Relationship between AMPK and the transcriptional balance of clock-related genes in skeletal muscle

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CIRCADIAN CLOCKS PROVIDE AN ADAPTIVE ADVANTAGE by coordinating physiological, behavioral, and biochemical events to predictable daily changes in the environment. Circadian rhythms in mammalian physiological functions are driven by a central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and this master clock is thought to synchronize slave oscillators in peripheral tissues (34). The central and peripheral oscillators share a common molecular circuitry, with a battery of transcriptional activators and repressors forming a self-sustained transcriptional feedback loop. CLOCK and ARNTL (alias BMAL1) are basic helix-loop-helix transcriptional activators that regulate Per and Cry gene expression. PER and CRY inhibit their own transcription, and their turnover allows this cycle to restart. The transcription factors BHLHB2 and BHLHB3 repress Per activation, whereas orphan nuclear receptors of the NR1D and ROR families maintain circadian clock function (26, 31). Importantly, circadian rhythms are associated with an increased risk for the development of metabolic disorders in humans (13).

AMPK is a heterotrimeric serine/threonine protein kinase composed of a catalytic α-subunit and noncatalytic β- and γ-subunits. The mammalian genome contains seven AMPK genes (22). Transgenic mice with Prkag3-isoforms. We have substitution in the AMPK α-subunit knockout (Prkag3-/-) mice or wild-type littermates were injected with saline or an AMPK activator, 5-amino-4-imidazole-carboxamide riboside (AICAR), and white glycolytic gastrocnemius muscle was removed for gene expression analysis. Genes involved in the regulation of energy homeostasis in eukaryotic cells (reviewed in Ref. 6). Circadian rhythms in mammalian physiological functions are driven by a central pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and this master clock is thought to synchronize slave oscillators in peripheral tissues (34). The central and peripheral oscillators share a common molecular circuitry, with a battery of transcriptional activators and repressors forming a self-sustained transcriptional feedback loop. CLOCK and ARNTL (alias BMAL1) are basic helix-loop-helix transcriptional activators that regulate Per and Cry gene expression. PER and CRY inhibit their own transcription, and their turnover allows this cycle to restart (17, 25). The stability and precision of circadian rhythms is further enhanced by the interplay between different modulators of the core clock genes. The helix-loop-helix transcription factors BHLHB2 (alias DEC1) and BHLHB3 (alias DEC2) repress CLOCK- and ARNTL-induced transactivation of the Per promoter while opposing activities of orphan nuclear receptors of NR1D (alias REV-ERB), and ROR families maintain circadian expression of Arntl (9, 10).

The master circadian pacemaker is primarily entrained by light, which illuminates through a retinohypothalamic tract linking the retina to the SCN (23). Peripheral circadian clocks can be synchronized by a master clock in SCN via chemical cues, such as rhythmically secreted hormones, or directly respond to the environment. Resistance exercise can directly affect expression levels of key clock components and downstream targets in skeletal muscle (35). Moreover, locomotor activity acts to phase-coordinate the expression of rhythmic genes in skeletal muscle (20). In nocturnal rodents fed exclusively during daytime, the phase of circadian gene expression becomes completely inverted in peripheral tissues, but not in the SCN (8, 28). Similarly, temperature cycles of the environment that oppose the natural rhythm can shift the phases of circadian oscillators in peripheral tissues, whereas the phase of the SCN master clock remains unaltered (5). The mechanism by which the peripheral circadian clock is regulated by external factors remains largely unexplored. Perturbations of circadian rhythms are associated with an increased risk for the development of metabolic disorders in humans (13). Importantly, glucose intolerance and obesity develop in mice with defective clock function (26, 31).

