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Effect of metformin and lipid emulsion on the circadian gene expression in muscle cells

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ABSTRACT

The circadian clock influences nearly all aspects of metabolism. However, little is known regarding the effect of the energy status on circadian rhythms. Our aim was to test the effect of two opposing energy situations, metformin and lipid emulsion (LE), on clock and metabolic circadian expression in differentiated C2C12 myotubes. Metformin treatment led to depleted ATP levels accompanied by elevated NADH levels, whereas LE treatment led to increased ATP and NAD^+ levels. Nevertheless, both LE and metformin treatments activated the AMP-activated protein kinase (AMPK) pathway. In contrast, the effect on circadian rhythms was completely different. LE led to disrupted clock and metabolic gene expression, whereas metformin led to mainly high-amplitude shifted rhythms. Combination of metformin and LE led to an antagonistic effect on circadian gene expression. Although metformin and LE have an opposing effect on circadian gene expression and on the cellular energy status, they both lead to AMPK activation.

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1. Introduction

The circadian clock, an endogenous time-tracking system, influences nearly all aspects of physiology (Lee et al., 2001; Reppert and Weaver, 2002). The core clock mechanism is composed of transcriptional–translational feedback loops that generate circadian rhythms via the concerted co-expression of specific clock genes (Schibler et al., 2003). The positive loop of the clock is composed of the transcription factor CLOCK which interacts with BMAL1 to drive transcription of a large number of genes. Among these genes are those that encode the PERIOD (PER1, PER2 and PER3) and CRYPTOCHROME (CRY1 and CRY2) proteins that serve as the negative feedback loop and inhibit CLOCK:BMAL1-mediated transcription (Reppert and Weaver, 2002). Other transcriptional loops involve the control of PERs and BMAL1 protein stability by casein kinase 1ε (CKIε) phosphorylation (Eide et al., 2005). *Bmal1* expression is negatively regulated by REV-ERBs and positively by retinoid-related orphan receptor α (RORα) (Preitner et al., 2002; Sato et al., 2004). The endogenous period produced by the circadian machinery is generally close, but not equal to, 24 h, therefore, it is entrained to external stimuli.

Metabolism is an important feedback to the circadian clock in peripheral tissues, synchronizing it to environmental cues, such as food availability (Froy, 2010). In addition, the core clock mechanism is tightly linked to metabolic pathways: (1) REV-ERBα and RORα, regulators of *Bmal1* transcription, regulate lipogenesis and lipid metabolism through interaction with PPARα (peroxisome proliferator-activated receptor α) (Preitner et al., 2002; Sato et al., 2004; Canaple et al., 2006). (2) PPARγ co-activator 1α (PGC-1α), which regulates energy metabolism, stimulates the expression of the clock genes *Bmal1* and *Rev-erbα*; mice lacking PGC-1α show abnormal diurnal rhythms of activity, body temperature and metabolic rate (Liu et al., 2007). (3) The $NAD(P)^+/NAD(P)H$ ratio, which depends on the metabolic state of the cell, dictates the binding of CLOCK:BMAL1 to DNA enhancer elements and leading to a change in the phase of cyclic gene expression (Rutter et al., 2001, 2002; Hirota and Fukada, 2004). (4) Activation of adenosine monophosphate-activated protein kinase (AMPK), a sensor of low energy and nutrient state in the cell, leads to altered circadian rhythms by destabilizing the negative limb of the circadian clock, PERs and CRYs (Um et al., 2007; Lamia et al., 2009).

AMPK is activated when cellular ATP levels are depleted and switches the cell from anabolic to catabolic pathways (Kahn et al., 2005). In peripheral tissues, following stress or hormonal signals, AMPK is phosphorylated and activated under elevated AMP levels by liver kinase B1 (LKB1) (Kahn et al., 2005). Consequently, phosphorylated AMPK activates PPARα and inactivates acetyl-CoA

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carboxylase (ACC), the rate limiting enzyme in fatty acid synthesis, leading to increased fatty acid oxidation and ATP production (Yoon et al., 2006). Targeted activation of AMPK with its indirect activator metformin shortened the circadian period of Rat-1 fibroblasts (Um et al., 2007). In peripheral tissues, intraperitoneal injection of metformin led to a phase advance in the circadian expression pattern of clock genes in wild-type mice (Um et al., 2007). However, oral administration of metformin affected the circadian clock and metabolic rhythms in a tissue-specific manner (Barnea et al., 2012).

As the levels of NAD(P)$^+$/NAD(P)H affect the core clock mechanism and modulation of the cellular energy sensor AMPK shifts circadian expression, the aim of this study was to evaluate the effect of two opposing energy situations, i.e., metformin and lipid emulsion, on circadian rhythms. Since skeletal muscle constitutes an important tissue whose metabolism is rapidly altered in response to different stimuli, we examined the effect of metformin and lipid emulsion on circadian clock and metabolic rhythms in C2C12 myotubes.

