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Circadian period of luciferase expression shortens with age in human mature adipocytes from obese patients

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ABSTRACT: Daily rhythms in physiology and behavior change with age. An unresolved question is to what extent such age-related alterations in circadian organization are driven by the central clock in the suprachiasmatic nucleus (SCN), modifying timing signals to contributing peripheral tissue oscillators, and are mediated by underlying changes in the local cellular oscillators themselves. Using a bioluminescence reporter approach, we sought to determine whether circadian clock function in human adipocytes from subcutaneous (SAT) and visceral (VAT) adipose tissues changes with age. SAT and VAT biopsies were obtained from obese individuals during gastric bypass surgeries [$n = 16$; body mass index: 44.8 ± 11.4 kg/m^2; age: 44 ± 9 yr (range: 30–58)]. Cells were isolated and transduced with a lentiviral circadian reporter construct [brain and muscle aryl hydrocarbon receptor nuclear translocator-like:luciferase (*BMAL:LUC*)], and bioluminescence was recorded over a period of 3 d. Human *BMAL1:LUC* adipocytes displayed a robust luminescence rhythm with comparable within-individual periods in mature and preadipocytes ($P > 0.05$). With increasing age, the circadian period decreased in mature adipocytes ($P = 0.005$) (β = 4 min/yr; $P < 0.05$). Our *ex vivo* approach indicated that ageing changes the organization of endogenous circadian oscillators in human adipocytes, independent of SCN signaling.—Kolbe, I., Carrasco-Benso, M. P., López-Mínguez, J., Luján, J., Scheer, F. A. J. L., Oster, H., Garaulet, M. Circadian period of luciferase expression shortens with age in human mature adipocytes from obese patients. FASEB J. 33, 175–180 (2019). www.fasebj.org

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Physiology is adapted to time-of-day–dependent needs that are anticipated by an internal time-keeping system composed of a network of cellular circadian clocks (1). These cell-autonomous clocks are driven by interlocking transcriptional–translational feedback loops of clock genes and proteins. One of these clock genes, brain and muscle aryl hydrocarbon receptor nuclear translocator-like (*BMAL*) *-1*, oscillates in its expression, and its function is essential for circadian timekeeping (2). Circadian clocks are present in virtually all cells, including those in adipose tissue (AT) (3). AT itself plays a leading role in systemic energy balance control and modulates food intake and substrate metabolism in other tissues (4).

Circadian rhythms deteriorate with age, and it is speculated that, in turn, age-related diseases are promoted by impaired clock function (5). Indeed, the association of age and circadian dysfunction can be directly observed in *Bmal1*-deficient mice that lack a functional circadian clock and show an accelerated aging phenotype (6). Furthermore, age-depended alterations in circadian behavior were observed in animal models where free-running periods (cycle length) of the rest–activity rhythms, body temperature, and drinking behavior shorten with age (7–11). In *ex vivo* conditions, a correlation between circadian period and age was reported for the master clock located in the suprachiasmatic nucleus (SCN) (12), but others found no age-related changes in the period of locomotor activity (13, 14) or in peripheral clocks, such as those of the cornea or the pituitary gland (12).

In humans, data are also contradictory. Although ageing is associated with an earlier timing of the daily activity phase (15) and a dampening of sleep-wake rhythms (16,

ABBREVIATIONS: AT, adipose tissue; BMAL, brain and muscle aryl hydrocarbon receptor nuclear translocator-like; BMI, body mass index; SAT, subcutaneous adipose tissue; SCN, suprachiasmatic nucleus; VAT, visceral adipose tissue

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17), these changes do not necessarily reflect alterations in the intrinsic circadian period. Forced desynchrony studies have shown that the period of melatonin secretion rhythms in healthy older subjects is not shorter than that in young adults, suggesting that the endogenous period of the central circadian clock does not change with age (18). One possibility is that, not the period of the central circadian clock, but that of the peripheral oscillators, changes with age. However, in humans, the analysis of age-related changes in peripheral clock regulation has been confined to skin-derived fibroblasts (19). To our knowledge, no study so far has determined the intrinsic circadian period in peripheral tissues with important metabolic functions, such as AT, or investigated the relation of peripheral clock periods to age in humans.

