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Food Entrainment of Circadian Gene Expression Altered in PPAR α ^{-/-} Brown Fat and Heart

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Abstract

The circadian clock is subject to food entrainment. Since PPAR α exhibits a circadian expression profile, we hypothesized that PPAR α deficiency would alter the food entrainable response of adipose, cardiac, and liver tissues. Wild type and PPAR α null mice were compared under *ad libitum* or restricted food access for the expression of circadian transcription factor-encoding mRNAs. Temporally restricted food access caused between a mean 5.8 to 11.5 hour phase shift in the expression profiles of the circadian genes *Bmal1*, *Per3*, and *Rev-erba* in all tissues of control mice. In contrast, these same conditions phase shifted the circadian genes in tissues of PPAR α null mice between a mean of 10.8 to 14.2 hr with amplitude attenuation. The food entrained phase shifts in the brown adipose and cardiac tissue circadian transcription factors of the PPAR α null mice were prolonged significantly relative to wild type controls. Likewise, PPAR α responsive genes in the livers of PPAR α null mice exhibited a significantly prolonged phase shift relative to control mice. These findings confirm and extend recent observations in the literature..

Keywords

Adipose Tissue; Circadian; Cardiac; Liver; Peroxisome Proliferator Activated Receptor α

Introduction

Circadian rhythms play a major role in many, if not all, physiological processes [1]. The circadian clock, comprised of a highly conserved group of transcription factors (*Clock*, *BMAL1*, *Period (Per)*, and *Cryptochrome (Cry)*), regulates the oscillatory expression profile of downstream targets including albumin D binding protein (*DBP*) and the orphan nuclear hormone receptors, *Rev-erba* and *Rev-erbb* [1,2]. While the suprachiasmatic nucleus (SCN) within the brain acts as the core circadian oscillator, recent findings indicate that autonomous circadian clocks exist within peripheral tissues [3,4]. When organ cultures were prepared with peripheral tissues isolated from *Per2* promoter/luciferase reporter transgenic mice, their luciferase expression profile displayed circadian rhythmicity for over 20 days *ex vivo* [4].

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Moreover, the peripheral tissues maintained their circadian rhythmicity even after SCN ablation *in vivo*. Thus, the circadian mechanisms throughout the body can display some degree of independence.

The circadian clock is entrained by food exposure and access in addition to photic stimuli [1, 2]. Temporal restriction of food access has been found to alter animal behavioral activities, leading to the hypothesis that a “food entrainable oscillator (FEO)” exists within the brain or some other site within the organism [5–7]. Restricted food access influences the expression profile of genes encoding the circadian transcription factors in the fat, liver, and other peripheral tissues [8–14]. When we restricted food access for AKR/J mice to the 12 hour lights on period for seven days, we observed a phase shift in the expression profile of multiple circadian transcription factors (*Bmal1*, *Clock*, *Cry1–2*, *Npas2*, *Per1–3*) and their immediate downstream targets (*DBP*, *E4bp4*, *Id2*, *Rev-erba*, *Rev-erbb*) in brown and white adipose tissues and liver [8]. This correlated with both a phase shift and amplitude attenuation in serum corticosterone levels [8].

Previous research has shown that the alpha isoform of peroxisome proliferators-activated receptors (PPAR α) exhibits circadian rhythmicity and plays a significant role in lipid metabolism [15–17]. PPAR α is known to be a major transcriptional regulator in fatty acid oxidation (FAO) through several processes such as fatty acid transport and activation of acyl-CoA esters [15,17]. Additionally, PPAR α has been shown to display patterns of expression that are in coordination with the core circadian genes’ profiles [16,18,19]. PPAR α plays a critical metabolic role as evidenced by the hypothermia and hypoglycemia displayed by PPAR α null mice relative to wild type controls under conditions of prolonged fasting [20,21]. Based on these findings, we postulated that the circadian response to food entrainment will differ in PPAR α null mice relative to their wild type controls. The current study tests this hypothesis by examining the impact of PPAR α deficiency on the expression genes encoding circadian transcription factors and PPAR α responsive proteins in peripheral tissues following temporally restricted food access. The results confirm and extend previous observations by Canaple et al [18].