2. Material and methods

2.1. Cell culture, differentiation and treatment

C2C12 myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% bovine calf serum and 5% CO_2 at 37 °C. Differentiation of cells to myotubes was achieved by allowing the cells to reach confluence in 0.1% gelatin-coated flasks. When cells were confluent the medium was replaced with DMEM supplemented with 2% horse serum. Every day thereafter, fresh medium (DMEM plus 2% horse serum) was added to the cells. Differentiation was achieved 72–96 h after medium change.

For circadian gene expression, differentiated C2C12 myotubes were synchronized with a 2-h pulse of medium supplemented with 1 μM dexamethasone at 08:00. After 2 h, the medium was replaced with fresh medium (Control) or medium-supplemented with 0.1% lipid emulsion (LE, Lipofundin 20%: soybean oil based lipid emulsion (Braun, Melsungen, Germany)), 1 mM metformin or their combination. Following 6 h of incubation ($t = 0$), the cells were then harvested in triplicates per treatment per time-point at 4-h intervals for 20 h and results were plotted. For the evaluation of protein and metabolite levels, cells were harvested following 26 h incubation with the treatments. Three independent experiments were performed.

2.2. Detection of intracellular triglyceride accumulation

Following 6 h incubation with 0.1% lipofundin, the medium was removed and cells were washed twice with warm phosphate-buffered saline (PBS), incubated for 10 min with 0.1 μg/ml Nile Red in PBS, and examined by fluorescence microscopy (excitation: 540 nm; emission: 605 nm, Eclipse TS100; Nikon, Tokyo, Japan). Increase in lipid accumulation, a characteristic of differentiated adipocytes, was evident by yellow droplets.

2.3. RNA extraction and quantitative real-time PCR

Cells were lysed in TRI-Reagent and total RNA was extracted. Total RNA was DNase I-treated and 3 μg DNase I-treated RNA were then reverse-transcribed using MMuLV reverse-transcriptase. One hundred ng DNA were then subjected to quantitative real-time PCR using the Sybr Green Master kit and the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primer design and qPCR were performed as was described (Barnea et al., 2010). Genes were normalized to mouse actin. The fold change in target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Applied Biosystems, USA). The primers used are listed in Supplemental Table S1.

2.4. Western blot analysis

Cells were lysed in 0.15 ml lysis buffer (20 mM Tris, 145 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 100 μM PMSF, 50 μM NaF, 1 mM sodium orthovanadate). Samples were run onto an SDS–polyacrylamide gel (10% for AMPK/LKB1, 7.5% for ACC), and after electrophoresis, proteins were transferred onto nitrocellulose membranes as was described (Froy et al., 2006). Blots were incubated with AMPK/pAMPK, ACC/pACC and LKB1/pLKB1 antibodies (Cell Signaling Technology, Beverly, MA, USA) and after several washes, with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA). The immune reaction was detected by enhanced chemiluminescence (ECL). Finally, bands were quantified by scanning and densitometry and expressed as arbitrary units.

2.5. NAD$^+$/NADH measurements

NAD$^+$ and NADH were measured as described before (Kato et al., 1973). Briefly, C2C12 myotubes grown in 25-cm flasks were homogenized in 500 μl of acid extraction buffer to obtain NAD$^+$, or 500 μl of alkali extraction to obtain NADH. Homogenates were then heated to destroy enzymatic activity, neutralized and protein concentrations were determined by the Bradford method. Low levels of NAD$^+$/NADH were amplified through a series of cycling steps and the concentration of nucleotides was measured in a 5 μl sample fluorimetrically (excitation: 340 nm, emission: 460 nm) by a microfluorometer plate reader (GENios, Tecan, Grödig, Austria). Values for both nucleotides were detected within the linear range of the standard curve and normalized to protein concentration. NAD$^+$/NADH ratios were calculated by comparing the ratios obtained from parallel cell dishes in each experiment.

2.6. ATP quantification assay

Following a 26-h treatment, C2C12 myotubes were washed twice with ice cold PBS (pH 7.4) and lysed in glycylglycine buffer (75 mM glycylglycine; 15 mM $MgCl_2$, pH 7.8). Samples were boiled (5 min at 95 °C) and centrifuged at 18,000 × g for 5 min. Assessment of ATP concentration in supernatant was performed by a firefly luciferase assay using a commercially available kit (ENLITEN®, Promega, Madison, WI, USA) and a luminometer (Tristar3 LB941, Berthold Technologies, Bad Wildbad, Germany). ATP concentrations from each sample were normalized to protein concentrations determined by the Bradford method.

2.7. Statistical analyses

All results are expressed as means ± SE. One-way ANOVA was used to analyze circadian pattern with several time-points. Statistical analysis was performed with JMP software (version 10, SAS Institute Inc. Cary, NC, USA). For accurate evaluation of rhythms, CircWave 1.4 (available at http://www.euclock.org) was used to produce a Fourier-curve that describes the data. Then the acrophase and p value of gene oscillation were calculated using the free Cosinor analysis software (Version 2.3 available at http://www.circadian.org/softwar.html). Levels were assessed by 1-way ANOVA and a least-significant difference Tukey–Kramer post hoc analysis was used for comparison among the groups. The significance level for all analyses was set at $p < 0.05$.

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