In mice, for the precise determination of the period *in vivo* and *ex vivo*, transgenic animals can be used that express luciferase reporters under the control of clock gene promoters or reporters fused to clock proteins (20, 21). Studying adipose rhythms in humans is more challenging and, so far, repeated biopsies, cultured primary adipocytes or explants that are sampled at different time points across the day and night have been necessary to analyze the rhythmic expression of circadian clock genes in AT (22). The precision of the assessment of period and phase of AT rhythms with these tools is considerably limited, as most of these experiments are performed within a 24 h timeframe with limited samples (typically, $n \leq 6$) and long intervals of several hours between sample time points (typically, $\geq$4 h). It is known that differences in period occur in the range of minutes. Therefore, to obtain the precise determination of the intrinsic period in human adipocytes, continuous clock expression measurements with a higher resolution over several cycles are needed.

The purpose of the current study was to investigate the intrinsic circadian period of adipocytes collected from patients during gastric bypass surgery and to study the relationship with age. To achieve this goal, we used a lentiviral approach to introduce a clock-driven luminescence reporter into human pre- and mature adipocytes of both subcutaneous and visceral origin. The implementation of this technique in human adipose cells allowed us to continuously monitor the expression of a key regulator of the circadian clock, such as *BMAL1*, at 22 min intervals for at least 3 consecutive days.

MATERIALS AND METHODS

Participants

AT biopsies from subcutaneous and visceral depots were obtained from 7 male and 9 female donors (n = 16) who had undergone gastric bypass surgery at the general surgery service of the Virgen de la Arrixaca University Hospital (Murcia, Spain). With the exception of 2 participants, all were morbidly obese [body mass index (BMI): 44.76 $\pm$ 11.38 kg/m^2; age: 44 $\pm$ 9 yr]. Weight, BMI, and total body fat percentage were determined the day before surgery by bioimpedance measurement with the SC-330 body composition analyzer (Tanita Corporation of America, Arlington Heights, IL, USA). The participants were also asked for habits of sleep and meal timing, and individual chronotypes were determined with the Munich ChronoType Questionnaire (23). The ethics committee of the Virgen de la Arrixaca University Hospital approved the protocols, and all participants signed a written, informed consent before biopsies were obtained.

Isolation and culture of adipocytes from human adipose tissue

Adipose tissue (AT) biopsies were obtained from subcutaneous (SAT) and visceral (VAT) adipose depots during the surgery and processed immediately. Biopsies were cut into small portions and digested with type-II collagenase (C6885, 1 mg/ml; MilliporeSigma, Burlington, MA, USA) for 45 min in a shaking water bath (37°C). Digestion was stopped by adding an equal volume of culture medium [DMEM with high-glucose (4.5 g/L), 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), and penicillin-streptomycin-glutamine (10378-016; Thermo Fisher Scientific)] to the suspension. Cells were passed through a 100 μm mesh and centrifuged at 1000 rpm for 5 min. Mature adipocytes were collected from the top layer in all participants and, after centrifugation at 1500 rpm for 5 min, preadipocytes were collected from the sediment in a subset of 8 participants. The number of cells was determined by Neubauer chamber counts, and the cells were cultivated in 6-well plates (Sarstedt A, Nümbrecht, Germany) at 37°C and 5% CO_2.

Luciferase assay

For transduction, 8 μg/ml polybrene (MilliporeSigma) and 6.5 $\times$ 10^7 infective units of virus were added to the medium of mature and preadipocytes and cultured overnight. On the next day, medium for the mature adipocytes was refreshed, and the cells were synchronized by adding 100 nm dexamethasone (MilliporeSigma) for 2 h at 37°C. For the recordings, medium was complemented with 100 nM D-luciferin sodium salt (MilliporeSigma). Preadipocyte medium was also exchanged on the next day, but they were further grown to confluence (3 d) and synchronized for 2 h with 50% fetal bovine serum at 37°C, and fresh medium with D-luciferin was added. Bioluminescence was measured (Fluostar Galaxy; BMG Labtech, Ortenberg, Germany) in triplicate for each individual preparation and recorded every 22 min at 37°C for 3 consecutive days.

Statistics

Raw luminescence data were baseline subtracted (24 h moving average) and period determined by sine wave fitting (Prism 5; GraphPad, La Jolla, CA, USA). Only rhythms with a fitted frequency of $<$0.3/h were considered. Significance of differences in period between adipocytes and preadipocytes were determined with paired Student's t test (see Table 2). The age of participants was normally distributed (Kolmogorov-Smirnov test), and correlations between rhythm characteristics and age of the participants were analyzed by Pearson correlation coefficient, with sleep duration and individual chronotype as covariates. Statistical analyses were performed with SPSS (IBM, Armonk, NY, USA).

