Review

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Adipose Circadian Rhythms: Translating Cellular and Animal Studies to Human Physiology

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Abstract (140 words; max 150)

Emerging links between circadian rhythms and metabolism promise much for the understanding of metabolic physiology and pathophysiology, in which white adipose tissue (WAT) plays a prominent role. Many WAT endocrine molecules, termed adipokines, display rhythmic plasma concentration. Moreover, similar to most other tissues, WAT exhibits widespread 24-hour variation in gene expression, with approximately 20% of the murine adipose transcriptome estimated to undergo daily variation.

A major limitation to human chronobiology research is the availability of physiologically defined peripheral tissues. To date most analyses of in vivo human peripheral clocks has been limited to blood leukocytes. However, subcutaneous adipose tissue represents a novel opportunity to study peripheral molecular rhythms that are of clearly defined metabolic relevance.

This review summarises basic concepts of circadian and metabolic physiology before then comparing alternative protocols used to analyse the rhythmic properties of human adipose tissue.

Keywords (max 6):

Circadian, clock gene, adipose, adipocyte, metabolism, translational

1. Introduction

One area of circadian biology that is currently receiving great attention is the interaction between circadian clocks and metabolism. Evidence linking these two processes is now widespread and covers multiple disciplines from epidemiology through to physiology and molecular biology (reviewed in e.g. Asher and Schibler, 2011; Bass and Takahashi, 2010; Garaulet et al., 2010; Froy, 2011). This review will initially describe some of the key elements of circadian rhythmicity and its metabolic interaction, before then focussing on the role of circadian timing within adipose tissue physiology.

2. Circadian clocks: molecular control and links with metabolism

Circadian clocks are present throughout the living world and regulate a diverse array of biological processes, including endocrine function (Hastings et al., 2007). In mammals, the anatomical framework of circadian rhythms is described by a complex circadian timing system in which the 'master' clock within the suprachiasmatic nuclei (SCN) of the hypothalamus responds to external photic stimuli and synchronises the relative phasing of additional clocks found throughout the brain and peripheral tissues (Dibner et al., 2010).

Understanding of the molecular basis of circadian timing has proceeded at great pace over the past fifteen years and detailed reviews of this subject can be found elsewhere (e.g. Baggs and Hogenesch, 2010; Ukai and Ueda, 2010). A dominant model of the

molecular clock is the transcriptional-translational feedback loop (TTFL) model. At its heart is a core autoregulatory feedback mechanism in which transcription factors CLOCK (or its paralogue NPAS2) and BMAL1 stimulate transcription of *Period (Per)* and *Cryptochrome (Cry)* genes. The translated PER and CRY proteins then feed back to repress their own transcription. The importance of these factors is revealed by the marked disruption of behavioural and molecular rhythms observed in mice that are homozygous for a dominant negative form of *Clock* (*Clock*-Δ19; Vitaterna et al, 1994), null mutation of the *Bmal1* gene (Bunger et al., 2000), or 'double knockout' for *Per1*/*Per2* (Bae et al., 2001) or *Cry1*/*Cry2* (van der Horst et al., 1999).

The simple loop described above is not by itself sufficient to generate a functioning circadian clock. Many additional components have now been identified that allow temporal control of the clock mechanism. For instance, the casein kinases 1 delta (CK1δ) and epsilon (CK1ε) provide post-transcriptional control of PER protein turnover (Lowrey et al., 2000; Akashi et al., 2002; Xu et al., 2005; Meng et al., 2008). Other key post-translational components within the mammalian TTFL mechanism include glycogen synthase kinase 3β (GSK3β), which phosphorylates multiple circadian proteins (Iitaka et al., 2005; Harada et al., 2005; Sahar et al., 2010); the F-box protein FBXL3, which is part of an ubiquitin ligase complex and destabilises CRY proteins (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007); and the nutrient-responsive enzyme AMP kinase (AMPK), which phosphorylates and destabilises CRY1 protein (Lamia et al., 2009).

