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Circadian rhythm of intracellular protein synthesis signaling in rat cardiac and skeletal muscles

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ABSTRACT

Intracellular signaling exhibits circadian variation in the suprachiasmatic nucleus and liver. However, it is unclear whether circadian regulation also extends to intracellular signaling pathways in the cardiac and skeletal muscles. Here, we examined circadian variation in the intracellular mammalian target of rapamycin (mTOR)/70 kDa ribosomal protein S6 kinase 1 (p70S6K) and extracellular signal-regulated kinase (ERK) pathways, which regulate protein synthesis in rat cardiac and skeletal muscles. Seven-week-old male Wistar rats were assigned to six groups: Zeitgeber time (ZT) 2, ZT6, ZT10, ZT14, ZT18, and ZT22 (ZT0, lights on; ZT12, lights off). The cardiac, plantaris, and soleus muscles were removed after a 12-h fasting period, and signal transducers involved in protein synthesis (mTOR, p70S6K, and ERK) were analyzed by western blotting. Circadian rhythms of signal transducers were observed in both cardiac (mTOR, p70S6K, and ERK) and plantaris (p70S6K and ERK) muscles ($p < 0.05$), but not in the soleus muscle. In the cardiac muscle, the phosphorylation rate of mTOR was significantly higher at ZT6 (peak) than at ZT18 (bottom), and the phosphorylation rate of p70S6K was significantly higher at ZT2 (peak) than at ZT18 (bottom). In contrast, in the plantaris muscle, the phosphorylation rate of ERK was significantly lower at ZT2 (bottom) than at ZT18 (peak). Our data suggested that protein synthesis via mTOR/p70S6K and ERK signaling molecules exhibits circadian variation in rat cardiac and fast-type plantaris muscles.

1. Introduction

Various physiological and metabolic processes, such as behavioral patterns, heart rate, blood pressure, and hormone secretion, exhibit a 24 h rhythm. These circadian rhythms are regulated by the central circadian clock, which is present in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN (central clock) plays a key role in the regulation of homeostatic functions and mediates the entrainment of the circadian rhythm to the environmental light/dark cycle [1].

Recent reports have demonstrated that the clock machinery is expressed not only in the SCN, but also in almost all other tissues, such as the heart, liver, kidney, and skeletal muscle [2,3]. Moreover, the peripheral clocks regulate diurnal changes in tissue-specific physiological processes. Numerous studies have indicated that clock genes, such as *CLOCK* and *BMAL1*, regulate the circadian rhythm in peripheral tissues [4,5]. The loss of these clock genes influences the function and growth of the liver, cardiac, and skeletal muscles of mice [3,6,7]. Additionally, more recent studies in mice have shown that the phosphorylation of molecules in intracellular signaling pathways, such as the extracellular signal-regulated kinase (ERK) and S6 ribosomal protein (rpS6) pathways, exhibits a circadian rhythm in the SCN [8,9] and regulates the output and input of the circadian clock. In the SCN, the ERK signaling pathway plays an important role in the clock-resetting mechanisms of mammalian circadian rhythms [10,11]. Moreover, rpS6 phosphorylation is regulated by the activation of mammalian target of rapamycin (mTOR) and its downstream target 70 kDa ribosomal protein S6 kinase 1 (p70S6K); the activation of this pathway leads to mRNA translation and protein synthesis [12]. However, it is not clear whether the phosphorylation of these pathways regulates the function of the circadian clock in peripheral tissues.

Interestingly, a recent report demonstrated that ERK signaling strictly regulates the circadian clock in mouse peripheral liver tissue, suggesting that the circadian oscillation of ERK activity regulates diurnal variation in liver function and homeostasis [13]. Furthermore, Lipton et al. [14] demonstrated that expression of the core clock protein BMAL1 in the mouse liver is mediated by the phosphorylation of mTOR/p70S6K and controls circadian timing. These pathways also play important roles in muscle growth and hypertrophy in the cardiac and skeletal muscles [15–17]; therefore, the circadian rhythm of these signaling pathways may be associated

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with dairy muscle protein turnover and muscle adaptation. However, circadian regulation of mTOR/p70S6K and ERK pathways in other peripheral tissues, such as the cardiac and skeletal muscles, is not well understood.

Therefore, in this study, we aimed to determine whether the activation of protein synthesis-related intracellular signaling pathways, such as the mTOR/p70S6K and ERK pathways, is involved in circadian rhythms in rat cardiac and skeletal muscles.

2. Materials and methods

2.1. Experimental animals and experimental design

The experiments in this study were approved by the Juntendo University Animal Care and Use Committee. Seven-week-old male Wistar rats were obtained from a licensed laboratory animal vendor (SLC Inc., Hamamatsu, Shizuoka, Japan). Rats were housed in an environmentally controlled room (temperature: 23 ± 1 °C; humidity: 55% ± 5%; 12 h dark/light cycle, with lights on at 18:00 and lights off at 6:00). Water and food were provided *ad libitum*.

Rats (n=48) were acclimated for 2 weeks and were then assigned to six Zeitgeber time points (ZT0, lights on at 18:00; ZT12, lights off at 6:00): ZT2 (n=8), ZT6 (n=8), ZT10 (n=8), ZT14 (n=8), ZT18 (n=8), and ZT22 (n=8). After 12 h of fasting, the rats were anesthetized with pentobarbital sodium (50 mg/kg) at each time point (ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22), and blood samples were collected from an abdominal vein. Cardiac, plantaris, and soleus muscles were quickly removed, weighed, and frozen in liquid nitrogen. Samples were stored at −80 °C until analysis.

Preparation of blood, cardiac muscle, and skeletal muscle samples.