RESULTS

The general characteristics of the cohort are presented in **Table 1**. Of the donors, 87.5% were morbidly obese at the

TABLE 1. *General, meal timing, and sleep characteristics of the participants*

Characteristics	Means ± SD	Minimum value	Maximum value
General			
Age (yr)	44 ± 9	30	58
BMI (kg/m^2)	45 ± 11	24.8	73.96
Total body fat (impedance; %)	46 ± 10	20.60	56.80
Meal timing (h:min)			
Breakfast onset	08:52 ± 01:24	06:30	11:50
Lunch onset	14:50 ± 00:31	14:00	15:30
Dinner onset	21:10 ± 00:41	20:00	22:00
Nighttime sleep characteristics			
Onset (h:min)	00:56 ± 01:06	23:50	03:00
Duration (h)	5.86 ± 1.11	4	7.5
Midpoint (h)*	3.64 ± 0.92	2.50	5.00

Total population (n = 16). *Munich ChronoType Questionnaire [Juda *et al.* (23)].

time they underwent gastric bypass surgery. With age ranging from 30 to 58 yr, the group covered almost 3 decades of age difference, but within that range, their ages were normally distributed. For the consideration of the influence of sleep on diurnal behavior and a possible influence on the cellular properties, habitual sleep characteristics were assessed, with an average midpoint of sleep at 03:38 ± 0:55 h. Meal times have a strong influence on metabolic tissues. In our cohort, the onset of food intake (breakfast) differed in extreme cases by up to 5 h, but on the day of surgery no breakfast was served, so the last consumed meals were lunch and dinner on the previous day and they started within a 1.5 and 2.0 h range for all donors, respectively.

AT cells displayed a robust luminescence rhythm in mature and preadipocytes of both subcutaneous and visceral origin (**Fig. 1** and Supplemental Fig. 1). This rhythm was maintained across the 3 d of analysis, with similar characteristics among the 3 d. In mature SAT, the average period was 21.28 h within a range of 20.51–22.22 h (**Table 2**). With a mean period of 21.36 h in mature VAT (range 20.13–22.23 h), the mature adipocytes of both tissue types were comparable in period. Likewise, preadipocyte period did not significantly differ in direct comparison to their corresponding mature adipocytes. The first acrophase in luciferase activity occurred around 10 h after synchronization and was stable across cell states (pre- *vs.* mature adipocytes) and tissue origins (subcutaneous *vs.* visceral).

The circadian rhythm period of the luminescence of all cell states and tissues depots did not depend on sleep timing or duration, nor were they influenced by meal timing. We found that luciferase rhythmicity in *Bmal1:luc* adipocytes was age dependent in mature adipocytes. Although the period within subjects did not differ significantly between depots or cell states, the analysis of all patient data revealed period shortening for mature adipocytes with increasing age (β = 4 min/yr) (**Fig. 2**). Separate analyses showed significant correlation for SAT, whereas correlation was not

