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Rapid paper

The circadian clock machinery controls adiponectin expression

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ABSTRACT

Adiponectin, an adipokine involved in glucose and lipid metabolism, exhibits a circadian manner of expression. Adiponectin expression is mediated by the helix–loop–helix transcription factor sterol regulatory element binding protein (SREBP)-1c. In this study, we tested whether the circadian clock helix–loop–helix transcription factors CLOCK and BMAL1 regulate adiponectin expression. We found that adiponectin expression is regulated by the clock through the circadian expression of its transcription factor *peroxisome proliferator-activated receptor* γ(PPARγ) and its co-activator PPARγ co-activator 1α (PGC1α) in mouse white adipose tissue and differentiated adipocytes. In addition, reconstitution of the core clock mechanism and siRNA experiments in cell culture suggest that the clock directly activates the adiponectin promoter and mediates its expression. In summary, adiponectin expression is regulated by the circadian clock and through the circadian expression of its transcription factor PPARγ and its co-activator PGC1α.

1. Introduction

Mammals have developed an endogenous circadian clock located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus that responds to the environmental light–dark cycle (Reppert and Weaver, 2002). Similar clock oscillators have been found in peripheral tissues, such as the liver, intestine, and adipose tissue (Froy, 2010). The core clock mechanism is encoded by the genes *Clock*, brain–muscle-Arnt-like 1 (*Bmal1*), *Period1* (*Per1*), *Period2* (*Per2*), *Period3* (*Per3*), *Cryptochrome1* (*Cry1*), and *Cryptochrome2* (*Cry2*) (Reppert and Weaver, 2002). The protein products CLOCK and BMAL1 are helix–loop–helix transcription factors that dimerize to activate transcription. CLOCK:BMAL1 heterodimers mediate transcription of a large number of genes including those of the negative feedback loop *Per*s and *Cry*s. When PERs and CRYs are produced in the cytoplasm, they oligomerize and translocate to the nucleus to inhibit CLOCK:BMAL1-mediated transcription. The circadian clock regulates metabolism and energy homeostasis in adipose tissues (Ando et al., 2005). This is achieved by mediating the expression and/or activity of certain metabolic enzymes and transport systems (Froy, 2010).

Adiponectin, an adipokine involved in glucose and lipid metabolism, increases fatty acid oxidation and potentiates insulin inhibition of hepatic gluconeogenesis, promoting insulin sensitivity (Berg et al., 2002). Adiponectin expression is mediated by *peroxisome proliferator-activated receptor* γ(PPARγ) and its co-activator PPARγ co-activator 1α (PGC1α) (Berg et al., 2002), whose expression is controlled by the circadian clock (Ando et al., 2005). In addition, the helix–loop–helix factor sterol regulatory element binding protein (SREBP)-1c is a known activator of adiponectin transcription, whereas Id3 inhibits adiponectin expression (Doran et al., 2008). As the adiponectin gene exhibits a circadian manner of expression and helix–loop–helix transcription factors regulate its expression, we tested whether the helix–loop–helix transcription factors CLOCK and BMAL1 directly regulate adiponectin expression.

2. Materials and methods

2.1. Animals and tissues

8-week-old C57BL/6 male mice were entrained to 12 h light and 12 h darkness (LD) for 2 weeks. Mice were placed on a 12-h fast and subsequently anesthetized every 3 h around the circadian cycle under dim red light. Epididymal adipose tissue was removed and immediately frozen in liquid nitrogen. Animals were humanely killed according to the strict guidelines of the Hebrew University.

2.2. RNA extraction and quantitative real-time PCR

RNA extraction and quantitative PCR were performed as was described (Barnea et al., 2013). Primers were designed with Primer

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express v.2 (Applied Biosystems, Foster City, CA) and validated by standard and dissociation curves of the product.

2.3. Cell culture and treatments

3T3-L1 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 5% CO_2 at 37 °C. Differentiation of pre-adipocytes to adipocytes was achieved, as was described (Barnea et al., 2013). Cells were harvested at 4-h intervals for 24 h.

2.4. Promoter isolation

Liver genomic DNA isolation was performed as was described (Sherman and Froy, 2008). Template genomic DNA was reacted by PCR with primers designed according to the adiponectin promoter (Seo et al., 2004).

2.5. Transfections and luciferase reporter assay

Gel-purified promoter sequences were ligated into the *KpnI*/*XhoI* sites of the pGL3-Basic vector (Promega, Madison, WI, USA) upstream of the luciferase gene. HEK-293 cells were transfected using jetPEI reagent (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions. 48 h after transfection, cells were harvested in Reporter Lysis Buffer (Promega, USA). Luciferase activities were assayed using a Tristar LB-941 (Berthold Technologies, Bad Wildbad, Germany) luminometer. β-galactosidase was co-transfected and used as a normalizer.