Methods and Materials

Experimental Design

Protocols were reviewed by the OCU and/or PBRC Institutional Animal Care and Use Committees. Founders of the breeding colony at OCU were provided by Dr. F. Gonzales (NCI, NIH) [15]. **Experiment 1** (March 2005) investigated differences in gene expression between *ad libitum* fed wild type and PPAR α -null mice. Eight to ten week old C57BL/6N x Sv/129 wild-type (WT) male mice were compared against 8–10 week old C57BL/6N x Sv/129 homozygous PPAR α -null male mice. Animals were acclimatized for 2 weeks to a regular chow diet (Purina 5015) with *ad libitum* access under a strict 12h light/ 12h dark cycle. After this period, groups of 3 animals from each genotype were sacrificed at 4 hour intervals over a 24-hour period. Liver, brown adipose tissue (BAT), epididymal adipose tissue (eWAT), and plasma were harvested, flash frozen in liquid nitrogen, and subsequently processed for total RNA isolation or cholesterol/triglyceride assay. **Experiment 2** (May of 2006) examined the effect of temporally restricted feeding (RF). The study was conducted with groups of 40 wild-type and 40 PPAR α -null male mice (age ranges, 7–25 weeks) acclimatized to a regular chow diet (Purina 5015) and maintained on a strict 12h light/ 12h dark cycle. The wild-type and PPAR α -null mice were then sub-divided into two cohorts; an *ad libitum* cohort that retained free access to food and an experimental cohort whose feeding time was restricted to the 12-hour lights on period. This feeding schedule was maintained for 8 days until the time of harvest. Body weights and food intake were monitored daily both cohorts. Animals from each cohort were then sacrificed as described in **Experiment 1**.

Real-Time PCR (RT-PCR)

Total RNA from the selected tissues was isolated and purified using TriReagent and 1-bromo-3-chloropropane, BCP (Molecular Research Center). 2 µg of RNA was then reversely transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT; Promega) with Oligo dT at 42°C for 1 hour in a 20 µl working volume. The cDNA was then diluted into 1:100 for the liver and 1:25 solutions for the heart and fat depots. Primers for the selected circadian and lipid metabolism genes were designed using Primer Express (Applied Biosystems) (Supplement Table 1). Real-Time PCR was then performed using the SYBR® Green PCR Master Mix (Applied Biosystems) on the 7900 Real Time PCR system (Applied Biosystems). Universal cycling conditions of 95°C for 10 min, 40 cycles of 95°C for 15 sec, and then 60°C for 1 min were used. Genes of interest were normalized against *Cyclophilin B*, which has been shown to maintain the consistency of expression required for a “housekeeping” control in a manner similar to *actin* and *GAPDH* [8, 22]. All RT-PCR assays were performed in triplicate and the *Cyclophilin B*-normalized ratio data presented in the figures represents the mean ± standard deviation.

Statistical Section

Oscillation patterns and periodicity of the qRT-PCR and serum data were evaluated using Time Series Analysis-Single Cosinor v. 6.0 software (Expert Soft Technologie) [23]. Comparisons between groups were performed using a one tailed Students t-test. Analyses with p values <0.05 were considered significant.

Results

Circadian core oscillator gene expression in wild type and PPARα-null mice with ad libitum food access

We examined RNA expression patterns in peripheral tissues harvested from wild-type and PPARα-null mice with *ad libitum* food access. Figure 1 displays 24 hour profiles of liver, BAT, and eWAT gene expression as a function of Zeitgeber Time (ZT) where ZT0 corresponds to the initiation of the 12-hour lights-on period. The positive circadian transcriptional regulators, *Bmal1* and *Npas2*, displayed similar expression profiles and amplitudes in all three tissues of wild type and PPARα-null mice. The profile of *Bmal1* and *Npas2* mRNA levels, normalized to *Cyclophilin B*, displayed mean acrophase values of ZT 20.9 and 21.7, respectively. The expression profile of the negative circadian transcriptional regulators, *Per1* and *Per3*, displayed mean acrophases of ZT11.4 and ZT10.6, respectively, reflecting an ~10 hr time difference relative to *Bmal1* and *Npas2*. The amplitudes of *Per1* and *Per3* were similar between wild type and PPARα-null mice in all tissues except eWAT. *DBP*, *Rev-erba*, and *Rev-erbb*, downstream transcriptional targets of *Bmal1* and *Npas2*, exhibited mean acrophases of ZT8.5, ZT 5.6, and ZT8.2 in all three tissues. The circadian rhythmicity of all genes in all tissues was statistically significant (p < 0.05) based on Cosinor analysis.

