in the ADRB1 promoter. The putative E-boxes located at -1005 bp in the ADRB1 promoter were barely detected in the precipitated DNA samples (**Figure 8B**). The data suggested that the E-box located at -741 bp and -806 bp in the ADRB1 promoter recruited the BMAL1 protein.

Finally, the functions of the three putative E-boxes of the ADRB1 promoter were examined using reporter gene assays. It was discovered that CLOCK-BMAL1 overexpression increased ADRB1 promoter activity. In contrast, E-box mutation suppressed the increase in ADRB1 promoter activity (**Figure 8D**). Taken together, the data suggested that BMAL1 binds to the E-box located at -741 bp and -806 bp positions and enhances ADRB1 promoter activity.

Discussion

The major study findings were as follows: (1) circadian oscillations of core circadian genes were not altered in CHF, which might explain the circadian oscillations of VAs in heart failure; (2) β1-AR exhibits a circadian rhythm in CHF

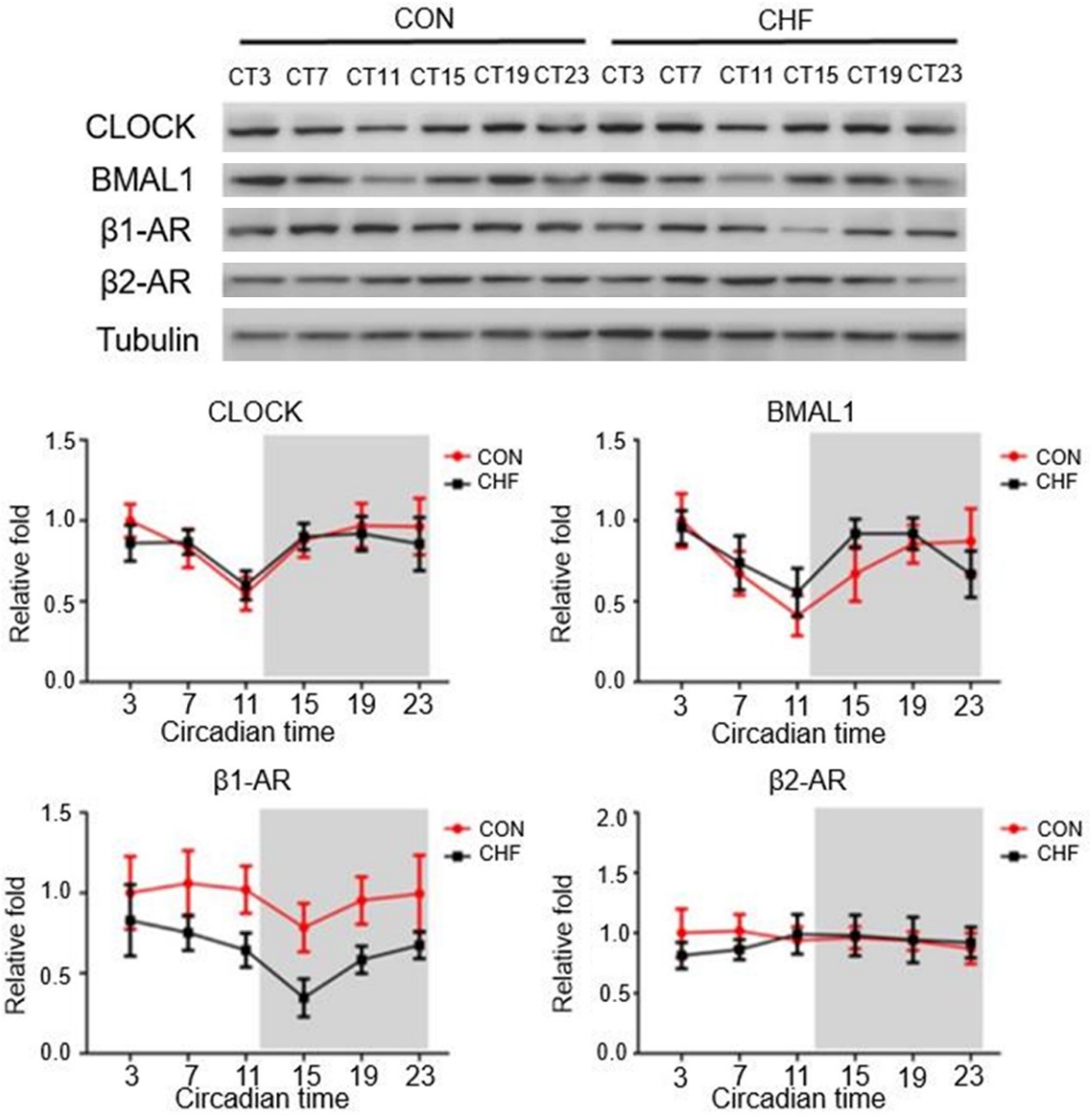

Figure 4. Circadian oscillations for protein level of CLOCK-BMAL1 and β-ARs in CHF model. BMAL1, CLOCK, and β1-AR exhibited circadian oscillations in control group. Compared to control group, Circadian rhythms of clock genes (BMAL1 and CLOCK) and β1-AR were maintained in CHF group ($P > 0.05$). However, the mean expression of β1-AR was attenuated in CHF group ($P < 0.01$). Nevertheless, β2-AR did not follow a circadian rhythm ($P > 0.05$). ($N = 5$ for each sampling time point). Data are expressed as mean ± SEM.

