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Regulation of Peripheral Molecular Clocks in Mammalian Tissues and In Vitro Skeletal Muscle Activation of AMP-Activated Protein Kinase via AICAR

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Abstract
Most organisms possess a common molecular machinery that governs cellular and tissue circadian rhythmicity through a roughly 24-hour transcription-translation feedback loop. It is estimated that up to 15 percent of human genes are influenced by the core clock machinery. It is likely, however, that the metabolic networks affected by the molecular clock differ according to body tissue.

Recent evidence suggests that peripheral molecular clocks are governed to a greater extent by energy availability than by light and dark cycles. AMP-activated protein kinase (AMPK) acts as a cellular fuel gauge within the cell and is activated in response to exercise and fasting. AMPK can also be pharmacologically activated by 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR). AMPK likely serves as an intermediary between metabolism and the molecular clock due to its activation of the rate-limiting enzyme in Nicotinamide adenine dinucleotide (NAD) biosynthesis, Nicotinamide phosphoribosyltransferase (NAMPT), and its role in PER and CRY degradation. The NAD-dependent histone deacetylase SIRT 1 inhibits the BMAL1-CLOCK complex in a NAMPT-dependent manner.

The complex interplay between metabolism and peripheral clocks mediated by AMPK is beginning to be unraveled. AMPK's tissue-specific influence on the molecular clock in skeletal muscles and other mammalian tissues requires further elucidation as it may provide insight into the etiology and treatment of metabolic disease. [*excerpt*]

Keywords
Circadian Rhythm

Disciplines
Anatomy | Cell Anatomy | Cell and Developmental Biology | Tissues

Comments
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Regulation of peripheral molecular clocks in mammalian tissues and *in vitro* skeletal muscle activation of AMP-activated protein kinase via AICAR

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Introduction

Most organisms possess a common molecular machinery that governs cellular and tissue circadian rhythmicity through a roughly 24-hour transcription-translation feedback loop. It is estimated that up to 15 percent of human genes are influenced by the core clock machinery. It is likely, however, that the metabolic networks affected by the molecular clock differ according to body tissue.

Tissue circadian rhythm is regulated by the cellular molecular clock machinery in interaction with a central nervous system clock. This cellular molecular clock machinery is composed of a transcription-translation feedback loop in which the transcription factors Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) and Circadian Locomotor Output Cycles Kaput (CLOCK) form a heterodimer complex that activates the transcription of their own suppressors cryptochrome (CRY) and period (PER) proteins (Figure 1).

Recent evidence suggests that peripheral molecular clocks are governed to a greater extent by energy availability than by light and dark cycles. AMP-activated protein kinase (AMPK) acts as a cellular fuel gauge within the cell and is activated in response to exercise and fasting (Figure 1). AMPK can also be pharmacologically activated by 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR; Figure 1). AMPK likely serves as an intermediary between metabolism and the molecular clock due to its activation of the rate-limiting enzyme in Nicotinamide adenine dinucleotide (NAD) biosynthesis, Nicotinamide phosphoribosyltransferase (NAMPT), and its role in PER and CRY degradation (Figure 1). The NAD-dependent histone deacetylase SIRT 1 inhibits the BMAL1-CLOCK complex in a NAMPT-dependent manner.

The complex interplay between metabolism and peripheral clocks mediated by AMPK is beginning to be unraveled. AMPK's tissue-specific influence on the molecular clock in skeletal muscles and other mammalian tissues requires further elucidation as it may provide insight into the etiology and treatment of metabolic disease.

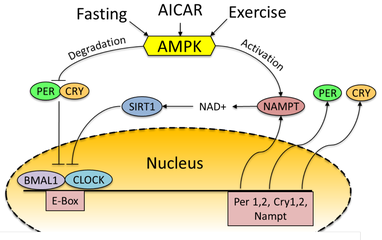


Figure 1. AMP-activated kinase influences the molecular clock by initiating pathways leading to the degradation of CRY and PER proteins (direct phosphorylation of CRY by AMPK leads to degradation). AMPK also activates NAMPT, which through SIRT 1 activation, represses BMAL1 and CLOCK initiated transcription.

Purpose

1. Investigate tissue-specific abundance of molecular clock machinery and NAMPT in order to determine which tissues may be sensitive to regulation by AMPK.

2. Design and fabricate an *in vitro* incubation model of isolated skeletal muscle in order to study the effects of acute metabolic disruptions on AMPK signaling.

Part A: Tissue Characterization of NAMPT, CLOCK, BMAL1

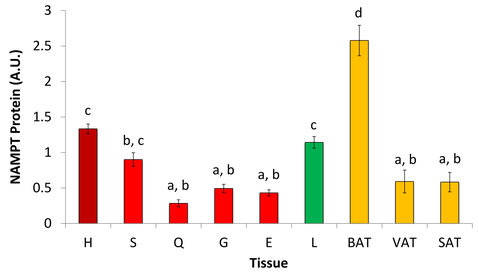
Methods

Tissue Collection and Processing. 10 male ICR mice were euthanized at 11:00AM. Heart (H), liver (L), quadriceps (Q), gastrocnemius (G), extensor digitorum longus (EDL), soleus (S), brown adipose tissue (BAT, visceral adipose tissue (VAT), and subcutaneous adipose tissue (SAT) were dissected and snap-frozen in liquid nitrogen. Tissues were pulverized, and homogenized with ice-cold lysis buffer.

Western Blotting. Tissues were blotted for NAMPT, BMAL1, and CLOCK proteins. Chemiluminescence was assessed using Image Lab software.

