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Genetic disruption of the cardiomyocyte circadian clock differentially influences insulin-mediated processes in the heart

Graham R. McGinnis [a], Yawen Tang [a], Rachel A. Brewer [a], Manoja K. Brahma [b], Haley L. Stanley [a], Gobinath Shanmugam [b], Namakkal Soorappan Rajasekaran [b], Glenn C. Rowe [a], Stuart J. Frank [c], Adam R. Wende [b], E. Dale Abel [d], Heinrich Taegtmeyer [e], Silvio Litovsky [b], Victor Darley-Usmar [b], Jianhua Zhang [b], John C. Chatham [b], Martin E. Young [a,*]

[a] Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA
[b] Division of Molecular Cellular Pathology, Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA
[c] Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, University of Alabama at Birmingham, Birmingham, AL, USA
[d] Division of Endocrinology and Metabolism, Department of Medicine and Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, IA, USA
[e] Division of Cardiology, Department of Internal Medicine, McGovern Medical School UT Health Science Center, Houston, TX, USA

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ABSTRACT

Cardiovascular physiology exhibits time-of-day-dependent oscillations, which are mediated by both extrinsic (e.g., environment/behavior) and intrinsic (e.g., circadian clock) factors. Disruption of circadian rhythms negatively affects multiple cardiometabolic parameters. Recent studies suggest that the cardiomyocyte circadian clock directly modulates responsiveness of the heart to metabolic stimuli (e.g., fatty acids) and stresses (e.g., ischemia/reperfusion). The aim of this study was to determine whether genetic disruption of the cardiomyocyte circadian clock impacts insulin-regulated pathways in the heart. Genetic disruption of the circadian clock in cardiomyocyte-specific Bmal1 knockout (CBK) and cardiomyocyte-specific Clock mutant (CCM) mice altered expression (gene and protein) of multiple insulin signaling components in the heart, including p85α and Akt. Both baseline and insulin-mediated Akt activation was augmented in CBK and CCM hearts (relative to littermate controls). However, insulin-mediated glucose utilization (both oxidative and non-oxidative) and AS160 phosphorylation were attenuated in CBK hearts, potentially secondary to decreased Inhibitor-1. Consistent with increased Akt activation in CBK hearts, mTOR signaling was persistently increased, which was associated with attenuation of autophagy, augmented rates of protein synthesis, and hypertrophy. Importantly, pharmacological inhibition of mTOR (rapamycin; 10 days) normalized cardiac size in CBK mice. These data suggest that disruption of cardiomyocyte circadian clock differentially influences insulin-regulated processes, and provide new insights into potential pathologic mediators following circadian disruption.

1. Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States and industrialized countries world-wide [1]. Diabetes mellitus increases the incidence of CVD, and worsens outcomes [2]. The most common form of diabetes is type 2 diabetes, which is characterized by both insulin resistance and insulin insufficiency [3]. Peripheral tissues exhibit differential insulin resistance, in terms of the impact on both distinct organs and intracellular signaling branches, which contributes to pathogenesis of the disease. In the case of the heart, insulin influences a host of processes central to cardiac function, ranging from glucose uptake and utilization, to the turnover of cellular constituents (e.g., protein, organelles) [4,5]. Despite hyperactivation of the insulin signaling pathway in type 2 diabetes, insulin-mediated glucose uptake is repressed. The contribution of aberrant cardiac insulin signaling towards the development of heart failure in diabetes has recently been reviewed [6].

Twenty-four-hour cycles have been observed for various biological functions, in essentially all species [7,8]. These oscillations are driven by both extrinsic and intrinsic factors. The latter includes the cell autonomous circadian clock, a sophisticated transcription-translation feedback loop mechanism that temporally partitions cellular processes. Two critical clock components include brain and muscle arnt-like 1 (Bmal1) and circadian locomotor output cycles kaput (Clock), which bind to conserved E-boxes in the promoter region of clock controlled

* Corresponding author at: Division of Cardiovascular Disease, Department of Medicine, University of Alabama at Birmingham, 703 19th St. S., ZRB 308, Birmingham, AL 35294, USA.
E-mail address: meyoung@uab.edu (M.E. Young).

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genes (as a heterodimer), resulting in 24-h oscillations in gene expression (and ultimately biologic processes) [9,10]. Although originally identified in the suprachiasmatic nucleus (SCN), this transcriptional network has been described in virtually all mammalian cells/organs, influencing processes ranging from the whole body (e.g., behaviors) to cellular levels (e.g., metabolism/signaling) [7,8]. In doing so, circadian clocks temporally orchestrate biologic/cellular functions, ensuring appropriate responses to predicted environmental stimuli/stresses.

Circadian disruption, whether environmental (e.g., shift work) or genetic (e.g., polymorphisms), increases the risk of obesity, diabetes, and CVD [11–14]. The clinical impact of abnormal 24-h rhythms is underscored by observations such as non-dipping hypertension, which results in greater risk of adverse cardiac events relative to dipping hypertension [15,16]. Consistent with these reports, animal studies indicate that germline knockout of circadian clock components increases adiposity, alters insulin secretion/sensitivity, impacts blood pressure, and impairs contractile function of the heart [17–20]. Moreover, circadian clocks are altered during various pathologies, including obesity, diabetes, and hypertension [21–23]. Collectively, these observations suggest that disruption of circadian clocks may play a causal role in the development of cardiometabolic diseases.