and CLOCK-BMAL1 regulates the circadian arrhythmic response upon ISO stimulation via β1-AR. Therefore, β1-AR blockers may blunt the circadian VAs in failing hearts. To the best of our knowledge, the study provides experimental evidence that CLOCK-BMAL1 regulates circadian oscillation of VAs in failing hearts via β1 adrenergic receptor.

Core clock genes conserve circadian oscillations in CHF, while clock output genes are attenuated. Several studies have reported that circadian rhythms of clock output genes are attenuated in TAC failing hearts and that core clock genes exhibit the same circadian pattern as control hearts [40]. In the present experiments, conservation of core circadian gene rhythms was also observed in remodeling hearts. Furthermore, clock output genes, including pyruvate dehydrogenase kinase 4 (Pdk4) and mitochondrial metabolism genes uncoupling protein 3 (Ucp3), were significantly

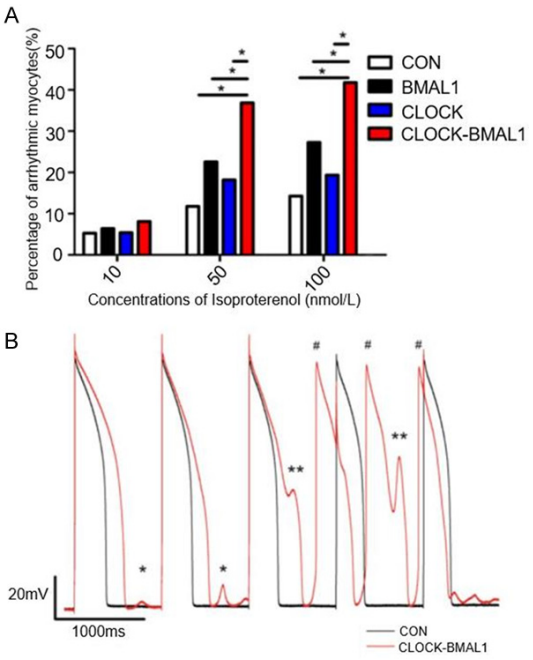

Figure 5. CLOCK-BMAL1 overexpression induced arrhythmic response to ISO stimulation in cardiomyocytes. A: Percentage of asynchronous activity in the control (white bars), BMAL1 overexpression (black bars), CLOCK overexpression (blue bars), and CLOCK-BMAL1 overexpression (red bars) groups after superfusion with Tyrode's solution containing ISO (10, 50, and 100 nmol/L) for 5 min. CLOCK-BMAL1 overexpression upregulated the percentage of arrhythmic myocytes. The number of cells = 80-120; hearts = 6; *P < 0.05. B: An example of action potential duration recorded from the control and CLOCK-BMAL1 overexpression groups showing the presence of early afterdepolarization (**), delayed afterdepolarization (*), and extrasystoles (#) during superfusion with ISO (100 nmol/L).

