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Circadian rhythmicity in murine pre-adipocyte and adipocyte cells

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Abstract

Adipose tissue is central to metabolic homeostasis, signalling in part through the secretion of molecules termed adipokines. Circadian rhythms play an important role in adipose physiology, with plasma adipokine concentration and ~20% of the murine adipose transcriptome undergoing 24 h variation. However, due to the heterogeneity of adipose tissue and rhythmical input from both neuronal and humoral signals, the cellular basis of adipose rhythms is unclear. We tested the hypothesis that adipocyte cells contain a circadian clock that drives rhythmic mRNA expression and adipokine secretion. From the murine pre-adipocyte 3T3-L1 cell line, we generated populations of both pre-adipocytes and differentiated adipocytes. Cells were then treated with a 2 h serum pulse and sampled every 4 h over a 48 h period. Expression of clock gene, 'metabolic' gene (*PPAR α* , *PPAR γ* , *SREBP1*) and adipokine mRNA was analyzed by quantitative real-time PCR, and secretion of the adipokines leptin and adiponectin was measured in culture medium from differentiated adipocytes. In pre-adipocytes, we observed robust rhythms of clock genes *Per2*, *Rev-erba*, and *Dbp*, but not of *Per1*, *Cry1*, *Bmal1*, or any of the 'metabolic' genes. Adipocytes produced similar temporal profiles of mRNA expression, albeit with a markedly reduced amplitude of *Per2* and *Dbp* rhythms. Despite no circadian rhythm of adipokine mRNA expression, leptin accumulation in the culture medium suggested circadian control of leptin secretion from adipocytes. Adiponectin secretion showed temporal variation, but without any apparent circadian rhythmicity. Our data, therefore, suggest that an endogenous adipocyte clock controls the rhythmic expression of only a subset of genes that are reported to exhibit 24 h rhythmicity in murine adipose tissue. Moreover, secretion of leptin may also be regulated by the adipocyte clock. (Author correspondence: j.johnston@surrey.ac.uk).

Keywords: clock gene, leptin, adiponectin, serum pulse

Introduction

Circadian clocks are present in multiple tissues throughout the body (Dardente & Cermakian, 2007). Moreover, many aspects of metabolic physiology are under circadian control (Bray & Young, 2007; Hastings et al., 2007; Zvonic et al., 2007; Green et al., 2008). Analysis of transgenic mouse models has recently provided compelling molecular links between components of the circadian clock and metabolism. Although the resulting phenotype appears partially dependent on genetic background, mutation of clock-related genes results in metabolic dysregulation (Turek et al., 2005; Oishi et al., 2006; Green et al., 2007; Kennaway et al., 2007). Similar genetic links have been found in humans, with clock gene polymorphisms being associated with obesity, metabolic syndrome, type 2 diabetes mellitus and circadian rhythm sleep disorders (Woon et al., 2007; Monteleone et al., 2008; Scott et al., 2008; Sookoian et al., 2008; Pereira et al. 2005; Lee et al., 2007; Pedrazzoli et al., 2007).

White adipose tissue (WAT) is a key metabolic structure. In addition to storing energy as triglycerides, WAT has endocrine activity and regulates many distal tissues. Among the molecules secreted by WAT is a group of metabolically active hormones, called the adipokines (Trujillo & Scherer, 2006). Within WAT, rhythmic gene expression is widespread, with approximately 20% of the murine WAT transcriptome exhibiting 24 h variation (Ptitsyn et al., 2006; Zvonic et al., 2006). Furthermore, circadian regulation of endocrine activity is likely to be an important output of this tissue. 24 h variation of adipokine concentration has been reported in blood (Sinha et al., 1996; Kalsbeek et al., 2001; Gavrilu et al., 2003); this rhythmicity may have a transcriptional basis, as adipokine mRNA is subject to 24 h variation in murine WAT (Ando et al., 2005).