AMPK-activated protein kinase (AMPK) is a critical regulator of energy homeostasis in eukaryotic cells (reviewed in Ref. 6). AMPK is a heterotrimeric serine/threonine protein kinase composed of a catalytic α-subunit and noncatalytic β- and γ-subunits. The mammalian genome contains seven AMPK genes encoding for two α-, two β-, and three γ-isofoms. We have provided evidence that AMPKγ3 subunit (Prkag3) is selectively expressed in glycolytic (white, fast-twitch type II) skeletal muscle, where it primarily forms heterotrimers with the α2- and β2-subunits (2). The dominant Rendement Napole phenotype identified in Hampshire pigs is caused by a R225Q substitution in the AMPKγ3 subunit (22). Transgenic mice with skeletal muscle-specific expression of the mutant (R225Q) form
of the mouse AMPKγ3 have elevated glycogen levels and increased glycogen resynthesis after exercise (2). The AMPK activator 5-amino-4-imidazole-carboxamide riboside (AICAR) failed to increase glucose uptake in isolated muscle of AMPKγ3 knockout mice, whereas contraction effects were preserved. Moreover, AMPKγ3 R225Q transgenic mice were protected against excessive triglyceride accumulation in skeletal muscle and dietary-induced insulin resistance, presumably because of an increase in lipid oxidation (2).

Here, we provide evidence that AMPK complexes are involved in regulation of the expression of clock-related genes in skeletal muscle. Thus AMPK appears to serve as a molecular link between the circadian oscillator and energy metabolism in peripheral tissues.

MATERIALS AND METHODS

Animal experiments. Prkag3−/− mice were created by conventional gene targeting techniques where a premature stop codon was introduced into the gene, skipping most of the 489 amino acids encoded by the wild-type transcript (2). Although conventional knockout technique leads to inactivation of AMPKγ3 gene is all tissues, the primary effect of the gene targeting is expected to be seen in the skeletal muscle, since APMKγ3 is selectively expressed in this tissue (18). Mice used in the study were bred into C57BL/6 genetic background. Mice were maintained in a 12:12-h light-dark cycle (lights on 6:00 AM to 6:00 PM, lights off 6:00 PM to 6:00 AM) and were cared for in accordance with regulations for the protection of laboratory animals. The study was performed after prior approval from the local ethical committee. Mice had free access to food before the experiment. Food was removed just before intraperitoneal injection of AICAR (0.25 g/kg dissolved in saline) or saline (0.9% NaCl). Blood samples were obtained via tail vein before and 15, 30, 60, and 120 min after injection for measurement of glucose concentration (One Touch Basic glucose meter; Lifescan). Glucose tolerance curves were performed, and the mean log_2 intensity for each group was calculated. The log_2 fold change was calculated by subtracting the mean log_2 intensity for one test group from the mean log_2 intensity for the test group. Statistical significance of the difference in gene expression was determined using two-sided Student’s t-test. A transcript was considered differentially expressed if the mean absolute fold change was >1.3 and the P value was ≤0.05. In addition, the mean intensity in the group showing highest expression should have been >50.

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) was performed on an extended set of samples, including 7–11 mice in each group, whereas RNA from 6 animals in each group was used in the gene array analysis. The primers were designed using the Primer Express 2.1 (Applied Biosystems; see Table 1 for primer information). PCR was performed in a final volume of 25 μl, consisting of diluted cDNA sample, 1× SYBR Green PCR Master Mix (Applied Biosystems), primers optimized for each target gene, and nuclear-free water. Of the three different housekeeping genes (Arbp, Gadph, Actb) tested in the muscle samples, Arbp (acidic ribosomal phosphoprotein PO) showed least variation and was selected as a normalization gene in qRT-PCR studies. Relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against Arbp using the standard curve method. AICAR vs. saline-treated mice were compared using two-sided Student’s t-test.