increased in TAC hearts during the light period (resting period) rather than the dark period (active period). In contrast, the apoptosis pathway gene of BCL2/adenovirus E1B interacting protein 3 (Bnip3) increased during the dark period [41]. Furthermore, recent studies confirmed that day/night gene rhythms can change during the progression of disease, making these circadian features predictive of heart failure. Increased active-period expression of Bnip3 and decreased resting-period expression of Ucp3 have been uncovered in heart failure [41]. This present study indicated that the mean expression of β1-AR decreased in CHF, which might be a promising chronobiomarker to predict the outcomes of heart failure.

Circadian arrhythmic response to ISO stimulation in failing hearts

In CHF patients, the incidence of VAs has a diurnal variation with the highest incidence in the morning (active period) [6, 9] that is often triggered by a peak in sympathetic activity [3, 5, 14, 16]. High sympathetic activity can result in VAs linked to afterdepolarization [17-19]. Not surprisingly, the present results showed that high concentrations of ISO lead to arrhythmic activity of cardiomyocytes. Moreover, a recent study showed that cardiomyocytes at CT3 (resting period) are more likely to exhibit arrhythmic activity than cardiomyocytes at CT15 (active period) during ISO stimulation [35].

Connections among circadian VAs, β-AR, and peripheral clocks

Previous studies have suggested that the peripheral clock regulates diurnal variation of β-AR functions. Durgan et al. reported that wild-type mice presented greater hypertrophic growth and hypertrophic marker with ISO stimulation at CT0 compared to CT12. Conversely, this variation was attenuated in CCM mice [28]. ISO-induced Fgf23 expression occurred in a circadian rhythm-dependent manner, but this dependence was not observed in BMAL1-deficient mice [42].

Moreover, peripheral clock control is evident in cardiac electrophysiological changes, which might account for circadian VAs due to direct modulation of various ion channels by cardiac circadian clocks. For example, the peripheral clock modulates KChIP2 via Krüppel-like factor 15 (Klf15) [26], while both the potassium channel gene (Kcnh2) and sodium channel gene (Scn5a) are regulated by the cardiomyocyte peripheral clock in mice [23, 24]. The present study supported the novel notion that peripheral clock also regulates β1-AR, which might account for circadian VAs.

The link between β1-AR and peripheral clocks

First, the expression of β1-AR exhibited circadian rhythms [38] and the β1-AR phase was delayed by 4-6 h compared to CLOCK-BMAL1 in both CON and CHF groups. Second, circadian variation of VAs was mediated by β1-AR. Third, overexpression of CLOCK-BMAL1 prolonged