Many secondary TTFLs have been identified in which the products of clock-controlled genes are able to feed back into the core loop; these secondary loops are thought to provide additional stability to the clock mechanism and/or provide pathways through which intracellular signals can regulate or synchronise clock function. Interestingly, a large number of secondary TTFLs include the regulation of metabolically active genes and proteins. The best-characterised secondary TTFL involves the nuclear receptor REV-ERBα (NR1D1), a heme sensor (Raghuram et al., 2007; Yin et al., 2007) that is rhythmically transcribed by CLOCK-BMAL1 complexes and acts to rhythmically repress *Bmal1* transcription (Preitner et al, 2002). Other secondary loops linked to metabolism involve peroxisome proliferator-activated receptor alpha (PPARα; Canaple et al., 2006), PPARγ co-activator 1α (PGC1α; Liu et al., 2007) and the nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase SIRT1 (Ramsey et al., 2009; Nakahata et al., 2009).

In addition to the TTFL model, there is a body of evidence indicating that circadian timing may employ mechanisms that are independent of the cell nucleus. Early data supporting such mechanisms included the observation of enzymatic rhythms in enucleate red blood cells (Mabood et al., 1978). Subsequent evidence came from the elegant demonstration of circadian rhythms of phosphorylation of the cyanobacterial protein KaiC when it is mixed with only two other proteins and ATP in vitro (Nakajima et al., 2005). More recently, it has been argued that circadian rhythms in multiple species are regulated by cytoplasmic signalling systems (reviewed in Hastings et al., 2008) and that eukaryotic rhythms of peroxiredoxin oxidation can persist in the absence of transcription and translation (O'Neill and Reddy, 2011;

O'Neill et al., 2011). A focus of future work in this area is likely to investigate the functional links between TTFLs and these extra-nuclear circadian mechanisms.

Strong genetic support for the link between circadian clocks and metabolism has come from observations that animals with lesions of clock-related genes display altered metabolic phenotypes. Examples include *Clock-Δ19* mice (Rudic et al., 2004; Turek et al., 2005; Oishi et al., 2006; Kennaway et al., 2007), and homozygous 'knockouts' for *Bmal1* (Rudic et al., 2004), *Nocturnin* (Green et al., 2007), *Per1* (Dallmann et al., 2006), *Per2* (Yang et al., 2009), *Per3* (Dallmann and Weaver, 2010; Costa et al., 2011), *Rev-erbα* (Raspe et al., 2002). Although the specific phenotype may be partially dependent upon genetic background (discussed in Johnston et al., 2009), the widespread nature of this clock-metabolic interaction is compelling. Building upon the animal data, numerous studies also report human genetic links between metabolic status and clock gene polymorphisms of *BMAL1* (Woon et al., 2007), *CLOCK* (Scott et al., 2008; Sookoian et al., 2008; Tsuzaki et al., 2010), *NPAS2* and *PER2* (Englund et al., 2009).

Finally, at the level of whole organism physiology, there is a clear relationship between abnormally timed feeding and metabolic dysfunction. In human populations, it is well documented that shift workers exhibit increased obesity and other markers of cardio-metabolic disease (Biggi et al., 2008; Suwazono et al., 2008; Pietroiusti et al., 2010). Although shift work includes a strong element of circadian disorder, it is also characterised by other disturbances, e.g. altered sleep, social interactions, and feeding quality/behaviour (reviewed in Lowden et al., 2010). However, human night eating syndrome is also associated with increased body weight (O'Reardon et al., 2004;

Colles et al., 2007), suggesting that eating outside the endogenous circadian day may at least partially explain metabolic disturbances in shift workers. This view is further supported by data from a laboratory forced desynchrony protocol (Scheer et al., 2009) and a wealth of human post-prandial studies demonstrating that nocturnal eating leads to elevated plasma triacylglycerol, glucose and insulin concentration (reviewed in Morgan et al, 2003). In rodent models, caloric intake at abnormal times of day induces increased body weight gain (Arble et al., 2009) whereas restricting food intake to the normal activity phase can prevent obesity in simulated 'night work' (Salgado-Delgado et al., 2010). Furthermore, housing mice on short 20-hour light-dark cycles accelerated body weight gain and caused elevated plasma leptin and insulin, without any significant change in glucose concentration, consistent with a reduction of insulin sensitivity (Karatsoreos et al., 2011). These data suggest that animal models may be informative in understanding the mechanisms linking timed feeding behaviour with metabolism.