Western blot analysis. Extensor digitorum longus (EDL) muscles were homogenized in ice-cold buffer [10% glycerol, 20 mM sodium pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% Nonidet polyoxyethylene (20) sorbitan monooleate (NP-40), 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 25 mM β-mercaptoethanol] and aliquots (10 μg) were subjected to SDS–PAGE. The proteins were transferred to nitrocellulose membranes and probed with primary antibodies against AMPKα1, AMPKα2, AMPKγ3, and β-actin. Blots were visualized using the enhanced chemiluminescence method (Amersham Life Science). The corresponding bands were quantified by densitometry using ImageQuant (Molecular Dynamics).

Table 1. Primer sequences used for quantitative real-time PCR

<table>
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<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Sense Primer (5’-3’)</th>
<th>Antisense Primer (5’-3’)</th>
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<td>Arbp</td>
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<td>GAGGAATCACTAGATCGAGATGATGGA</td>
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<td>Clock</td>
<td>NM_007715</td>
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<td>Arntl</td>
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<tr>
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<td>AGATCTTGTGGAAGATGGACGATGATGGA</td>
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<tr>
<td>Cry2</td>
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<td>AGGCCCCGACGCGAGGAA</td>
<td>GTTTTTACGGACCGACTCATTCT</td>
</tr>
<tr>
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Fig. 1. Effect of systemic 5-aminoo4-imidazole-carboxamide riboside (AICAR) administration on blood glucose profile and skeletal muscle gene expression in wild-type and Prkag3−/− mice. A: WT mice were randomized to wild-type and Prkag3−/− mice. AICAR (0.25 g/kg) or saline was administered to wild-type and Prkag3−/− mice by tail vein injection, as described in MATERIALS AND METHODS. Blood glucose was measured at the indicated time points. Data are means ± SE for n = 6 wild-type and n = 6 Prkag3−/− mice. B: White gastrocnemius muscle was collected at 3.5, 6.5, and 9.5 h after an ip injection of either AICAR (0.25 g/kg) or saline from wild-type mice (n = 4 for AICAR and n = 4 for saline treatment at each time point). mRNA expression levels of known transcriptional targets for AICAR (Ppargc1a, Hk2, Slc2a4, and Ucp3) were assessed by quantitative real-time PCR (qRT-PCR). Results are fold change of expression (mean ± SE) for AICAR- vs. saline-treated mice at each time point. The expression level in the saline-treated mice is set to 1, and the error bar represents the average SE for the saline-treated groups over the 3 time points. Statistical differences comparing AICAR vs. saline-treated mice were determined by 2-sided Student’s t-test (*P < 0.05, **P < 0.01, and ***P < 0.001).

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Fig. 2. Effect of systemic AICAR administration on clock gene expression in wild-type and Prkag3−/− mice. White gastrocnemius muscle was obtained 6 h after AICAR (0.25 g/kg) or saline administration from wild-type (A) or Prkag3−/− mice (B). mRNA expression (mean ± SE) of Bhlhb2, Cry2, Nr1d1, and Per1 was determined by qRT-PCR; n = 7–11 mice in each group. Statistical differences were determined by 2-sided Student’s t-test (*P < 0.05 and **P < 0.01).

**Whole body energy homeostasis.** Oxygen consumption, respiratory exchange ratio (RER), and locomotor activity were measured using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH). Male Prkag3−/− mice and wild-type littermates (n = 8) were housed individually with ad libitum access to standard chow and water. Mice were acclimatized to the metabolic cages for 24 h before a 120-h period of automated recordings every 20 min. Oxygen consumption was determined by an open-circuit Oxymax. Sample air from individual cages was passed through sensors to determine O2 and CO2 content. Oxygen consumption was calculated as the difference between the input and output oxygen flow. RER was calculated as the ratio between the CO2 production and the O2 consumption. An RER of 1.0 indicates high utilization of carbohydrates for energy, and an RER of 0.7 indicates increased fatty acid oxidation (19). Ambulatory locomotor activity was measured by consecutive beam breaks in adjacent beams under the 24-h period.