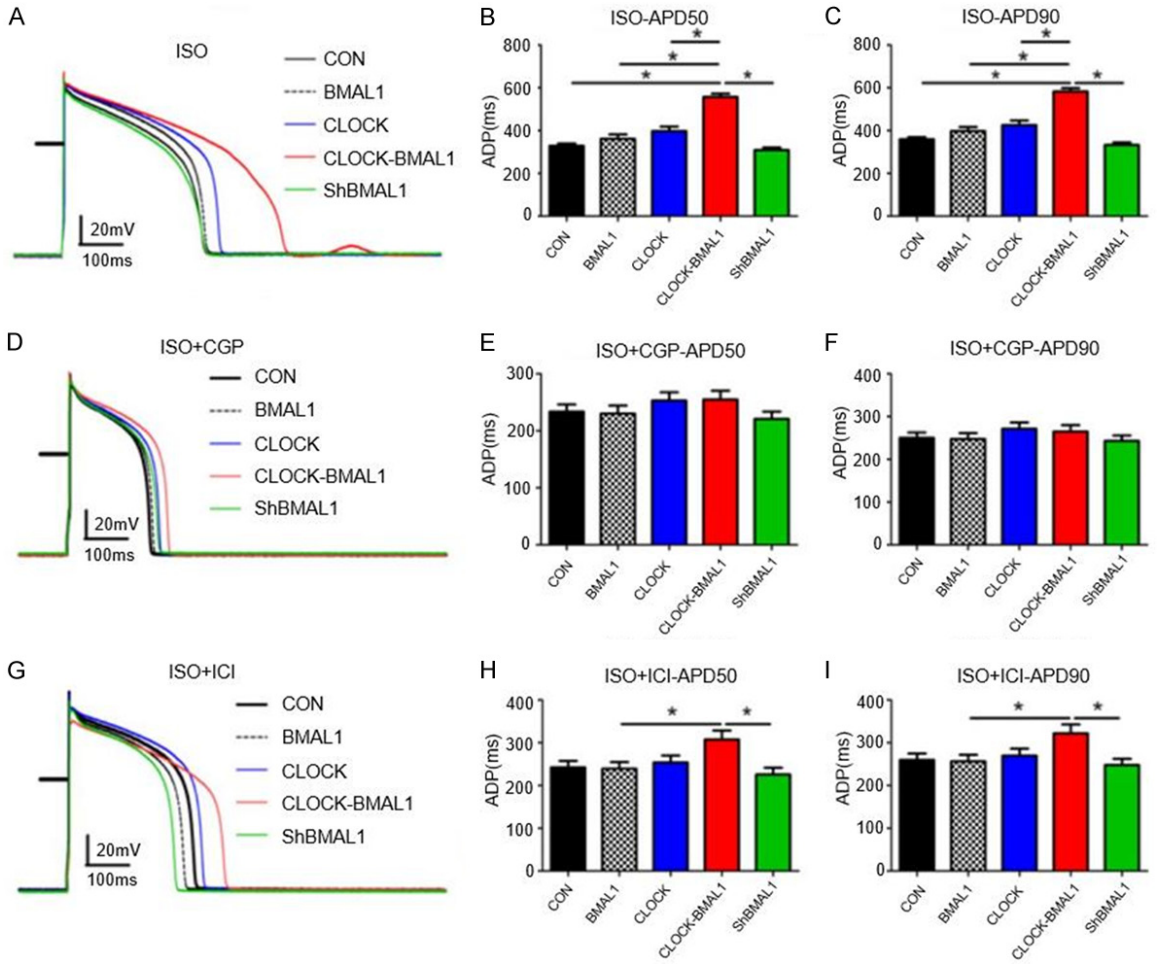

Figure 6. CLOCK-BMAL1 overexpression prolonged cardiomyocyte APD with ISO stimulation through β1-AR. A-C: Representative APDs (1-Hz) from myocytes with added ISO in the control, BMAL1 overexpression, CLOCK overexpression, CLOCK-BMAL1 overexpression, and sh-BMAL1 groups; APD at 50% (APD50) and 90% (APD90) repolarization. D-F: Representative APDs from myocytes with added ISO and highly selective β1 antagonists (CGP-20712A). G-I: Representative APDs from myocytes with added ISO and highly selective β2 antagonists (ICI-118,551). N = 10 cells from eight hearts per group. Data are expressed as mean ± SEM; *P < 0.05.

the APD and induced arrhythmic activity to ISO stimulation of cardiomyocytes, which was biased through β1-AR activation. Fourth, CLOCK-BMAL1 co-infection induced the expression of β1-AR in myocytes. Fifth, CLOCK-BMAL1 overexpression increased ADRB1 promoter activity, while mutation of E-boxes abolished increases in ADRB1 promoter activity *ex vivo*. Finally, it was confirmed that BMAL1 was precipitated by the E-box located at -741 bp and -806 bp in the ADRB1 promoter using ChIP *in vivo*. Therefore, these data supported β1-AR as a CLOCK-BMAL1-regulated gene.

In addition, Motif centrality analysis of ChIP-Seq demonstrated that spacer lengths of tandem CLOCK-binding motifs were in the range of 60 bp around each CLOCK-binding site [43, 44]. The present data showed that BMAL1 binds to the E-box elements located at -741 bp or -806 bp, which are called double E-box elements or tandem E1-E2 box elements [43-45]. As proper spacing is implicated in CLOCK- and BMAL1-mediated transcription [43, 44], the double E-box elements of ADRB1 can significantly enhance the expression of β1-AR. Here, it was found that β1-AR was regulated by circadian cardiomyocyte CLOCK-BMAL1 in guinea pigs. The study also confirmed that CLOCK-BMAL1 enhances the expression of β1-AR by transcriptionally binding to E-box.