Recent studies suggest that the cardiomyocyte circadian clock influences numerous processes essential for maintenance of cardiac function, including metabolism, electrical activity, and cell signaling [24–26]. In the latter case, we have reported that the cardiomyocyte circadian clock influences responsiveness of the heart to both physiologic (e.g., fatty acids) and pathologic (e.g., ischemia/reperfusion) stimuli/stresses [27–29]. We now show that genetic disruption of the cardiomyocyte circadian clock leads to alterations in gene/protein expression of multiple insulin signaling components. This results in chronic activation of the Akt/mTOR/S6 signaling axis, increased protein synthesis, and increased cardiac size; the latter are normalized by pharmacologic inhibition of mTOR. Chronic mTOR activation is also associated with inhibition of autophagy. Conversely, despite chronic activation of Akt, insulin-mediated glucose utilization was attenuated following circadian disruption. These studies highlight a critical role of the cardiomyocyte circadian clock in regulating myocardial insulin signaling/sensitivity, and suggest that circadian disruption differentially influences distinct branches of the insulin signaling cascade known to regulate critical cardiac processes.

2. Methods

2.1. Mice

The cardiomyocyte circadian clock was genetically ablated by targeting BMAL1 and CLOCK, as described previously [24,25]. Twelve to sixteen week old male cardiomyocyte-specific Bmal1 knockout (CBK; BMAL1$^{flox/flox}$/α-MHC-CRE$^{+/-}$) and littermate control mice (CON; BMAL1$^{flox/flox}$/MHC-CRE$^{-/-}$) on the C57BL/J6 background were utilized, as well as cardiomyocyte-specific Clock mutant (CCM; α-MHC-dnCLOCK$^{+/-}$) and littermate control mice (WT; α-MHC-dnCLOCK$^{-/-}$) on the FVB/N background. Mice were kept in temperature, humidity, and light-controlled rooms on a strictly enforced 12-h light/12-h dark schedule, where ZT0 refers to the beginning of the light phase and ZT12 refers to the beginning of the dark phase. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham. It is noteworthy that the current study utilized CCM mice as a secondary model of cardiomyocyte-specific circadian clock disruption, and as such the data obtained for this model is located in Supplemental figures.

2.2. Rapamycin feeding

In select experiments, mice were provided food supplemented with encapsulated rapamycin at 42 ppm (eRAPA, Rapamycin Holdings Inc., San Antonio, TX); diet containing encapsulation material was used as a control.

2.3. In vivo insulin challenge

Mice were fasted for 6 h prior to anesthetization (400 mg/kg chloral hydrate), followed by administration of insulin (0.167 U/kg; a sub-maximal insulin level, determined by initial dose-response studies [data not shown]) or vehicle (saline) via the inferior vena cava (at ZT0 or ZT12). Hearts were isolated and snap frozen in liquid nitrogen 5 min after insulin/saline administration. Frozen hearts were stored at −80 °C, and subsequently utilized for Western blot analysis.

2.4. Ex vivo myocardial insulin challenge

Hearts were isolated (at ZT0 or ZT12) and perfused in the non-recirculating working mode as described previously [24,25]. A sub-set of hearts were challenged with insulin (100 μU/mL) for 5 min, and subsequently utilized for Western blot analysis. A second set of hearts were challenged with insulin (0, 10, or 100 μU/mL) for 30 min, during which time metabolic fluxes were measured via addition of metabolic tracers: D-[U-^{14}C]-glucose (0.20 mCi/L; oxidative and non-oxidative glucose utilization) and L-[2,3,4,5,6-^{3}H]-phenylalanine (0.20 mCi/L; protein synthesis) [30]. More specifically, the following was assessed: 1) full oxidation of exogenous glucose by following the release of [^{14}C]-CO_2 into the perfusate (i.e., exogenous glucose oxidation; [31]); 2) the anaerobic catabolism of exogenous glucose to lactate by following the release of [^{14}C]-lactate into the perfusate (i.e., a non-absolute measure that correlates with glucose uptake plus glycolysis; [32]); 3) the net incorporation of exogenous glucose into glycogen by following the generation of [^{14}C]-glycogen (i.e., net glycogen synthesis; [33]); and 4) the net incorporation of exogenous phenylalanine into protein by following the generation of [^{3}H]-protein (i.e., net protein synthesis; [34]). In the case of [^{14}C]-CO_2 and [^{14}C]-lactate release, coronary effluent was collected at 5-min intervals during the perfusion, and data are presented as steady state values (i.e., the last 10 min of the perfusion protocol). In the case of net glycogen and protein synthesis, hearts were freeze clamped at the end of the perfusion protocol, followed by extraction of [^{14}C]-glycogen and [^{3}H]-protein, as described previously [33,35].

2.5. In vivo protein synthesis

Mice were injected via tail vein with 150 mM Phenylalanine plus L-[2,3,4,5,6-^{3}H] Phenylalanine (0.18 mCi/kg), according to the flooding dose technique [35]. Myocardial protein was precipitated and washed with 10% trichloroacetic acid, neutralized with 1 N NaOH, and counted in a scintillation counter (Beckman Coulter).

2.6. Quantitative RT-PCR

RNA was extracted from hearts followed by assessment of gene expression using standard procedures as described previously [36,37]. Gene expression analysis of insulin signaling and autophagic components (*Insr, Irs1, Irs2, Pik3ca, Pik3r1, Pdpk1, Akt1, Akt2, As160, Gsk3β, I-1, Mtor, Ulk1, Lc3, P62*) was performed using conventional methods with SYBR Green; primer sequences are included in Supplemental Table 1. All RT-PCR data were normalized to the average expression of three different housekeeping genes: acidic ribosomal phosphoprotein P0 (*36B4*), hypoxanthine phosphoribosyltransferase 1 (*Hprt*), and TATA-binding protein (*Tbp*), as described previously [38]. ΔΔCt values are represented as fold change from a designated control group.