3. Identification and function of adipose rhythms

Adipose tissue is found in localised depots throughout the body and has multiple functions. For many years, it was believed that the roles of white adipose tissue were restricted to physical cushioning and energy storage. However, the cloning of the hormone leptin was pivotal in the identification of fat as an important endocrine tissue (Zhang et al., 1994). There are now a large number of identified adipose signalling molecules, termed adipokines (reviewed in Trujillo and Scherer, 2006; Galic et al., 2010), many of which exhibit 24-hour variation in plasma concentration (Sinha et al.,

1996; Gavrila et al., 2003; Parlee et al., 2010; Scheer et al., 2010). In rodents, the diurnal leptin rhythm is dependent upon an intact SCN (Kalsbeek et al., 2001) demonstrating a functional link with the circadian timing system. Although these leptin rhythms could result from numerous factors (e.g. acute response to feeding, rhythmic clearance), their presence may reflect a circadian component to adipokine secretion residing locally within adipose tissue. Indeed, preliminary data from synchronised murine 3T3-L1 adipocyte cells indicates that one of the functions of the adipocyte clock may be to regulate leptin secretion (Otway et al., 2009). Although this finding needs to be validated using a perfusion culture system, it is nonetheless consistent with the identification of a circadian component to daily variation in human plasma leptin data (Shea et al., 2005).

Molecular evidence for the presence of adipose circadian clocks has come from the identification of rhythmic clock gene expression in serial murine tissue samples (e.g. Ando et al, 2005; Zvonic et al., 2006; Kohsaka et al., 2007). In addition to these focussed analyses of clock gene expression, array-based approaches have been applied to adipose tissue in order to estimate the breadth of influence of rhythmical physiology. Microarray experiments have provided comprehensive analysis of 24-hour transcriptome regulation in multiple tissues (reviewed in Duffield, 2003), and it is often regarded that approximately 5-10% of the transcriptome of a given tissue exhibits daily rhythms. Only a small number of these genes are rhythmic in multiple tissues, suggesting that most rhythmic genes are tissue-specific outputs of the core circadian clock. Analysis of inguinal and epididymal murine adipose tissue has estimated that up to 20% of the adipose transcriptome undergoes 24-hour variation (Ptitsyn et al., 2006; Zvonic et al., 2006). Identification of the rhythmically expressed

192 genes strongly suggests that many of the key metabolic functions of adipose tissue are
193 under temporal control. However, a caveat in the interpretation of mRNA data comes
194 from the liver; the hepatic proteome in fact poorly matches its corresponding
195 transcriptome (Reddy et al., 2006) and thus rhythmicity in the adipose transcriptome
196 may not accurately reflect protein expression and/or function.
197
198 Tissue-specific disruption of circadian clock function has been successfully
199 accomplished in transgenic mouse models. For instance, using this technique, it has
200 been demonstrated that both the hepatic and pancreatic clocks play key roles in whole
201 body glucose homeostasis (Lamia et al., 2008; Marcheva et al., 2010; Sadacca et al.,
202 2011). A similar transgenic approach has used expression of CLOCK-Δ19 driven by
203 the aP2 promoter to suppress clock function in adipocytes (Bray and Young, 2009).
204 However, this approach targets not only adipocytes but also other cell types, such as
205 cardiomyocytes and macrophages. It is therefore difficult to ascribe a definitive
206 physiological role to the adipose clock at present. Recent work has identified a new
207 adipocyte-specific promoter cassette (Wang et al., 2010) and so it is likely that an
208 adipocyte-clock knockout mouse will soon be available.
209
210
211 **4. Molecular analysis of human adipose rhythms**
212
213 A major technical limitation that has hindered the study of human circadian rhythms
214 has been the difficulty in identifying a tissue that can be used to study peripheral clock
215 function in vivo. Although visceral adipose depots are not readily accessible,
216 subcutaneous adipose tissue offer the possibility of sampling that is tolerable for the

experimental subjects. Varied experimental approaches have been employed to assess human adipose rhythms (summarised in Table 1).