Blood samples were centrifuged at 3000 rpm for 10 min to obtain serum and stored at −80 °C. Growth hormone (GH) and corticosterone concentrations were estimated by commercial laboratories (Shibayagi Co., Ltd., Shibukawa, Japan and Oriental Yeast Co., Ltd., Tokyo, Japan).

For protein analyses, the cardiac and plantaris muscles were powdered in liquid nitrogen. Samples were then homogenized in ice-cold homogenization buffer (50 mM HEPES [pH 7.4], 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 1% Triton X-100) containing a protease inhibitor cocktail (Complete EDTA-free; Roche, Penzberg, Germany) and phosphatase inhibitor cocktail (PhosSTOP; Roche). Homogenates were centrifuged at 12,000×*g* for 15 min at 4 °C, and the protein concentrations of the supernatants were determined using a BCA Protein Assay Kit (Thermo, Rockford, IL, USA).

2.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting, and immunodetection

To determine the protein expression and phosphorylation status in the muscles, SDS-PAGE and immunodetection were performed using techniques previously described by Yoshihara et al. [18]. Briefly, protein extracts were solubilized in sample buffer [99% glycerol, 2-β-mercaptoethanol, 20% SDS, 1 M Tris-HCl (pH 6.8), and bromophenol blue] and incubated at 95 °C for 5 min. Equal amounts of protein were then loaded onto 10% TGX FastCast acrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA) and run at 150 V for 50 min. Separated proteins were transferred to PVDF membranes (Bio-Rad Laboratories) using a Bio-Rad Mini Trans-Blot cell at 100 V for 60 min at 4 °C in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). After transfer, membranes were blocked for 1 h using blocking buffer (5% nonfat dry milk in Tween-Tris-buffered saline [T-TBS: 40 mM Tris-HCl, 300 mM NaCl, and 0.1% Tween 20, pH7.5]) at room temperature. The membranes were then incubated with the following primary antibodies: anti-phospho-mTOR Ser2448 (1:2000; #2971; Cell Signaling Technology, Danvers, MA, USA), anti-mTOR (1:2000; #2972; Cell Signaling Technology), anti-phospho-p70S6K Thr389 (1:2000; #9234; Cell Signaling Technology), anti-p70S6K (1:2000; #9202; Cell Signaling Technology), anti-phospho-p44/42 ERK1/2 Thr202/Thr204 (1:5000; #4370; Cell Signaling Technology), and anti-p44/42 ERK1/2 (1:5000; #4695; Cell Signaling Technology) in dilution buffer for 2 h at room temperature. After several washes in T-TBS, membranes were incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000; #7074; Cell Signaling Technology) in dilution buffer for 1 h at room temperature. After several washes in T-TBS, proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA), and signals were recorded using an ATTO Light Capture System (Tokyo, Japan). Analyses were performed using CS ANALYZER 3.0 (ATTO, Amherst, NY, USA). The ratio of phosphorylated protein to total protein expression was determined using arbitrary units.

2.3. Statistical analysis

All values are presented as means ± standard errors (SEs). Boxplots were used to identify outliers (Q1−(1.5×IQR) or Q3+(1.5×IQR)) in the data set. Circadian rhythms were analyzed statistically using the modified cosinor analysis (nonlinear least-squares [NLLS] Marquardt−Levenberg algorithm) [19]. The function was defined as $f(x)=M+A\times\cos(2p/T[x-ø])$, where M is the mesor (the average cycle value), A represents the amplitude (half the distance between peaks of the fitted waveform), T represents the circadian period, and ø is the acrophase (time point in the cycle of highest amplitude in radians). The circadian period (T) was 24 h under 12 h light and 12 h dark periods. Acrophase is expressed as hours elapsed from ZT0. The significance of circadian rhythmicity was assessed using the zero-amplitude test; *p*-values of less than 0.05 were considered statistically significant for the given period of the cosine curve approximation. Differences among groups were analyzed using a one-way analysis of variance (ANOVA). When a significant difference was observed, a post-hoc Scheffe's test was performed. Differences with *p*-values of less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Signal transduction

Fig. 1 shows the circadian variation in the mTOR, p70S6K, and ERK1/2 phosphorylation rate in the cardiac (A, B, and C), plantaris (D, E, and F), and soleus (G, H, and I) muscles. The total protein expression levels of mTOR, p70S6K, and ERK1/2 did not differ significantly among the experimental groups in the cardiac, plantaris, and soleus muscles (data not shown).

The cosinor analysis indicated that the rates of mTOR, p70S6K, and ERK1/2 phosphorylation displayed robust circadian rhythms in the cardiac muscle. The rate of mTOR phosphorylation was higher at ZT2 than at ZT18 ($p < 0.01$, bottom). The phosphorylation rate at ZT6 (peak) was significantly higher than those at ZT14 ($p < 0.01$), ZT18 ($p < 0.001$, bottom), and ZT22 ($p < 0.01$). Moreover, the phosphorylation rate of p70S6K at ZT2 (peak) was significantly elevated compared with those at ZT14 ($p < 0.01$) and ZT18 ($p < 0.01$, bottom). The phosphorylation rate at ZT6 was significantly higher than that at ZT18 ($p < 0.05$, bottom) in the cardiac muscle. No significant differences were observed among time points in the phosphorylation rate of ERK1/2 in the cardiac muscle.

In the plantaris muscle, a cosinor analysis confirmed circadian rhythms in the phosphorylation rates of p70S6K and ERK1/2. There were no significant differences in the phosphorylation rates of mTOR and p70S6K among time points. However, the ERK1/2 phosphorylation rate was significantly lower at ZT2 ($p < 0.001$, bottom), ZT6 ($p <$

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