However, rhythmicity in adipocyte cells, *per se*, is poorly understood. WAT contains many cell types, including adipocytes, pre-adipocytes, fibroblasts, macrophages, and vascular endothelial cells that may contribute to the reported rhythms of gene expression in murine WAT (Balsalobre et al., 1998; Hayashi et al., 2007; Takeda et al., 2007; Wu et al., 2007). Moreover, WAT, like other peripheral tissues, receives many rhythmic humoral and neuronal inputs (Balsalobre et al., 2000; Bartness et al., 2001; Pevet et al., 2006), which can directly regulate clock gene expression (Balsalobre et al., 2000; Johnston et al., 2006; Kornmann et al., 2007; Reddy et al., 2007; Wagner et al., 2007). This rhythmical input may be driven both by endogenous circadian timing and also from masking effects of the light-dark cycle in which animals were kept in previous studies. Here, we have used an *in vitro* approach to study rhythmicity in murine pre-adipocyte and adipocyte cells.

Materials and Methods

All experimental procedures conformed to published ethical standards (Portaluppi et al., 2008).

Cell culture

3T3-L1 pre-adipocytes (ATCC, LGC Standards, Teddington, Middlesex, UK) were cultured in growth medium [DMEM (Invitrogen Ltd, Paisley, UK) supplemented with 10% foetal bovine serum (Invitrogen), antibiotic/antimycotic (Invitrogen), and sodium pyruvate (Sigma-Aldrich Co Ltd, Poole, Dorset, UK)]. Two days post-confluence (day 0), differentiation was induced by adding 0.5mM IBMX (Sigma-Aldrich), 1µM dexamethasone (Sigma-Aldrich) and 10µg/ml insulin (Invitrogen). After 48 h, medium was exchanged for growth medium containing 10µg/ml insulin. 48 h later, this was exchanged for normal growth medium, which was replaced every two days thereafter. To characterize differentiation, RNA was extracted

using TRIzol (Invitrogen) for semi-quantitative reverse transcriptase PCR, and triglyceride analyses were carried out on days 0, 2, 4, 6, 8, and 10. 3T3-L1 pre-adipocytes (two days post-confluence) and differentiated adipocytes (day 8 post-differentiation induction) were pulsed with 50% horse serum (Invitrogen) in DMEM for 2 h, as described previously (Balsalobre et al., 1998). Medium was then replaced with serum-free DMEM for the remainder of the experiment. Every 4 h from initiation of the serum pulse (0 h), RNA was extracted for real-time quantitative PCR, and medium was collected for analysis of adipokine secretion.

Oil red O stain

Cells were washed with 10% formalin in 1xPBS and then incubated in fresh formalin solution for 1 h at room temperature. Cells were washed twice with 60% ethanol, twice with distilled water, and incubated in 0.18% oil red O solution (Sigma-Aldrich) for 10 min. Cells were washed three times with distilled water, and then photographed.

Quantitative triglyceride analysis

Cells were rinsed twice with 1xPBS, scraped into 0.1M SDS solution in 1xPBS and lysed by vortex mixing and sonication. Triglyceride was measured using an Alfa Wassermann one-step triglyceride assay (Randox Laboratories Ltd, Crumlin, County Antrim, UK) adapted for 96-well plates. Intra-assay coefficient of variance was 3.8%.

Reverse transcriptase PCR

Total RNA was reverse transcribed using random primers and M-MLV Reverse Transcriptase (Promega Corporation, Southampton, UK). cDNA was then used for semi-quantitative reverse-transcriptase PCR using GoTaq Green Master Mix (Promega) according to the manufacturer's instructions. Primers are shown in Table 1.

Quantitative real-time PCR (qPCR)

cDNA was synthesized as described above. cDNA was then used for qPCR using ABsolute™ QPCR ROX Mix (Abgene, Epsom, UK). Primer-probe sets are shown in Table 2. Standard curves ($R^2 > 0.99$) were generated using murine genomic DNA (Promega).