Effect of temporally restricted food access on the circadian gene expression profiles in wild type and PPARα-null mice

In *Experiment 2*, all cohorts displayed comparable body weights at the initiation of the study. During the first two days, the wild type and PPARα-null mice with temporally restricted food access exhibited reduced food intake relative to their *ad libitum* fed counterparts; however, by day 3, all cohorts displayed comparable food intake and body weights in all cohorts were comparable at the conclusion of the study (data not shown). The PPARα mRNA levels displayed a statistically significant circadian profile in all tissues in wild type mice under *ad libitum* conditions (Supplement Figure 1).

Under conditions of *ad libitum* food access, the amplitudes and acrophase for representative circadian gene markers (*Bmal1*, *Per3*, *Rev-erba*) were similar in the BAT and cardiac tissue of wild type and PPAR α -null mice (Figure 2). When food access was temporally restricted, the wild type mice shifted the acrophase of circadian gene expression by means ($\pm$ S.D.) of 5.8 ± 2.0 hrs (heart) and 7.1 ± 8 hrs (BAT) relative to the *ad libitum* state. In contrast, with temporally restricted food access, the PPAR α -null mice shifted the acrophase of circadian gene expression between 14.2 ± 2.5 hrs (heart) and 11.4 ± 1.2 hrs (BAT); relative to the wild type controls, these values were statistically significant. In BAT, temporally restricted food access reduced the amplitude of circadian gene expression in both wild type and PPAR α -null mice (Figure 2). In cardiac tissue, temporally restricted food access reduced the amplitude of wild type *Bmal1* and PPAR α -null *Rev-erba* expression and only increased this parameter for PPAR α -null *Bmal1* (Figure 2).

Temporal restriction of food access shifted the acrophase of circadian gene expression in eWAT by 8.7 ± 1.6 hrs in wild type mice and 10.8 ± 2.2 hrs in PPAR α -null mice ($p = 0.176$); this was associated with a change in amplitude in some but not all genes. (Figure 3 and Supplement Tables 4 and 5) In liver, temporal restriction of food shifted the acrophase of circadian gene expression by 11.5 ± 0.8 hrs in wild type mice and 13.2 ± 0.5 hrs in PPAR α -null mice ($p = 0.075$) (Figure 3). The amplitude of gene expression under temporal restricted food access was not significantly changed relative to the *ad libitum* state for either wild type or PPAR α -null mice. In all tissues, the period of circadian gene expression ranged between 23.0 to 25.1 hours and achieved statistically significant rhythmicity in nearly all instances. The one exception was *Rev-erba* expression in the liver of wild type mice with temporally restricted food access; this displayed a statistically significant ultradian period of 12.2 hours (Supplement Tables 4 and 5).

Circadian oscillations in hepatic expression of PPAR α downstream target genes

The circadian expression profile of representative genes regulated by PPAR α were evaluated in the liver of wild type and PPAR α -null mice (Figure 4). The mRNAs of the lipid metabolic enzymes acyl Co-A oxidase (ACOX1), fatty acid desaturase 2 (FADS2), and elongation of very long chain fatty acid 5 (Elovl5) displayed statistically significant circadian oscillations in wild type and PPAR α -null mice; however, the amplitude of ACOX1 was markedly attenuated in the PPAR α -null mice. With restricted feeding, the mean shift acrophase shift in these three genes was significantly greater in PPAR α -null (12.6 ± 2.8 hrs) relative to wild type mice (6.7 ± 4.1 hrs) (Supplement Tables 4 and 5).

Discussion

Temporally restricted food access entrains a phase shift in circadian transcription factor gene expression profiles in peripheral tissues of wild type mice [8–14]. The current work reports that PPAR α deficiency significantly modulates this phenomenon in BAT and cardiac muscle, shifting the acrophase of circadian gene expression by up to an additional 8 hrs. While eWAT and liver displayed a prolonged food entrained acrophase shift of 1.5–2 hrs of circadian gene expression in the PPAR α null mice relative to wild type controls, these differences did not achieve statistical significance. However, the expression of PPAR α responsive genes in the liver showed evidence of a prolonged food entrained acrophase shift in the PPAR α null mice relative to controls with some degree of amplitude attenuation.

