Activation of 5′-AMP-activated Kinase with Diabetes Drug Metformin Induces Casein Kinase Iε (CKIε)-dependent Degradation of Clock Protein mPer2*

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Metformin is one of the most commonly used first line drugs for type II diabetes. Metformin lowers serum glucose levels by activating 5′-AMP-activated kinase (AMPK), which maintains energy homeostasis by directly sensing the AMP/ATP ratio. AMPK plays a central role in food intake and energy metabolism through its activities in central nervous system and peripheral tissues. Since food intake and energy metabolism is synchronized to the light-dark (LD) cycle of the environment, we investigated the possibility that AMPK may affect circadian rhythm. We discovered that the circadian period of Rat-1 fibroblasts treated with metformin was shortened by 1 h. One of the regulators of the period length is casein kinase 1ε (CKIε), which by phosphorylating and inducing the degradation of the circadian clock component, mPer2, shortens the period length. AMPK phosphorylates Ser-389 of CKIε, resulting in increased CKIε activity and degradation of mPer2. In peripheral tissues, injection of metformin leads to mPer2 degradation and a phase advance in the circadian expression pattern of clock genes in wild-type mice but not in AMPK α2 knock-out mice. We conclude that metformin and AMPK have a previously unrecognized role in regulating the circadian rhythm.

Animal behavior, including spontaneous locomotion, sleeping, eating, and drinking, follows a 24-h light-dark (LD)2 cycle of the environment. The master pacemaker for the rhythmic behavior lies in the suprachiasmatic nucleus (SCN) of the hypothalamus (1). The SCN neurons, cued by the LD cycle, orchestrate the circadian rhythms of peripheral clocks that reside in most cells of the body. The pacemaker, both in the SCN and in peripheral tissues, consists of a self-sustaining near 24-h rhythm in the expression of core clock genes. A central component of this pacemaker is the negative-feedback loop, which results from Per and Cry proteins suppressing their own transcription with a precisely timed lag. A key regulator of the period length is casein kinase 1ε (CKIε) (2). CKIε and its homolog CKIδ regulate the circadian period by phosphorylating mammalian Per proteins (3–6). The role of CKIδ in mammalian circadian rhythm is best illustrated by the semidominant mutation in hamster CKIδ, tau (7). CKIδαβ is a highly specific gain-of-function mutation that increases the CKIδ kinase activity on Per proteins. CKIδ-mediated phosphorylation induces proteasome-mediated degradation of Per proteins, leading to circadian phase advance and shortened period length (8).

AMPK, by sensing the rise in AMP level under energy-deprived conditions (9), maintains energy homeostasis by stimulating ATP production and suppressing ATP-consuming processes such as synthesis of macromolecules (10). The catalytic subunit of AMPK has two isoforms, α1 (11) and α2 (12). Mice with a knockout (KO) of either AMPK α1 or AMPK α2 are viable (13, 14), but mice with a KO of both α1 and α2 are not viable, indicating that the two isoforms have partially redundant functions. In the hypothalamus, AMPK maintains energy homeostasis at the whole body level by stimulating food intake (15). Activation of AMPK in peripheral tissues such as liver and muscle decreases serum glucose level. Metformin, which activates AMPK (16), is one of the most widely used drugs for treatment of type II diabetes and is, in most cases, the agent of choice for initial therapy (17).

Here, we report that metformin and AMPK have a surprising role in modulating the circadian rhythm. We find that CKIε is activated by AMPK-dependent phosphorylation and stimulates mPer2 degradation. Activation of AMPK by metformin injection shifted the circadian expression pattern of clock genes in wild-type mice but not in AMPKα2 KO mice.