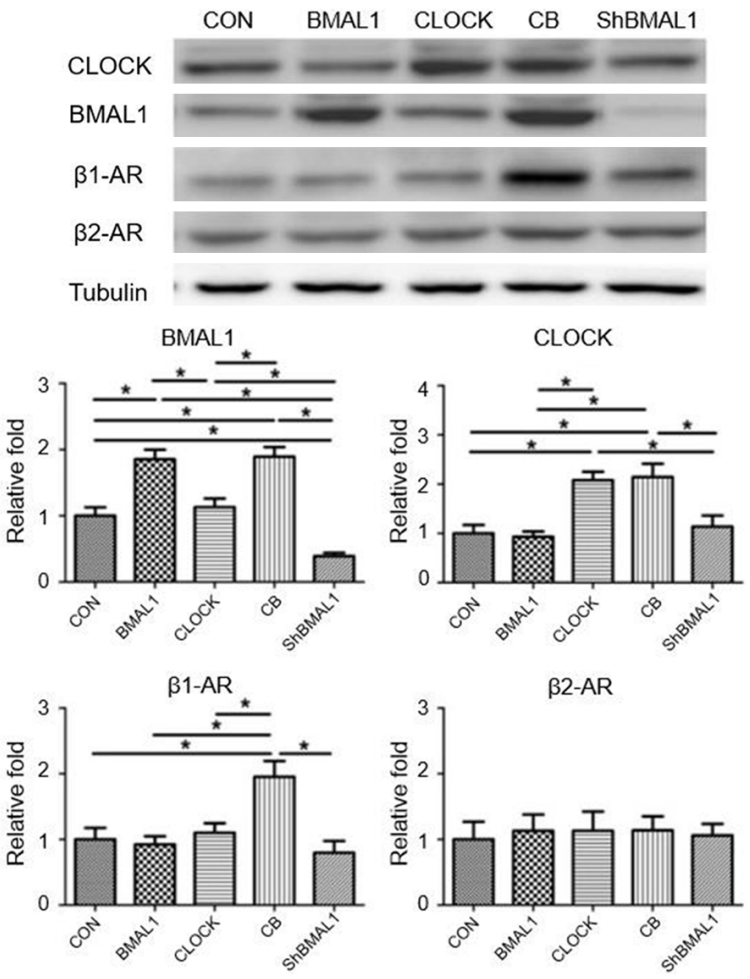

Figure 7. CLOCK-BMAL1 overexpression induced the expression of β1-AR rather than β2-AR. Altered protein expression of CLOCK, BMAL1, β1-AR, and β2-AR were determined with western blotting in the control, BMAL1 overexpression, CLOCK overexpression, CLOCK-BMAL1 overexpression (CB), and sh-BMAL1 groups. N = 5 per group. Data are expressed as mean ± SEM; *P < 0.05.

Clinical implications of CLOCK-BMAL1-regulated β1-AR circadian rhythm

Due to β1-AR circadian regulation, chronotherapy should be taken into consideration to optimize medical intervention time and decrease adverse cardiovascular effects [46]. For example, Nebivolol taken in the evening and not in the morning attenuated morning pre-awakening systolic BP [47]. In addition, studies have demonstrated a circadian influence of β-blockers on heart rhythm (HR). For example, propranolol taken in the morning needs less time to have the greatest suppressive effects on HR [48] and have more significant suppressive effects on the increase of HR caused by exercise [49]. In hypertensive patients, bisoprolol taken in the morning reduces the 24-h ambulatory HR [50]. In coronary heart disease patients, myocardial ischemic episodes caused by HR rise tend to happen during the daytime than in the dark and propranolol blunts the diurnal variations of HR-related episodes [51].

Given the importance of the cardiac circadian system in heart disease, researchers have focused on novel pharmacological compounds to target the ROR and/or REV-ERB nuclear receptors [52] or other core circadian genes [53]. Although these new chronobiology drugs have not yet been well examined in heart diseases, they are promising candidates that can benefit cardiovascular physiology and pathophysiology, especially due to their influence on the circadian phase [54] and muscle metabolism [55].