4.1. Single time point analysis

The simplest way to estimate the association between clock gene expression and metabolism is to measure the expression of different clock genes at a single time point. Although this approach may appear counter-intuitive, the relationship between expression of a given clock gene and either metabolic status or the expression of other gene(s) may be informative if biopsies are collected at an equivalent time point. Furthermore, the use of a single biopsy permits the analysis of visceral tissue, which cannot be repeatedly sampled.

In one study, *BMAL1*, *PER2* and *CRY1* were measured in visceral and subcutaneous biopsies taken from obese men with BMI $\geq$ 40 (Gómez-Abellán et al., 2008). The expression of all three genes in subcutaneous adipose exhibited significant correlations with plasma concentration of total and low density lipoprotein (LDL) cholesterol; in visceral adipose *PER2* expression was negatively correlated with waist circumference. A second study (Wu et al., 2009) compared subcutaneous adipose mRNA expression with BMI in subjects ranging from lean (BMI < 25) to obese (BMI > 30). Expression of most clock genes was found to correlate with that of adipocyte biomarker genes. However, correlations between clock gene expression and BMI were more complex with marked differences found in different subject clusters, possibly reflecting effects of subject age.

Although these experiments provide useful preliminary data linking regional adipose clock gene expression with metabolic status, they are nonetheless subject to the disadvantage that a single time point analysis does not permit interpretation of circadian or diurnal rhythms.

4.2. Cultured biopsies

An imaginative method used to circumvent the problems of single time point sampling has been to culture adipose biopsies taken from human volunteers. In these experiments, adipose biopsies have been collected from both subcutaneous and visceral regions of metabolically characterised subjects. A single biopsy is split into pieces which are cultured and then collected for analysis, typically at 6-hourly intervals over a 24 hour period. Using this protocol, temporal variation has been demonstrated for the expression of genes involved in glucocorticoid function, circadian rhythms and adipokine signalling (Hernández-Morante et al., 2009; Gómez-Santos et al., 2009; Gómez-Abellán et al., 2010). Cosinor curve fits through the four time points indicate that there are marked phase differences in mRNA rhythms between subcutaneous and visceral adipose depots. This intriguing possibility suggests that adipose clocks may be depot-specific and thus warrants further investigation into the coupling and function of adipose clocks.

In addition to the ability to compare subcutaneous and visceral adipose depots from the same individuals, this technique permits the identification of endogenous adipose rhythms. However the technique does also provide some disadvantages. Firstly, temporal resolution of the analysis is limited by the amount of tissue that can be

surgically removed. Secondly, by moving the tissue into an in vitro environment, it is difficult to relate results to in vivo physiology; given the importance of endocrine and other methods of inter-tissue communication in controlling whole-body metabolism, the variation between in vivo and in vitro adipose rhythmicity may have important consequences.

4.3. Serial biopsies

Despite tissue sampling restrictions, there has been some progress in the study of human peripheral rhythms. To date, the most commonly studied tissue has been blood leukocyte cells (e.g. Boivin et al., 2003; Fukuya et al., 2007), the rhythms in which correlate with those of melatonin and cortisol, in addition to sleep-wake timing (Archer et al., 2008). More recently, it has been reported that samples of human hair follicles from head or chin can be used as a marker of human peripheral rhythms (Akashi et al., 2010). However, one disadvantage of both leukocyte and hair follicle rhythms is that they do not have a clear link with metabolic physiology.

Two studies have now described temporal changes in gene expression using serial subcutaneous adipose biopsies. In the first of these studies, three biopsies were collected over 10.5 hour period; using array analysis of adipose from the umbilical region of overweight-obese male subjects (BMI range 27-35), they estimated that approximately 25% of the human adipose transcriptome undergoes diurnal regulation (Loboda et al., 2009). Interestingly, there were small but significant differences in gene signatures in the subjects, dependent upon whether they ate a meal 1-hour after the first biopsy or not. In a subsequent study, we have analysed diurnal gene

expression in individuals who are lean, mildly obese or obese with type 2 diabetes (Otway et al., 2011). Following a controlled pre-laboratory routine lasting one week, volunteers were brought into the laboratory for a night of acclimation. Biopsies were then collected every 6-hours across a 24-hour period from the upper buttock region, which is known to contain metabolically active adipose tissue (Khan et al., 2002). We observed robust rhythms in all clock genes measured and also in genes that have been linked to both circadian and metabolic function. Surprisingly, and in contrast to data from a similar mouse experiment (Ando et al., 2005), we found no significant differences in gene expression between our three experimental groups.