MATERIALS AND METHODS

Real-time Luminescence Reporting—Rat-1 cells with mPer2-luciferase reporter (18) were grown to confluency. Rat-1 cells were then treated with forskolin, and 30 min later, the medium was replaced with the recording medium, containing 10% of fetal bovine serum and HEPES-buffered Dulbecco’s modified Eagle’s medium. Luminescence was measured for 6 days by counting the photon every 60 s. The period length was determined with regression analyses by using ClockLab (Actimetrics). Four to six peaks detected from the baseline-subtracted

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‡ The abbreviations used are: LD, light-dark; AMPK, 5′-AMP-activated kinase; CKIε, casein kinase Iε; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; MEF, murine embryo fibroblasts; aa, amino acids; WT, wild-type; SCN, suprachiasmatic nucleus; GST, glutathione S-transferase; KO, knock-out; CA, constitutively active; DN, dominant-negative.

3 B. Viollet, unpublished data.
data (the baseline drift determined by an adjacent averaging method with 24 h was corrected by subtracting it from the original data) were used for the period determination. A more detailed description of this technique has been previously published (19).

**Mice**—Animals were maintained in barrier facilities with sterile food, water, and bedding on a 12-h light/dark (6 a.m./6 p.m.) cycle and were allowed free access to food. All experiments were approved by NHLBI Animal Care and Use Committee.

**In Vitro AMPK Kinase Assay**—GST-CKI fusion protein fragments and mutants were expressed and purified using the GST purification kit (Amersham Biosciences) as described by the manufacturer. Reactions (20 μl) were performed in kinase buffer (20 mM HEPES-NaOH, pH 7.0, 0.4 mM dithiothreitol, 0.01% Brij-35 with or without 300 μM AMP and 15 μCi of [γ-32P]ATP with 50 milliunits of AMPK (Millipore) and 2 μg of GST-CKI fusion protein substrates. The mixture was incubated 30 °C for 20 min and analyzed by SDS-PAGE.

**Immunoprecipitation Kinase Assay**—Myc-tagged CKI, transiently expressed in 293T cells, was immunoprecipitated with anti-Myc antibody and 10 μl of protein A-agarose beads in 200 μl of total volume overnight after preclearing the lysates with protein A-agarose beads for 2 h at 4 °C. For the kinase reaction, a His-tagged mPer2 (amino acids 550–763) fragment was expressed in and purified from Escherichia coli. Kinase reactions were performed as described previously (3).

**In Vivo 32P Labeling Assay**—V5-tagged CKI fusion fragment 2 and its S389A mutant were transiently expressed in 293T cells as described above. 36 h after transfection, cells were preincubated with phosphate-free Dulbecco’s modified Eagle’s medium for 1 h and labeled with 500 μCi/ml [32P]orthophosphate (Amersham Biosciences) for 2 h. Cells were washed with phosphate-buffered saline, after which they were lysed in radio-immune precipitation buffer containing protease and phosphate inhibitors. Equal amounts of protein were preincubated by incubating them with protein A-agarose beads for 2 h at 4 °C. The samples were immunoprecipitated with V5 antibody and protein A-agarose bead overnight at 4 °C. The precipitates were washed four times with the cell lysis buffer and dissolved in SDS sample buffer for SDS-PAGE.

**RESULTS AND DISCUSSION**

Since food intake and energy metabolism are synchronized to the LD cycle, we suspected that some pathways regulated by food intake and energy metabolism may affect the circadian rhythm. Since AMPK plays a central role in food intake and energy metabolism, we investigated the possibility that an AMPK-dependent pathway can affect circadian rhythm. The ability to visualize circadian oscillations of gene expression in vitro has been facilitated by the development of a cell culture-based luminescent reporter assay that can monitor the expression levels of the reporter in real time (20). To monitor the circadian variation in the activity of the mPer2 promoter, we used Rat-1 fibroblasts that have been stably transfected with an mPer2-luciferase reporter. After these cells were synchronized with forskolin (21), we treated them with metformin and monitored the luciferase activity in real-time. As shown in Fig. 1, metformin shortened the period length by approximately 1 h (p < 0.01).