**RESULTS AND DISCUSSION**

To characterize the functional groups of genes, which are transcriptionally regulated in an AMPKγ3-dependent manner, a gene expression analysis was conducted in white gastrocnemius muscle of wild-type and Prkag3−/− mice after systemic...
AICAR administration. Gene array approach was applied for global expression profiling, offering an advantage of unbiased data collection, compared with candidate gene-based approaches. AICAR activates AMPK in vitro and in vivo and increases skeletal muscle glucose transport via the AMPK system (4, 15, 21). In isolated glycolytic skeletal muscle from Prkag3^-/- mice, AICAR-induced glucose uptake was abolished, providing an essential role of AMPK3 subunit in AICAR-mediated glucose transport in skeletal muscle (2). Prkag3^-/- mice and wild-type littermates were injected with AICAR (0.25 g/kg) or saline (0.9% NaCl), and blood glucose concentration was determined for up to 120 min. AICAR injection reduced blood glucose concentration in wild-type mice at 15 min (P < 0.001), with maximal effect observed at 30 min (P < 0.001), compared with the saline group. Similar results were obtained in Prkag3^-/- mice [significant reduction at 15 min (P < 0.01) and maximal reduction at 30 min (P < 0.001)]. Blood glucose levels were normalized at 120 min after AICAR administration in wild-type and Prkag3^-/- mice (Fig. 1A).

Although AICAR-mediated glucose transport in isolated EDL muscle of Prkag3^-/- mice is abolished (2), the hypoglycemic effect of systemic AICAR injection was identical in Prkag3^-/- and wild-type mice (Fig. 1A). The increase in skeletal muscle glucose transport via GLUT4 translocation and the suppression of glucose production in the liver via regulation of hepatic gluconeogenic genes have been shown to contribute to the AMPK-mediated hypoglycemic effect of AICAR (3, 4, 7, 14, 15). The magnitude of AICAR-stimulated glucose transport into skeletal muscle is further dependent on nutritional status of the animal and muscle fiber type (1, 11). Importantly, AICAR is known to reduce gluconeogenesis in the liver also by AMPK-independent inhibition of fructose-1,6-bisphosphatase (33). The complex question of the relative contribution of skeletal muscle vs. liver in mediating the glucose-lowering effect of AICAR in vivo remains unclear. AMPK3 subunit is expressed only in skeletal muscle (18) and, whereas in vitro data indicate a key role for this subunit in AICAR-mediated glucose transport in the muscle (2), AMPK3 is unlikely to be involved in regulation of glucose metabolism in the liver. Therefore, the suppression of the hepatic glucose production by AICAR may possibly override the reduced muscle-specific response in AMPK3 knockout mice, which would explain the similar blood glucose profile in Prkag3^-/- and wild-type mice observed in this study.

In a pilot study, the time frame for AICAR-induced changes in skeletal muscle gene expression was determined. White gastrocnemius muscle was dissected at 3.5, 6.5, or 9.5 h after intraperitoneal injection of AICAR or saline, and expression profiles of known targets for AICAR (12, 29) were determined by qRT-PCR analysis. From the targets analyzed, Pparge1a, Slc2a4 (alias Glut4), and Ucp3 had a highest peak of expression at 6.5 h postinjection. Hk2 was maximally induced at 3.5 h after AICAR administration, although the significant upregulation was observed at the 6.5-h time point as well (Fig. 1B).

Based on these results, the skeletal muscle samples for gene array experiments were collected 6 h after AICAR administration to identify the AICAR-derived changes in global gene expression profile.