In general, our results confirm those reported by Canaple et al. [18]. Both studies show food entrainment of circadian transcription factor gene expression in the liver of both the wild type and PPAR α null mice and of the *PPAR α* mRNA level in the liver of wild type mice. Both studies demonstrate a comparable shift in the hepatic expression of *Per 3* and *Rev-erba* between daytime and nighttime fed PPAR α null and wild type mice [18]. Likewise, independent studies

by Satoh et al. have demonstrated that murine energy metabolism and hepatic circadian transcription factor mRNA expression profiles acclimated within six days to a similar time-restricted feeding regimens [24].

Mice deficient in PPAR α display altered cardiac tissue metabolism, relying primarily on glycolysis rather than fatty acid oxidation, resulting in decreased energy reserves and reduced contractile function under stress [20,25,26]. With prolonged fasting, PPAR α null mice are prone to hypothermia, consistent with altered BAT function [20,21]. These findings may be linked to the current observation that BAT and cardiac tissue of PPAR α null mice show altered food entrainable circadian transcription factor gene expression. However, further studies will be necessary to define the mechanism connecting circadian transcription factors and PPAR α in these peripheral tissues. Nevertheless, analyses of the circadian profile of PPAR α null mice have increased significance in light of recent observations that mice deficient in the genes encoding PPAR gamma co-activator 1 (PGC1) α and β , co-activators of both PPAR α and γ [27], also exhibit a disrupted circadian rhythm and altered metabolic function [28,29].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Circadian Gene Expression Profiles in Metabolic Tissues of Wild Type and PPAR α Null Mice

The mRNA expression profile of “positive” (*Npas2*, *Bmal1*) and “negative” (*Per1*, *Per3*) circadian transcriptional regulators and their immediate downstream targets (*DBP*, *Rev-erba*, *Rev-erbβ*) are displayed over a 24-hour period in brown adipose tissue (BAT), liver, and epididymal white adipose tissue (eWAT) mRNAs pooled from groups of $n = 3$ mice per time point. In this and all subsequent figures, the 24-hour expression profile has been duplicated to visually display evidence of circadian rhythmicity. The qRT-PCR values (mean $\pm$ S.D. of triplicate determinations) were normalized relative to *Cyclophilin B*. Cosinor analysis documented p values < 0.05 for all measurements in all tissues with the exception of *Per1* (wild

type and PPAR α null BAT and wild type eWAT) and *Per3* (wild type BAT) (Supplement Table 2).

Figure 2. Circadian Gene Expression in Brown Adipose and Cardiac Tissue of Wild Type and PPAR α Null Mice under Ad Libitum and Temporally Restricted Food Access

The mRNA expression of *Bmal1*, *Per3*, and *Rev-erba* was determined in brown adipose tissue (BAT) and heart isolated from wild type (solid line) and PPAR α null (dotted line) mice with either *ad libitum* (ad lib) or temporally restricted (RF) food access. Values are the mean $\pm$ S.D. of triplicate qRT-PCR assays conducted using mRNA pools from $n=3$ animals per time point and normalized relative to *Cyclophilin B*. Cosinor analysis documented p values < 0.05 for all measurements in both tissues (Supplement Table 3).

Figure 3. Circadian Gene Expression in Liver and Epididymal White Adipose Tissue of Wild Type and PPAR α Null Mice under *Ad Libitum* and Temporally Restricted Food Access

The mRNA expression of *Bmal1*, *Per3*, and *Rev-erba* was determined in liver and epididymal white adipose tissue (eWAT) isolated from wild type (solid line) and PPAR α null (dotted line) mice with either *ad libitum* (ad lib) or temporally restricted (RF) food access. Values are the mean $\pm$ S.D. of triplicate qRT-PCR assays conducted using mRNA pools from $n=3$ animals per time point and normalized relative to *Cyclophilin B*. Cosinor analysis documented p values < 0.05 for all measurements in both tissues with the exception of *Rev-erba* in wild type liver RF ($p = 0.112$) and wild type eWAT ad lib ($p = 0.081$) and RF ($p = 0.072$) (Supplement Table 3).

Figure 4. Circadian expression profile of hepatic genes regulated by PPAR α

The mRNA level of ACOX-1, Elovl-5, and FADS2 were determined in the liver of wild type (solid line) and PPAR α null (dotted line) mice under ad libitum and restricted feeding conditions. Cosinor analyses documented statistically significant rhythms ($p < 0.05$) for all expression profiles except for WT-ad lib ACOX1 ($p = 0.32$) and FADS2 ($p = 0.96$) and KO-restricted feeding ACOX1 ($p = 0.53$) (Supplement Table 3).