Adipokine assays

Adipokine concentration was measured using Quantikine ELISA kits (R&D Systems Europe Ltd, Abingdon, UK). Intra-assay coefficient of variance was 2.2% and 6.5% for leptin and adiponectin assays, respectively.

Statistics

Quantitative data represent the mean $\pm$ SEM of n=3 groups of cells from a representative experiment. Serum pulse experiments were repeated two (pre-adipocyte) or three (adipocyte) times, with similar data obtained from each replicate experiment. Triglyceride accumulation and qPCR data were analyzed by one-way or two-way ANOVA, with Least Square Difference post-hoc test, as appropriate. Rate of adipokine secretion was measured by testing the departure of hormone accumulation from linearity, using a runs test. Significant difference was defined as $p < 0.05$.

Results

Differentiation of 3T3-L1 cells

Lipid droplets were first observed four days after onset of differentiation and, by day 8, many cells had large unilocular droplets (Figure 1A). Quantitative analysis revealed triglyceride elevation above undifferentiated cells by day 4, followed by rapid accumulation

thereafter (Figure 1B). *PPAR γ* mRNA was detectable in undifferentiated cells, but was elevated from day 2 through to day 10. In contrast, expression of *Glut-4* and *Leptin* was only detected after two and four days of differentiation, respectively (Figure 1C).

Clock gene expression in pre-adipocytes and adipocytes

Following a serum pulse, significant ($p < 0.001$, one-way ANOVA) temporal variation of mRNA expression was observed in pre-adipocytes for all genes examined except *Per1* ($p > 0.05$, one-way ANOVA). For *Per2* and *Dbp* a rapid (within 4 h) effect was followed by a peak around 24 h post-pulse and an additional elevation in gene expression 24 h later. *Rev-erba* exhibited two peaks, approximately 20 and 44 h post-pulse. By contrast, *Cry1* and *Bmal1* expression was acutely stimulated by the serum pulse, but then showed no clear circadian variation (Figure 2 left).

Treatment of differentiated adipocytes with a serum pulse induced significant ($p < 0.001$, one-way ANOVA) variation in expression of all clock genes (Figure 2 right). As in pre-adipocytes, expression of *Per1*, *Cry1*, and *Bmal1* in serum pulsed adipocytes did not appear to exhibit circadian rhythms. Temporal variation of *Per2*, *Dbp*, and *Rev-erba* expression was similar to that previously observed in the pre-adipocytes. However, for all three genes there were significant effects of both time and cell type ($p < 0.001$, two-way ANOVA) around the main peak, as measured between 16 and 32 h post-pulse. There was also a significant ($p < 0.001$) interaction between time and cell type for *Per2* and *Dbp*. Subsequent post-hoc analysis revealed that, although there was no effect of cell type on gene expression at 16 h post-pulse, *Per2* and *Dbp* expression was significantly elevated in pre-

adipocytes over the next three to four time points, indicating that the amplitude of *Per2* and *Dbp* rhythms in differentiated adipocytes was greatly attenuated (Figure 3).

Metabolic gene expression in 3T3-L1 pre-adipocytes and adipocytes

Following a serum pulse, both pre-adipocytes and adipocytes exhibited significant ($p<0.05$) temporal variation in *SREBP1*, *PPAR α* , and *PPAR γ* mRNA expression. In pre-adipocytes, *SREBP1* and *PPAR γ* expression gradually decreased following the pulse, whereas *PPAR α* exhibited an acute, transient suppression (Figure 4 left). In adipocytes, expression of *SREBP1* and *PPAR α* gradually increased, with expression of *PPAR α* decreasing again by 44 h post-pulse, but remaining constant in *SREBP1* (Figure 4 right). However, none of the genes showed circadian variation of expression in either cell type.

