Original article

MOLECULAR METABOLISM

Muscle insulin sensitivity and glucose metabolism are controlled by the intrinsic muscle clock

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ABSTRACT

Circadian rhythms control metabolism and energy homeostasis, but the role of the skeletal muscle clock has never been explored. We generated conditional and inducible mouse lines with muscle-specific ablation of the core clock gene *Bmal1*. Skeletal muscles from these mice showed impaired insulin-stimulated glucose uptake with reduced protein levels of GLUT4, the insulin-dependent glucose transporter, and TBC1D1, a Rab-GTPase involved in GLUT4 translocation. Pyruvate dehydrogenase (PDH) activity was also reduced due to altered expression of circadian genes *Pdk4* and *Pdp1*, coding for PDH kinase and phosphatase, respectively. PDH inhibition leads to reduced glucose oxidation and diversion of glycolytic intermediates to alternative metabolic pathways, as revealed by metabolome analysis. The impaired glucose metabolism induced by muscle-specific *Bmal1* knockout suggests that a major physiological role of the muscle clock is to prepare for the transition from the rest/fasting phase to the active/feeding phase, when glucose becomes the predominant fuel for skeletal muscle.

Keywords Bmal1; Circadian rhythms; Glucose metabolism; Glucose uptake; Skeletal muscle; Muscle insulin resistance

1. INTRODUCTION

Circadian rhythms and energy metabolism are inextricably linked. While many behavioral and physiological processes linked to energy metabolism show daily fluctuations under the control of an endogenous circadian clock and environmental cues, the circadian clock is itself influenced by feeding and activity rhythms, and ultimately metabolic state [1–3]. The regulation of body metabolism by normal circadian rhythms has important clinical implications, since chronic circadian misalignment, as occurs in shift work, is associated with a higher prevalence of insulin resistance, obesity, metabolic syndrome and diabetes [4–6].

The circadian timing system is composed of a central pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus and autonomous molecular oscillators present in all cells of the body. At the cellular level, the core of this 24-h rhythm generating oscillator is composed of molecular feedback loops [7]. In the major loop, the transcription factors BMAL1 and CLOCK bind as heterodimers to the promoters of *Per* and *Cry* genes inducing their transcription. Through negative feedback, PER and CRY proteins translocate to the nucleus and repress the CLOCK:BMAL1 complex. In a second regulatory loop, the expression of *Bmal1* (*Arntl*) is controlled by CLOCK:BMAL1-dependent transcriptional activators of the ROR family, such as RORα, and repressors of the REV-ERB family, such as REV-ERBα. In addition to the core clock genes, all tissues contain a large number of oscillating genes, most of which are tissue-specific and regulated either directly by the core oscillator or by extrinsic circadian signals [7].

An important role for circadian clocks in the regulation of glucose metabolism is supported by genome-wide DNA-binding analyses and by ablation of clock genes. A time-resolved and genome-wide map of BMAL1 binding in mouse liver, derived from ChIP-seq analyses, has shown that carbohydrate metabolism is a major output of the circadian clock in this tissue [8]. Global inactivation of different clock genes leads to altered glucose metabolism: *Clock* mutant mice display hyperglycemia, obesity and metabolic syndrome [9]; *Per1*/*Per2* double knockout mice and *Cry1*/*Cr2* double knockout mice exhibit glucose

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Abbreviations: BSA, bovine serum albumin; 2-DG, 2-Deoxyglucose; GSEA, Gene Set Enrichment Analysis; imKO, inducible muscle-specific *Bmal1* knockout; HK2, hexokinase 2; KHB, Krebs–Henseleit buffer; mKO, muscle-specific *Bmal1* knockout; PDH, pyruvate dehydrogenase; PDK, PDH kinase; PDP, PDH phosphatase; SCN, suprachiasmatic nucleus; ZT, *Zeitgeber* time

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intolerance [10,11]; *Bmal1* null mice are also glucose intolerant, and show altered gluconeogenesis [10,12]. In addition, the normal circadian rhythm of increased insulin sensitivity during the active/feeding phase is completely abolished in global *Bmal1* null mice [13].

Interpretation of whole body knockout studies is complicated by the fact that all tissues are affected, including both the central pacemaker and peripheral clocks. The hierarchical role of the central pacemaker was demonstrated by the finding that circadian rhythms of blood glucose, insulin and glucagon, as well as glucose tolerance, are all abolished by SCN lesion [14]. An equally important role for peripheral oscillators has been demonstrated by tissue-specific knockout of clock genes, with distinct effects depending on the tissue. Selective knockout of *Bmal1* in the liver causes hypoglycemia [10], while ablation of the same gene in the endocrine pancreas leads to fasting hyperglycemia and severe glucose intolerance [15]. Local insulin sensitivity was found to be normal in mice lacking *Bmal1* selectively in the liver or adipose tissue [10,16]. Although skeletal muscle is a primary site of insulin-dependent glucose disposal, the specific role the muscle clock plays in regulating glucose uptake and metabolism is not known. A daily rhythm in plasma glucose concentration has been described in both animals and humans, with a peak just before the start of the main activity period, reflecting fluctuations in glucose export, glucose uptake and insulin sensitivity [14]. However, it is not clear whether these circadian rhythms are controlled by peripheral clocks in muscle and adipose tissue, or by humoral or neural signals emanating from the SCN. A circadian rhythm of glucose uptake was also demonstrated in muscle cell cultures, but these daily fluctuations were observed both in basal and insulin-stimulated conditions [17]. To understand the physiological role the intrinsic muscle clock plays in glucose metabolism, we have generated two skeletal muscle-specific *Bmal1* knockout models. We report here that muscle-specific *Bmal1* ablation causes impaired insulin-dependent glucose uptake and reduced glucose oxidation in skeletal muscle, and we identify potential mechanisms involved in mediating these effects.