Study limitations

Unlike human beings, guinea pigs are nocturnal animals and have a completely different circadian rhythm. Moreover, cardiomyocytes during isolation and culture processes might behave differently. However, internal controls for each set of the experiments were included. Furthermore, we did not directly observe the effects of CLOCK-BMAL1 on cardiac electrophysiological features using cardiac-specific transgenic animal model. Finally, blockades of β3, β4, and β5-ARs was not examined in the study.

Conclusion

In summary, our study revealed that CLOCK-BMAL1 regulates circadian oscillation of VAs in failing hearts via the β1 adrenergic receptor. The novel circadian mechanism regulating

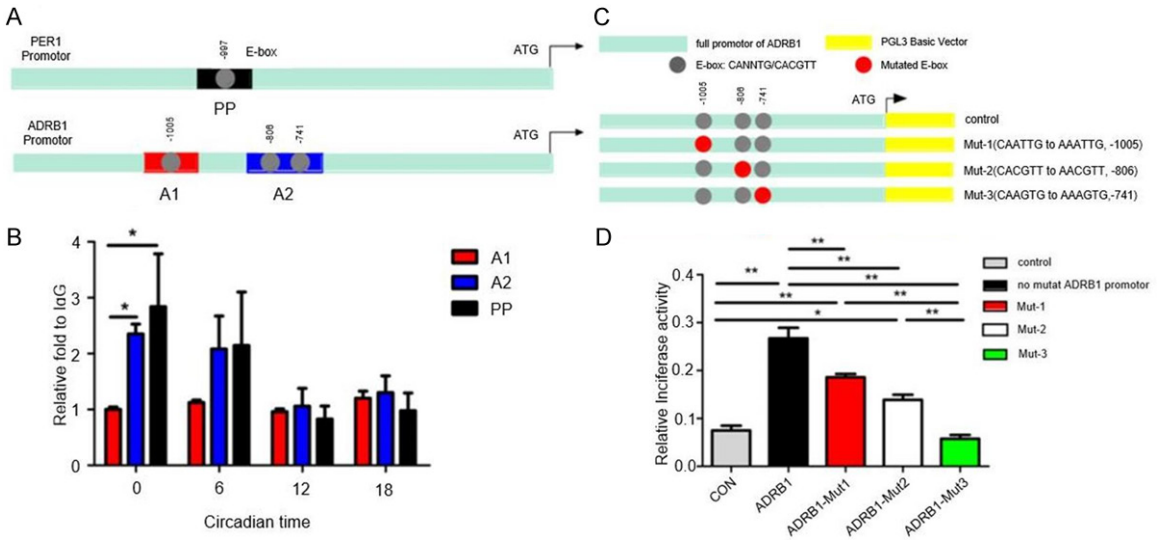

Figure 8. CLOCK-BMAL1 bound to the enhancer of β1-AR and upregulated β1-AR expression. A: Three putative E-box sequences in ADRB1 promoter located at -741 bp (A1), -806 bp, and -1005 bp (A2); PP: PER1 promoter E-box area. B: DNA expressions of A1 and A2 in anti-BMAL1 antibody-precipitated DNA samples; Rabbit IgG was used as a negative immunoprecipitation control. C: Three mutated E-box sequences in ADRB1 promoter. D: Luciferase activity of ADRB1 promoter and/or mutant ADRB1 promoters in overexpression of CLOCK-BMAL1. N = 4 for each group. Data are expressed as mean ± SEM; *P < 0.05, **P < 0.01.

β1-AR expression provides a molecular basis for circadian control of arrhythmogenesis in CHF.

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Disclosure of conflict of interest

None.