Adipokine mRNA expression and secretion from differentiated adipocytes

Following a serum pulse, leptin and adiponectin concentration increased in the culture medium in a non-linear fashion ($p<0.05$; Figure 5A). The rate of secretion was estimated by plotting the difference in mean concentration between adjacent time points (Figure 5B). Leptin secretion was low between 8-12 h post-pulse and then appeared to exhibit circadian rhythmicity, with a second nadir 24 h later (Figure 5B). We were unable to detect circadian changes in adiponectin secretion. Expression of *Leptin* (Figure 5C left) and *Adiponectin* (Figure 5C right) mRNA exhibited significant temporal variation ($p<0.001$) following a serum pulse. However, neither showed evidence of circadian rhythmicity.

Discussion

In order to directly address adipocyte rhythmicity, we studied the well-characterized 3T3-L1 cell model. Although these cells are known to express clock genes (Shimba et al.,

2005; Wang & Lazar, 2008), there are no available quantitative data describing temporal changes in their physiology. Consistent with the recent finding of circadian rhythms in human adipose-derived stem cells (Wu et al., 2007; Huang et al., 2009), we observed circadian variation of *Per2*, *Dbp*, and *Rev-erba* expression in both pre-adipocytes and differentiated adipocytes. However, we surprisingly failed to observe circadian rhythmicity of *Per1*, *Cry1*, *Bmal1*, *PPAR α* , *PPAR γ* , *SREBP1*, *Leptin*, and *Adiponectin* mRNA, which are all rhythmic in murine WAT (Ando et al., 2005; Ptitsyn et al., 2006; Yang et al., 2006; Zvonic et al., 2006). Our data, therefore, suggest that some of the reported rhythmicity in murine WAT may derive from non-adipocyte cell types and/or rhythmical input signal(s).

Following the original description of circadian rhythms in a serum pulsed cell line, it was suggested that the effect of the pulse was either to initiate circadian rhythms in individual cells or synchronize a population of cells, each of which contains a pre-existing intracellular clock (Balsalobre et al., 1998). Elegant single cell imaging studies later provided strong experimental support for the second of these possibilities (Nagoshi et al., 2004; Welsh et al., 2004). The fact that we observed rhythms of *Rev-erba*, *Per2*, and *Dbp* in this study demonstrates that the serum pulse employed was successful in synchronizing our cell population. The lack of rhythmicity of other genes was thus unexpected. Although very low-amplitude rhythms of these genes may exist, our data suggest that an adipocyte clock is insufficient to drive rhythmic expression of these mRNAs in 3T3-L1 cells. Future work will investigate the molecular basis of this surprising finding.

The amplitude of *Per2* and *Dbp* rhythms in adipocyte cells was clearly less than that of the equivalent pre-adipocytes. As circadian clocks reside within individual cells (Welsh et al., 1995; Nagoshi et al., 2004; Welsh et al., 2004), the dampened rhythms observed in our

experiments may conceivably be due to attenuated intra-cellular rhythm amplitude or a decreased ability of the serum pulse to synchronize the cell population. Although we cannot yet rule out poorer inter-cellular synchronisation behind the reduced rhythmicity in the 3T3-L1 adipocytes, attenuated amplitude of the rhythmicity in adipose tissue from obese mice (Ando et al., 2005; Kohsaka et al., 2007) is consistent with a possible inhibition of the molecular clock mechanism by intra-cellular triglyceride accumulation.