2. MATERIALS AND METHODS

2.1. Animals

Muscle-specific inactivation of *Bmal1* (mKO) was obtained from the cross between a C57BL/6 mouse line with floxed *Bmal1* [18] and a C57BL/6 mouse line carrying a Cre recombinase transgene under control of the *Mlc1f* promoter (*Mlc1f–Cre*) [19]. In the resulting mKO mice, the region coding for the BMAL1 basic helix–loop–helix DNA binding domain is excised. Cre-negative littermates were used as controls.

A second knockout model with inducible muscle-specific inactivation of *Bmal1* (imKO) was obtained by crossing the *Bmal1* floxed line with mice carrying an α-skeletal actin driven Cre recombinase fused to a mutated estrogen receptor [20], which can be activated by treatment with tamoxifen (i.p. 1 mg/day for 5 days). Cre negative littermates, also receiving tamoxifen treatment, were used as controls. All strains had been backcrossed a minimum of 6 times with C57BL/6 mice. Experimental protocols were reviewed and approved by the local Animal Care Committee, University of Padova. Animals were housed in a temperature-controlled room (22 °C) under a 12 h light–dark regimen, with lights on at ZT0 (6 am), lights off at ZT12 (6 pm), with standard chow diet (Mucedola, Settimo Milanese, Italy) and water provided *ad libitum*. All tissues were collected immediately after cervical dislocation at ZT0, 4, 8, 12, 16, and 20, snap frozen in liquid nitrogen and stored at −80 °C until subsequent use. To monitor locomotor activity levels, a telemetric sensor (PhysioTel, TA10TA-F20, Data Sciences, Inc.) was implanted i.p. under general anesthesia. Mice were allowed to recover for at least 2 weeks, and locomotor activity was recorded continuously for 12 days.

2.2. Immunofluorescence

Cryosections of fast and slow muscles were stained with monoclonal anti-myosin heavy chain (MyHC) antibodies produced in our lab [21], and now distributed by the Developmental Studies Hybridoma Bank (DSHB, University of Iowa): BA-D5 (IgG2b, supernatant, 1:100 dilution) specific for MyHC-I, SC-71 (IgG1, supernatant, 1:100 dilution) specific for MyHC-2A and BF-F3 (IgM, purified antibody, 1:100 dilution) specific for MyHC-2B. Type 2X fibers are not recognized by these antibodies, and so appear black. Three different secondary antibodies (Jackson ImmunoResearch) were used to selectively bind to each primary antibody: goat anti-mouse IgG1, conjugated with DyLight488 fluorophore (to bind to SC-71); goat anti-mouse IgG2b, conjugated with DyLight405 fluorophore (to bind to BA-D5); goat anti-mouse IgM, conjugated with DyLight549 fluorophore (to bind to BF-F3). Muscle sections, 8 μm thick, were incubated with M.O.M. IgG blocking solution (Vector) for 1 h at room temperature, then briefly washed twice with PBS for 2 min. A solution with all the primary antibodies in phosphate-buffered saline (PBS) containing 0.5% of bovine serum albumin (BSA) was then prepared, and sections were incubated for 1 h at 37 °C. After 3 washes (5 min each) with PBS, sections were incubated for 30 min at 37 °C with a solution with the three different secondary antibodies, diluted in PBS containing 0.5% of BSA and 5% of goat serum. After 3 washes with PBS (5 min each) and a brief rinse in water, sections were mounted with Elvanol. A control incubation with no primary antibodies was performed, and also control incubations with each primary antibody and non-specific secondary antibodies to exclude any possible cross-reaction. Pictures were collected with an epifluorescence Leica DM5000 B equipped with a Leica DFC 300 FX camera. Single-color images were merged to obtain a whole muscle reconstruction with Adobe Photoshop CS2 (Adobe Systems Inc.).

2.3. Electron microscopy

Muscles were fixed at resting length in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. During subsequent washing in phosphate buffer, small bundles of fibers were dissected from the superficial regions of the muscles and postfixed in 1% OsO_4 in phosphate buffer and treated with uranyl acetate before dehydration and embedding in Epon. Ultrathin sections were stained with lead citrate.

2.4. Force measurements

In vivo force measurements were performed as described previously [22]. Briefly, mice were anesthetized and stainless steel electrode wires were placed on either side of the sciatic nerve. Torque production of the plantar flexors was measured using a muscle lever system (Model 305c; Aurora Scientific, Aurora ON, Canada). The force–frequency curves were determined by increasing the stimulation frequency in a stepwise manner, pausing for 30 s between stimuli to avoid effects due to fatigue. Muscle force was normalized for the weight of the gastrocnemius muscle.

2.5. RNA isolation and qPCR

Total RNA was isolated using TRIzol (Invitrogen) followed by cleanup with the RNeasy Mini Kit (Qiagen). RNA integrity was evaluated with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and quantified with a NanoVue spectrophotometer (GE Healthcare Life