Since proteasomal degradation of mPer2 that is stimulated by CKI phosphorylation can shorten the circadian period length (4), we explored the possibility that AMPK regulates the period length by stimulating CKI-mediated degradation of mPer2. To do this, we transiently transfected NIH3T3 cells with expression vectors for V5-tagged mPer2 and either CKI or dominant-negative CKI (K38R) and treated them with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) (22), an activator of

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**FIGURE 1. Metformin treatment shortens circadian period length.** A, real-time luminescence reporting of Rat-1 fibroblasts that have been stably transfected with an mPer2-luciferase reporter (pGL-2). The tracings of cells treated with metformin (2 mM) are shown. B, the calculated period length after metformin treatment (n = 4). Results are mean ± S.E.; **, p < 0.01.

**FIGURE 2. Activation of AMPK stimulates CKI-mediated degradation of mPer2.** A, V5-tagged mPer2 was cotransfected with an empty vector (--), Myc-CKI, or dominant-negative Myc-CKI K38R into NIH3T3 cells. After treating with AMPK activator AICAR (1 mM) for 2 or 4 h, the V5-mPer2 level was detected by immunoblotting with V5 antibody. Activation of AMPK was visualized by immunoblotting with antibody specific for phosphorylated AMPK. B, infection of HeLa cells with adenovirus expressing CA or DN AMPK or green fluorescent protein (GFP). The levels of transiently expressed V5-mPer2 in adenovirus infected cells were detected by immunoblotting with V5 antibody. C, metformin-induced degradation of transiently expressed V5-mPer2 does not occur in AMPKα1/2 KO MEFs. Green fluorescent protein expression is shown to demonstrate similar transfection efficiency in KO cells. D, the experiment shown in panel B was repeated in the presence of the proteasomal inhibitor MG132. E, the effect of AICAR on mPer2 levels either with or without MG132 is shown.
AMPK. As shown in Fig. 2A, mPer2 protein levels decreased dramatically in the presence of CK1ε, but this was blocked by dominant-negative CK1ε (K38R), suggesting that AICAR treatment decreased mPer2 levels in a CK1ε-dependent manner. To demonstrate that the reduction in mPer2 levels was caused by AMPK activation, we overexpressed constitutively active (CA) AMPKα1/ε2 and dominant-negative (DN) AMPKα2 in HeLa cells by infecting them with adenovirus carrying the expression vector for CA AMPK or DN AMPK. The levels of transiently expressed V5-mPer2 were significantly reduced by infection with CA AMPK adenovirus but were increased by infection with DN AMPK adenovirus (Fig. 2B). To further establish that activation of AMPK reduces mPer2 levels, we examined AMPK-dependent reduction in mPer2 level using murine embryo fibroblasts (MEFs) developed from AMPKα1/ε2 double KO and WT embryos (23). As shown in Fig. 2C, activation of AMPK with metformin reduced mPer2 levels in WT MEFs but not in AMPKα1/ε2 double KO MEFs. Activation of AMPK with metformin also reduced mPer1 levels in WT MEFs but not in AMPKα1/ε2 double KO MEF (data not shown). To demonstrate that AMPK reduced mPer2 levels through proteasome-dependent degradation, we repeated the experiment shown in Fig. 2B in the presence of the proteasome inhibitor MG132 (Fig. 2D). Indeed, MG132 blocked the reduction of mPer2 level induced by CA AMPK. Consistent with this, AICAR-induced reduction in mPer2 level was also blocked with MG132 (Fig. 2E). Taken together, these results suggest that activation of AMPK induces CK1ε-mediated degradation of mPer2 by proteasome.