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Table S1. Echocardiography and pathology for control and CHF

	CON (n)	CHF (n)
Echocardiography	(7)	(7)
LVID;d (mm)	6.33 ± 0.422	8.00 ± 0.300**
LVID;s (mm)	3.66 ± 0.257	5.72 ± 0.244**
LVPW;d (mm)	2.06 ± 0.130	1.69 ± 0.022*
FS (%)	42.18 ± 0.854	28.55 ± 0.625**
EF (%)	72.15 ± 1.092	53.26 ± 1.020**
Pathology	(8)	(8)
HW/Tibia (g/cm)	0.43 ± 0.023	0.65 ± 0.025**
LW/Tibia (g/cm)	0.78 ± 0.013	0.92 ± 0.059*
mRNA	(8)	(8)
ANP	1.31 ± 0.326	9.14 ± 2.493*

Values are mean ± SEM. Control (CON): sham-operated guinea pigs. LVID;d: left ventricular internal dimension diastolic. LVID;s: left ventricular internal dimension systolic. LVPW;d: left ventricular posterior wall diastolic. EF: ejection fraction. FS: fractional shortening. HW/Tibia: heart weight/tibia length ratio. LW/Tibia: lung weight/tibia length ratio. ANP: atrial natriuretic factor. n: number of guinea pigs. *P < 0.05, **P < 0.01 vs. CON.

Table S2. Primers used for RT-PCR

Gene name	Primer	Sequence
ADRB1	Sense	5'-CAGCACTTGGGATCGTTGTAGC-3'
	Antisense	5'-CGCTGGGAGTACGGTTCCTT-3'
ADRB2	Sense	5'-CACAACCCATACCATCAAG-3'
	Antisense	5'-GCAAACGGTCACCAACTA-3'
ANP	Sense	5'-CGTGGTGCTGAAGTTTATTTGT-3'
	Antisense	5'-GGCTGTATTGTGGTTCTCCC-3'
BMAL1	Sense	5'-TCCCTCGGTCACATCC-3'
	Antisense	5'-AGCAAACTACAAGCCAACT-3'
β-actin	Sense	5'-TTGCTGCGTTACACCCTT-3'
	Antisense	5'-GTCACCTTCACCGTTCCA-3'
CLOCK	Sense	5'-TCCAGATTCCATCCAGTATG-3'
	Antisense	5'-AAGTTGCTGACCTTGAGA-3'
DBP	Sense	5'-AGAAGCAGGGTCCTCTTTCC-3'
	Antisense	5'-TTGGCTGCGGTTTAAGTTCC-3'
NR1D2	Sense	5'-TGTGGCATCAGGATTCCACT-3'
	Antisense	5'-CATCCCCACAGACAGACACT-3'
PER1	Sense	5'-TCCCAGTGTTCTCCCCTAGA-3'
	Antisense	5'-CATAGGGGTAGCTGGATGGG-3'
ADRB1 area1	Sense	5'-CGCTCAAAGGTCGATCAGGAGAC-3'
	Antisense	5'-GTTCCCTTGCGTCCTTCCCTTAC-3'
ADRB1 area2	Sense	5'-TTTGAGGAGGTCGGGGTCACTTG-3'
	Antisense	5'-GCGCACTAAGGAAAACGCGTCTC-3'
PER1 promotor	Sense	5'-CCACTGACAACACCCAGAGGACG-3'
	Antisense	5'-GCGTGATCGGATCTAGGGTCGGTGA-3'