In addition to control of mRNA expression, our data indicate that adipocyte clocks may drive circadian secretion of leptin, but not adiponectin. Although the reduction of leptin secretion between 8 and 12 h post-pulse coincides with reduced mRNA expression, later temporal fluctuations of leptin secretion do not parallel mRNA levels. This finding is consistent with a previous observation that hepatic protein rhythms do not necessarily reflect temporal mRNA expression in the same tissue (Reddy et al., 2006). 24 h rhythms of plasma leptin and adiponectin have previously been reported in humans and rodents by some investigators (Sinha et al., 1996; Kalsbeek et al., 2001; Gavrilu et al., 2003). However, this is the first indication of an adipocyte-based mechanism driving rhythmic plasma leptin concentration, as opposed to other possible contributory factors, such as changes in hormone clearance or secretory control by input pathway(s). Based on our data, such mechanisms may contribute towards the 24 h variation of plasma adiponectin concentrations that have been reported *in vivo*. Future analysis of rhythmic adipokine secretion from adipocyte cultures will need to consider hormone half life. Previous studies of plasma and intracellular hormone dynamics have estimated half-lives of approximately 30 min for leptin (Klein et al., 1996; Harris et al., 1997) and 3 h for adiponectin (Hoffstedt et al., 2004; Clasen et al., 2005), but their half-life in culture medium is unclear.

Although this study increases current knowledge of the interaction between circadian clocks and adipocyte biology, there are a number of ways in which the work could be usefully extended. Despite the 3T3-L1 cell line being frequently used as a model for adipocyte physiology, it is quite possible that there are differences in circadian biology between these cells and primary mouse adipocytes. Thus, we have employed some caution when comparing our data with published data describing murine WAT gene expression. It would also be beneficial to lengthen the time course of the study in order to allow more detailed analysis of the nature of adipocyte rhythmicity, including calculation of rhythm period and phase. One way of achieving this aim would be to employ real-time reporter imaging technology. Indeed, real-time analysis of *Bmal1*, *Per1*, and *Cry1* promoter activity in living cells may reveal circadian changes of gene expression that were not detected using our standard serum pulse protocol.

In summary, this study provides evidence for an endogenous adipocyte clock that controls rhythmic expression of a subset of genes found to be rhythmic in WAT. The adipocyte clock may also regulate endocrine activity. These data, therefore, strengthen the view that circadian rhythms have functional relevance to adipose biology, lipid metabolism, and related pathophysiology.

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Figure Legends

Figure 1. Differentiation of 3T3-L1 cells. Pre-adipocytes were differentiated into adipocytes as described in Materials and Methods. At two-day intervals, cells were analyzed by (A) Oil Red O histological stain, (B) triglyceride quantification, (C) gene expression by reverse transcriptase PCR. (B) Data show mean $\pm$ SEM of n=3 groups of cells/time point.

Figure 2. Temporal profiles of clock gene expression in pre-adipocytes and adipocytes following a serum pulse. Cells were treated with a serum pulse and gene expression was measured by qPCR. Data show mean $\pm$ SEM of n=3 groups of cells/time point.

Figure 3. Differential amplitude of *Per2* and *Dbp* expression in pre-adipocytes and adipocytes. Cells were treated with a serum pulse and gene expression was measured by qPCR. Data represent mean $\pm$ SEM for n=3 groups of pre-adipocyte (black bars) and adipocyte (white bars) cells between 16-32 h post-pulse. ** $p < 0.01$, *** $p < 0.001$ pre-adipocyte vs adipocyte gene expression at a given time point.

Figure 4. Temporal profiles of metabolic gene expression in pre-adipocytes and adipocytes following a serum pulse. Cells were treated with a serum pulse and gene expression was measured by qPCR. Data show mean $\pm$ SEM of n=3 groups of cells/time point.

Figure 5. Temporal expression and secretion of leptin and adiponectin following a serum pulse of differentiated adipocytes. Adipokine concentration was measured in culture medium by ELISA and gene expression measured by qPCR. (A) Concentration of leptin and adiponectin in culture medium following the serum pulse (mean $\pm$ SEM of n=3 groups of

cells/time point). Dashed lines show the linear regression for the data. (B) Rate of secretion, estimated by plotting the difference in mean adipokine concentration between consecutive time points from (A). (C) Adipokine gene expression measured by qPCR (mean $\pm$ SEM of n=3 groups of cells/time point).