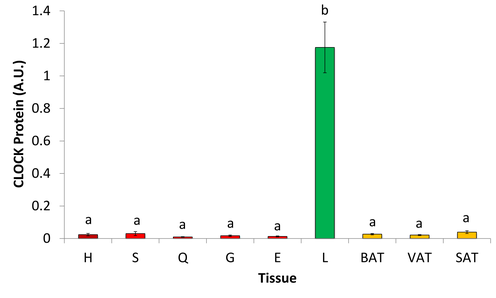
Results

Figure 2. NAMPT protein expression is elevated in mitochondria-dense murine tissues.



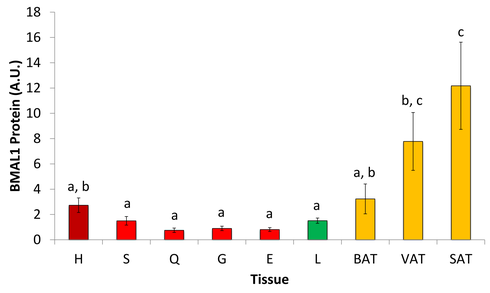
Data shown as means +/- SEM. n=10.
A.U., arbitrary units; H, heart; S, soleus; Q, quadriceps; G, gastrocnemius; E, extensor digitorum longus; L,liver; BAT, brown adipose tissue; VAT, visceral adipose tissue; SAT subcutaneous adipose tissue. Common letters denote sample means that do not reveal a statistically significant difference.

Figure 3. CLOCK protein is relatively abundant in mouse liver.



Data shown as means +/- SEM. n=10.
A.U., arbitrary units; H, heart; S, soleus; Q, quadriceps; G, gastrocnemius; E, extensor digitorum longus; L,liver; BAT, brown adipose tissue; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue. Common letters denote sample means that do not reveal a statistically significant difference.

Figure 4. BMAL 1 protein expression in mouse tissues.



Data shown as means +/- SEM. H, S, Q, G, E, L n=9, BAT, VAT, SAT n=6. A.U., arbitrary units; H, heart; S, soleus; Q, quadriceps; G, gastrocnemius; E, extensor digitorum longus; L,liver; BAT, brown adipose tissue; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue. Common letters denote sample means that do not reveal a statistically significant difference.

Part B: In vitro incubation of Skeletal Muscle

Methods

Tissue Collection and Processing.
Skeletal muscles from female ICR mice mice were dissected and mounted on custom-made incubators.

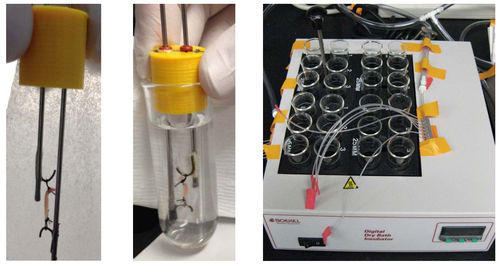
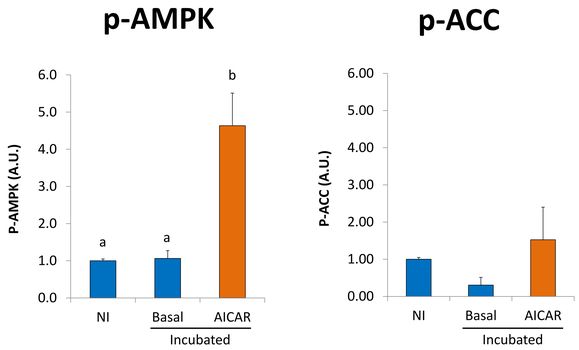


Figure 5. Mounted skeletal muscle (left), *in vitro* incubation apparatus (middle), and heat block (right).

Incubation of Skeletal Muscle with AICAR.
- Skeletal muscles were preincubated at 37 degrees Celsius in oxygenated Krebs Ringer bicarbonate (KRB) buffer for 20 minutes.
- Muscles were transferred to KRB buffer with 2mmol AICAR or maintained in KRB buffer (basal control).
- Following incubation, tissues were removed from incubators and immediately frozen in liquid nitrogen.
- Muscles were processed in ice-cold lysis buffer and Western blotted for p-AMPK and p-ACC.
- Chemiluminescence was assessed using Image Lab software.

Results

Figure 6. Incubation of skeletal muscle with AICAR phosphorylates and activates AMPK and ACC



Data shown as means +/- SEM. NI, Basal, AICAR n=2-4. A.U., arbitrary units; NI, non-incubated; Basal, incubated control; AICAR, incubated AICAR treatment. Common letters denote sample means that do not reveal a statistically significant difference.

Conclusions

- The soleus and other highly oxidative tissues (heart, liver, brown adipose tissue) contained the highest amount of Nampt. Given that NAD and thus Nampt-dependent sirtuins such as SIRT1 are not only part of circadian regulation, but also help regulate mitochondrial biogenesis, this raises the question whether circadian variations are especially pronounced in mitochondria-dense tissues.

- CLOCK protein expression is relatively abundant in the liver. Since the liver is the central regulator in many metabolic processes and systemic energy homeostasis, these findings could further underline the relationship between metabolism and circadian clock function.

- BMAL1 was most prevalent in visceral adipose tissue and subcutaneous adipose tissue.

- Relative abundances of CLOCK and BMAL 1 proteins are not clearly correlated in a tissue-specific manner, even though these proteins form a heterodimer complex to initiate transcription of circadian-regulated genes. Perhaps, these data indicate that these proteins have additional functions within the cell that do not require their interaction.

- *In vitro* skeletal muscle treatment with AICAR activated AMPK and ACC through phosphorylation.

Future Directions

- Investigate the effect of AMPK activation by AICAR on tissue circadian protein expression in isolated skeletal muscles.
- Nutritional intervention such as fasting/overfeeding and AMPK activation.

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