Although these studies are limited to sampling subcutaneous adipose tissue, which is not as good a predictor of metabolic disease as visceral adipose (Després and Lemieux, 2006), they nonetheless now validate a protocol for analysis of in vivo rhythms in a metabolically active tissue. Furthermore, it is clear that subcutaneous adipose tissue can be used as a marker of physiologically relevant peripheral clock in studies of in vivo human chronobiology.

4.4. Cell culture and real-time analysis of gene expression

As discussed previously (Johnston et al., 2009; Gimble et al., 2011), one important consideration when studying adipose tissue is its heterogeneous nature. Most, if not all, of the adipose cell types contain their own endogenous clock. Furthermore, the relative composition of adipose tissue varies depending upon metabolic state; for instance, obesity is characterised by increased macrophage infiltration into the tissue.

316 A potential limitation of adipose tissue analysis is therefore the uncertainty of which
317 cell type(s) are contributing towards the observed rhythmicity.
318
319 In addition to work with the murine 3T3-L1 cell line (Otway et al., 2009) described
320 above, investigators have studied rhythms in human adipocyte cells differentiated
321 from adipose-derived stem cells (ASCs). Using a serum pulse technique, such as
322 pioneered by Balsalobre et al. (1998), followed by analysis of cell lysates collected
323 every four hours, circadian rhythms of gene expression have been identified in
324 undifferentiated (Wu et al., 2007; Huang et al., 2009) and adipocyte differentiated
325 (Wu et al., 2007) ASCs. Together these data have provided strong support for the
326 presence of circadian clock within adipocyte cells, the key metabolic cell type within
327 adipose tissue.
328
329 Published data of adipocyte cell rhythms have thus far been restricted by the sampling
330 resolution of the methodology described above. Further understanding of adipocyte
331 circadian biology will likely require genetic manipulation of the core clockwork
332 and/or real-time reporter gene analysis to provide superior temporal resolution of
333 molecular rhythms. By using lentiviral constructs to transfect primary cells, real-time
334 reporter imaging has been successfully applied to the study of human skin fibroblasts
335 in vitro (Brown et al., 2005). As well as the improved temporal resolution provided
336 by this approach, it provides the advantage of permitting analysis of multiple
337 circadian cycles from a small amount of source tissue. As a result, it may well
338 therefore be ideal for comparing the circadian properties of multiple adipose depots
339 taken from both subcutaneous and visceral regions.
340

5. Conclusions

The links between circadian and metabolic physiology are becoming clearer and promise much for the understanding of metabolic disease. Moreover, the identification of subcutaneous adipose tissue as an accessible human peripheral clock will aid the translation of chronobiology to human subjects.

Although much research into the physiology of circadian metabolism has to date focussed on the liver and pancreas, there is mounting interest in understanding the function of adipose and adipocyte clocks. By using transgenic approaches, a key step forward will be the analysis of mice bearing a localised ablation of adipose clock function. In terms of human physiology, a number of complementary techniques are now available for experimental use. However, due to the likely effects of parameters such as feeding time (Zvonic et al., 2006; Loboda et al., 2009) and subject age (Wu et al., 2009) on clock gene expression, it will be extremely important for experimental groups to be tightly controlled in future studies.

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777 Table 1. Comparison of methods used to study human adipose clocks.

778

Method	Advantage	Disadvantage
Analysis of single time point biopsy	Study of subcutaneous and visceral fat	Difficult interpretation of temporal changes
Culture of single time point biopsy	Study of subcutaneous and visceral fat	Does not reflect in vivo state of tissue
	Identification of endogenous adipose rhythms	Limited tissue/sampling resolution
Serial sampling of biopsies	Analysis of in vivo rhythms	Analysis limited to subcutaneous fat
	Useful as a general marker for human metabolic rhythms	Limited tissue/sampling resolution
Cell culture	Study of subcutaneous and visceral fat	Does not reflect in vivo state of cells
	Viral transfection of reporter constructs permits high temporal resolution from a small amount of tissue	
	Identification of endogenous cellular rhythms	