We investigated whether AMPK stimulated CK1ε-mediated degradation of mPer2 by directly phosphorylating CK1ε. To test this possibility, we performed in vitro kinase reactions using fragments F1 (aa residues 1–230) and F2 (aa 211–416) of CK1ε as AMPK substrates (Fig. 3A). AMPK did not phosphorylate F1 but did phosphorylate F2 very strongly in an AMP-dependent manner. In vitro kinase assays using fragments derived from F2 as substrates of AMPK indicated that the AMPK phosphorylation site resides in aa 377–404 near the C terminus of CK1ε (Fig. 3B). Examination of the amino acid sequence in this region revealed that Ser-389 is part of a motif that resembles the consensus AMPK phosphorylation site (S/T)XXNL (Fig. 3C). Ser-389 and
noprecipitated CKIε containing the S389A mutation and performed a kinase reaction using mPer2 as substrate (Fig. 3E). Although the basal activity of CKIε (S389A) was similar to that of CKIε, the activity of CKIε (S389A) did not change with AICAR treatment, whereas the activity of CKIε consistently increased by about 3-fold within 2 h of AICAR treatment (p < 0.05). If activation of AMPK stimulates mPer2 degradation by phosphorylating Ser-389 of CKIε, mPer2 degradation should be blocked in S389A mutant. Indeed, AICAR-induced degradation of mPer2 did not occur when CKIε (S389A) was overexpressed (Fig. 3F). The activity of the endogenous CKIε immunoprecipitated from Rat-1 fibroblasts (Fig. 1) was also increased after metformin treatment (Fig. 3G), and mPer2 in Rat-1 fibroblasts was degraded after metformin treatment (Fig. 3H).

Since the oscillation in mPer mRNA expression results from mPer proteins suppressing their transcription through negative feedback, degradation of mPer2 during the period when mPer2 transcription is increasing should cause the mPer2 mRNA level to rise faster and peak earlier. To test this possibility, we injected WT mice with metformin, which has a short half-life (2.7 h) in mice (24), at 8 a.m. and measured mRNA levels of core clock genes mPer1, mPer2, Clock, and Bmal1 at 8 a.m. (no injection), 1 p.m., 6 p.m., 10 p.m., 2 a.m., and 7 a.m. in the heart, skeletal muscle, and fat. Consistent with our earlier results, metformin injection induced AMPK activity and led to mPer2 degradation (Fig. 4A). Since metformin injection causes transient hypoglycemia, we also injected insulin to another group to evaluate the effect of hypoglycemia alone. Metformin and insulin both decreased serum glucose by ~40% after 1 h (data not shown). The peak mPer1 and mPer2 mRNA levels in heart occurred at 6 p.m. in saline- and insulin-injected mice, but it occurred at 1 p.m. in metformin-injected mice (Fig. 4B). The expression patterns of Bmal1 and Clock mRNAs were also shifted forward in the tissues of metformin-injected mice (Fig. 4B). Phase advance was also induced in skeletal muscle (Fig. 4C) and fat (data not shown). To determine whether AMPKα2 was required for the metformin-induced phase advance, we performed these experiments in AMPKα2 KO mice. As shown in Fig. 4D, the levels of mPer2 protein in AMPKα2 KO mice were higher when compared with WT mice but did not change with metformin injection. Moreover, metformin injection did not significantly alter mPer1 and mPer2 mRNA expression patterns in AMPKα2 KO mice as it did in WT mice (p = 0.2–0.9 between saline- and metformin-injected samples) (Fig. 4E). These results indicate that metformin-induced mPer2 degradation and phase advance were mediated primarily by AMPKα2.

In summary, we provide evidence that activation of AMPK can modulate the circadian rhythm by targeting the CKIε-mPer pathway. From what is known about metformin or AMPK, their linkage to the CKIε-mPer pathway and circadian rhythm was unexpected. However, previous work has shown that altering the eating behavior affects the circadian rhythm. For example, leptin-deficient mice, which overeat and develop morbid obesity, have altered sleep regulation and circadian rhythmicity (25). Moreover, locomotor activity and the circadian clock in the peripheral tissues can be entrained by feeding (26). It would be interesting to test whether AMPK functions as the feeding sensor for this type of entrainment.

Although metformin caused a dramatic shift in the circadian phase in the peripheral tissues, we have not been able to demonstrate that the circadian rhythm of behavior can be shifted by
metformin because metformin does not cross the blood brain barrier. However, our work provides a proof of principle that chemical manipulation of the circadian phase, at least in the peripheral tissues, may be possible.

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