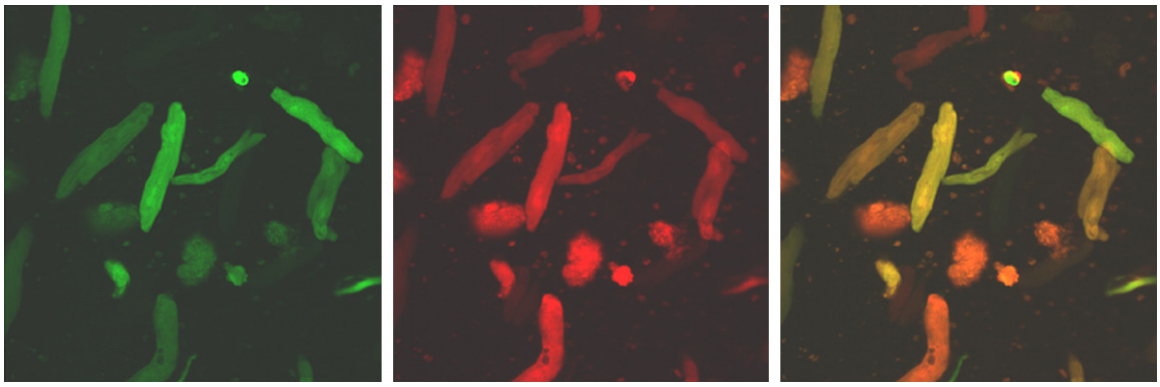

Figure S1. Expressions of CLOCK and BMAL1 adenovirus-infected cardiomyocytes (confocal microscope; magnification, ×200). Ad-CLOCK were labeled with red fluorescent protein while Ad-BMAL1 with green. Red and green fluorescence images were merged.

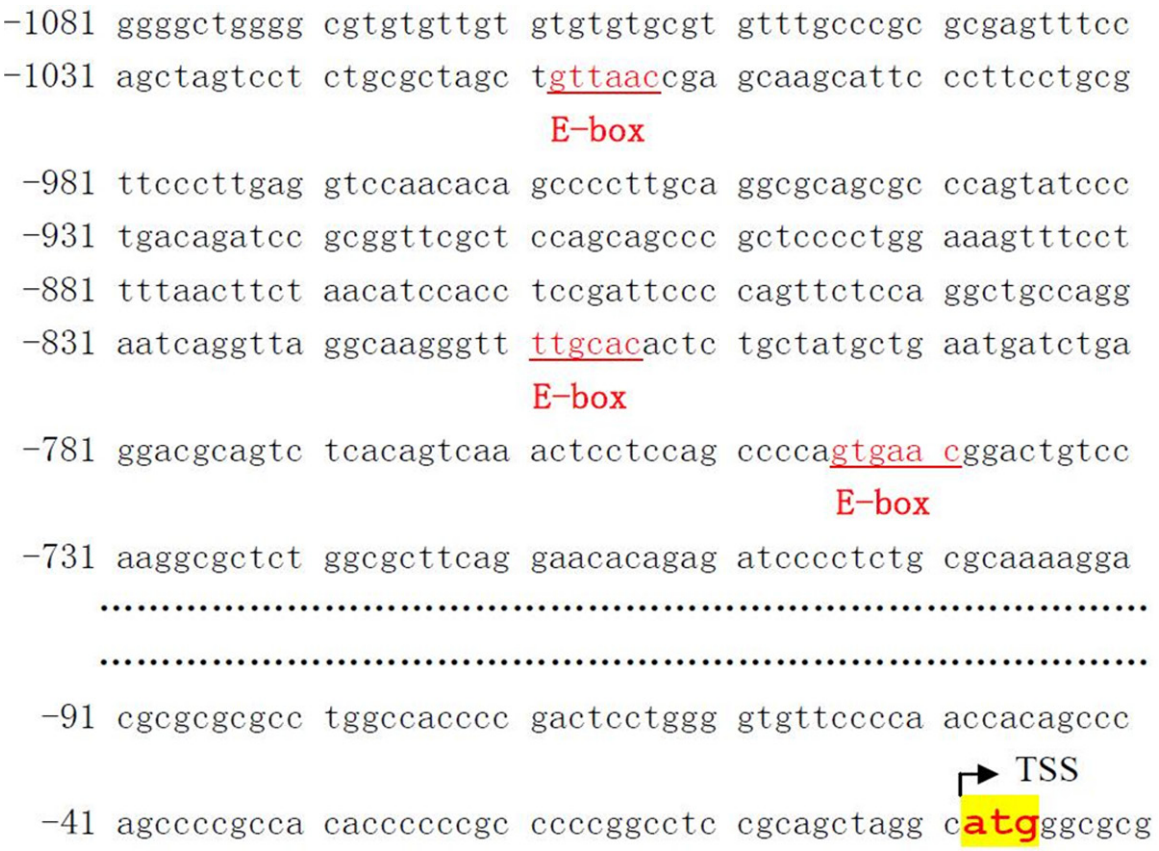

Figure S2. Putative BMAL1 binding sites for ADRB1 promoter in guinea pig. 2000-bp length sequence upstream of the transcription start site of ADRB1 promoter. (Translation start site is highlighted in yellow).