One functional group of targets, which appeared differentially regulated in response to AICAR in wild-type mice, was genes involved in the regulation of circadian rhythms. To minimize erroneous conclusions due to technical variability and multiple testing effects inherent to the microarray technology, qRT-PCR analysis was applied to validate expression profiles of selected genes involved in regulation of clock
function. PER and CRY proteins are known to form the negative feedback loop by inhibiting their own transcription through the association with the CLOCK-ARNTL (17, 25). AICAR injection increased mRNA expression of Cry2 gene in skeletal muscle from wild-type mice compared with the saline-treated mice (P < 0.01) (Fig. 2A). However, mRNA expression of Cry1, Per1/2, Clock, and Arntl was unaltered in response to the AICAR injection (data not shown). Bhlhb3 and Nr1d1 genes are negative regulators of clock function. BHLHB2 and -3 were shown to repress CLOCK- and ARNTL-induced transcription activation through direct protein-protein interactions with ARNTL and/or competition for promoter elements (10). In our experiments, skeletal muscle mRNA expression of Bhlhb2 was suppressed after AICAR injection in wild-type mice (P < 0.005; Fig. 2A), whereas the Bhlhb3 expression was unaltered. The members of the orphan nuclear receptor NR1D and ROR families are known to repress or activate the Arntl gene transcription, respectively (9). Only the Nr1d1 and Nr1d2 display a rhythmic expression, with clear amplitude in skeletal muscle (9). Interestingly, AICAR downregulated Nr1d1 and Nr1d2 gene expression in wild-type mice (P < 0.05; Fig. 2A), whereas expression of Nr1d2, Rora, and Rorc was unchanged (data not shown). AICAR injection in Prkag3+/− mice did not significantly alter Bhlhb2, Cry2, or Nr1d1 mRNA levels (Fig. 2B). When the basal level of core clock gene expression in Prkag3+/− mice was compared with the wild-type littermates, only Per1 expression was higher in knockout mice (P < 0.05), although this gene was unaltered in response to AICAR treatment. No differences in protein expression were detected in AICAR vs. saline-treated groups when the levels of Per1, Cry2, and BHLHB1 were compared in samples collected 6 h posttreatment (data not shown). This was unsurprising since the time point chosen for sample collection in this study was estimated to be the most optimal to quantify differences at the transcription level (Fig. 1B) and was unlikely to be the optimum time point for assessing translational differences.

To determine whether the AMPKγ3 subunit plays a role in the circadian regulation of metabolism, RER was measured by indirect calorimetry in Prkag3+/− mice and wild-type littermates over 24 h. RER was elevated in wild-type mice during the dark compared with the light phase of the day (P < 0.001; Fig. 3A), corresponding to the switch in substrate utilization previously suggested (19, 30). Importantly, the light-dark cycle variation of RER was unchanged in Prkag3+/− littermates, indicating the knockout mice are unable to undergo a diurnal shift in energy utilization. The 24-h rhythm of spontaneous locomotor behavior was unaltered in Prkag3+/− mice vs. wild-type mice, with both groups exhibiting clear diurnal activity patterns showing increased nocturnal activity. Thus the difference in the RER in the Prkag3+/− mice is unrelated to changes in locomotor activity (Fig. 3B).

Energy homeostasis, including glycolysis, oxidative phosphorylation, and lipid metabolism, is subject to circadian regulation, synchronizing both energy intake and expenditure to changes in the external environment. More recently, a causal effect of metabolism on peripheral circadian rhythms has become appreciated. The DNA-binding activity of the CLOCK-ARNTL was found to be regulated by the redox state of NAD (27), which is directly influenced by energy metabolism. PPARGC1A (alias PGC-1α), a transcriptional coactivator that regulates energy metabolism, influences the circadian clock in skeletal muscle and liver (16). Interestingly, metformin induces the degradation of PER2 protein in an AMPKα2-dependent manner (32).

Here we demonstrate that AICAR, a pharmacological AMPK activator, regulates skeletal muscle expression of several clock-related genes in an AMPKγ3 subunit-dependent manner (Fig. 4). Moreover, the diurnal shift in RER observed in wild-type mice is blunted in Prkag3−/− littermates. In conclusion, AMPK heterotrimeric complexes containing the γ3-subunit play a role in linking peripheral circadian oscillators and energy metabolism in skeletal muscle while the molecular signaling pathways underlying the relationship between AMPK and clock gene expression remain to be elucidated.

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