2.6. siRNA experiments

Clock siRNA (Santa Cruz, Santa Cruz, CA, USA) was transfected into differentiated 3T3-L1 cells using Interferin (Polyplus Transfection, USA) according to the manufacturer's instructions. Two days later, cells were harvested, reacted with TriReagent (Sigma, Rehovot, Israel) and RNA was extracted.

2.7. Statistical analyses

All results are expressed as means ± SE. One-way ANOVA was used to analyze the circadian pattern with several time-points. Levels were assessed by 1-way ANOVA and Student's t-test for comparison between 2 groups. The correlation of adiponectin to *Clock* expression in 3T3-L1 cells was assessed by linear regression. Statistical analysis was performed with JMP software (version 10, SAS Institute Inc. Cary, NC, USA).

3. Results

3.1. Circadian adiponectin expression in white adipose tissue and differentiated adipocytes

We first set out to establish the rhythmicity of the adiponectin gene (*AdipoQ*). We tested its expression in white adipose tissue (WAT) and 3T3-L1 differentiated adipocytes. *AdipoQ* exhibited circadian expression in both mice and cell culture ($p < 0.01$, 1-way ANOVA test) (Fig. 1A and B). The gene encoding PPARγ, a positive regulator of adiponectin expression, whose expression is regulated by the core clock mechanism, also showed circadian rhythmicity (Fig. 1). *Pgc1α*, the gene encoding PPARγ co-activator, also showed circadian oscillation in WAT and differentiated adipocytes (Fig. 1). In WAT, there was a 3 h phase difference among adiponectin, *Pparγ* and *Pgc1α* mRNA peak (*Pgc1α* at circadian time (CT) 6, *Pparγ* at CT9 and *AdipoQ* at CT12) exemplifying a circadian hierarchy of expression. In 3T3-L1 adipocytes, *Pgc1α* and *Pparγ* mRNA peaked at the same time, but 3 h earlier than *AdipoQ* mRNA (Fig. 1). Thus, these finding suggest that adiponectin circadian expression is mediated either directly by the core clock mechanism or indirectly via its clock-controlled transcription factors.

3.2. CLOCK:BMAL1 heterodimer regulates AdipoQ expression

To determine whether CLOCK:BMAL1 heterodimer regulates adiponectin expression directly by binding to E-box-like sequences located on the adiponectin gene promoter (Fig. 2A), we isolated this region from liver genomic DNA based on a previously reported sequence (Seo et al., 2004). This region (459 bp) was used to generate an adiponectin–luciferase fusion construct and contained 2 E-box-like sequences (5′-CATGTG-3′ and 5′-CAGCTG-3′) (Fig. 2A). We tested whether CLOCK:BMAL1 activates adiponectin promoter using transient transfection into HEK-293 cells. Co-transfection of plasmids harboring the genes encoding CLOCK and BMAL1 together with the adiponectin promoter fused to the luciferase reporter gene revealed that CLOCK:BMAL1 heterodimer up-regulated the expression by ~2 fold ($p < 0.05$, 1-way ANOVA) (Fig. 2B). This up-regulation was similar to that achieved with PPARγ and RXR, the well established transcription factors involved in adiponectin expression (Seo et al., 2004). Co-expression with CRY1 inhibited CLOCK:BMAL1-mediated expression of the adiponectin promoter (Fig. 2B). Treatment of differentiated 3T3-L1 adipocytes with *Clock* siRNA significantly reduced endogenous *Clock* mRNA expression (Fig. 2C). Transfection of *Clock* siRNA also reduced *AdipoQ* mRNA expression ($p < 0.05$, t-test) (Fig. 2D). Reduction in endogenous *Clock* mRNA expression showed high correlation with the reduced levels of *AdipoQ* mRNA expression (Fig. 2E). These results show that the CLOCK:BMAL1 heterodimer is recruited to the adiponectin promoter and up-regulates its expression and CRY1 suppresses this effect.

4. Discussion

This study provides evidence that the clock through the circadian expression of the transcription factor PPARγ and its co-activator PGC1α regulates adiponectin expression. In addition, the core clock mechanism may directly bind to the adiponectin promoter and mediate its expression. Previous studies have shown that helix–loop–helix transcription factors regulate adiponectin expression (Doran et al., 2008). Whereas the helix–loop–helix factor sterol regulatory element binding protein (SREBP)-1c is a known activator of adiponectin transcription, Id3 inhibits adiponectin expression (Doran et al., 2008). As previous studies have shown that the adiponectin gene exhibits a circadian manner of expression and that clock disruption correlated with altered expression of adiponectin (Ando et al., 2005), we tested whether the clock helix–loop–helix transcription factors CLOCK and BMAL1 regulate directly adiponectin expression. Herein, we show that a reconstitution of the core clock mechanism leads to increased transcriptional activity of the adiponectin promoter and that co-transfection of the negative feedback loop component CRY1 leads to transcriptional suppression. Although we did not use any synchronizer for the 3T3-L1 cells, we have previously shown that these cells are synchronized through the differentiation process *in vitro* (Barnea et al., 2013). Addition of dexamethasone and insulin during differentiation leads to cell clock