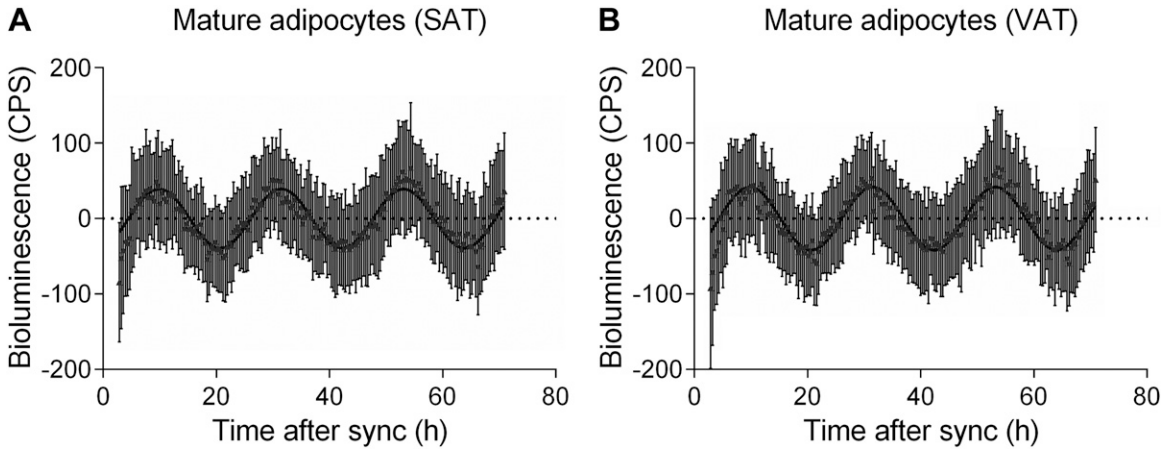

Figure 1. Circadian rhythms of bioluminescence in human subcutaneous and visceral mature adipocytes. Bioluminescence was assessed over 3 d (72 h) in subcutaneous and visceral adipocytes from 16 participants after the tissue was infected with a lentiviral vector expressing a circadian *BMAL1:luc* reporter. Rhythm of mature adipocytes of subcutaneous adipose tissue (SAT; n = 16) (*A*) and mature adipocytes of visceral adipose tissue (VAT; n = 15) (*B*). Curve indicates group cosine fit. CPS, counts per second. Data were normalized by subtracting the midline estimating statistic of rhythm. Vertical error bars, SEM. $P < 0.001$.

TABLE 2. *Circadian rhythm characteristics of mature adipocytes and preadipocytes of human AT*

Characteristic	AT depot	Mature adipocytes (n = 16)			Preadipocytes (n = 8)			P*
		Means ± SD	Minimum value	Maximum value	Means ± SD	Minimum value	Maximum value	
Period (h)	SAT	21.28 ± 0.61	20.51	22.22	20.64 ± 0.88	19.20	21.69	0.21
	VAT	21.36 ± 0.54	20.13	22.23	20.51 ± 0.93	18.87	21.67	0.09
Acrophase (h:min)	SAT	10:20 ± 1:15	07:41	11:58	10:06 ± 1:15	07:12	11:08	0.80
	VAT	10:22 ± 1:18	08:00	12:04	10:19 ± 1:17	07:25	11:31	0.90

The acrophase refers to the time of maximum expression of *BMAL1* after synchronization of the cell cultures. Rhythm for mature adipocytes and preadipocytes, $P < 0.001$. *Differences between adipocytes and preadipocytes.

significant for VAT or for preadipocytes from both AT depots (data not shown).

DISCUSSION

Viral transduction of human adipocytes with a lentiviral circadian luciferase reporter, a technique that was first introduced for human primary skin fibroblasts (24), allowed us to assess real-time clock function in freshly isolated adipocytes. In mature adipocytes of patients who underwent gastric bypass surgery, we were able to demonstrate a local deceleration of the adipose circadian clock rhythm with increasing age at an average rate of 4 min/yr. After transduction, adipocyte cells exhibited a robust rhythm of bioluminescence throughout the 3-d experiment, allowing for a precise calculation of the endogenous circadian period. Our results indicate that the local circadian rhythmicity in human mature adipocytes is associated with aging, with a shortening of the circadian period in mature adipocytes of older subjects. A similar correlation was also seen in rats, where a shortening of the circadian period with age was shown in several tissue explants (12).

In general, expression experiments for human circadian rhythm analysis require a large amount of tissue or repeated invasive extractions from participants. In our own experience, for adipose tissue analysis, at least 12 g of tissue is needed to establish a reliable circadian expression profile (22, 25–27). In this case, the profile is obtained by taking AT samples at least every 4 h along a 24 h cycle with a total of 6 time points in the analysis. In contrast, the bioluminescence technique that we used requires only a single biopsy of <4 g. This amount of AT was sufficient to obtain enough mature adipocytes and preadipocytes for a several-day recording with continuous measurements every 22 min (~200 determinations per explant, in total). This is a relevant advantage for human studies, in particular when studying nonobese subjects with less available AT mass. Extended monitoring allows for a much more accurate determination of the period of the circadian free-running rhythm.

Our bioluminescence results indicate that the endogenous circadian period of the AT clock of morbidly obese patients is ~21 h, similar to that found in other clock genes in human neuroblastoma cells (28). In contrast, other studies have shown that the circadian period was ~24–25 h in pancreatic islet cells of subjects who had brain death (*BMAL1* period: 23.9 h) (29), in human primary fibroblasts mostly from healthy subjects (*BMAL1* period: 24.5 h) (24), and in mouse immortalized adipocyte cell lines (*BMAl1* period, 25.0 h) (30). The differences in period among these studies could be explained by differences in: *1*) species (mice *vs.* humans), *2*) pathologic conditions (*e.g.*, healthy *vs.* brain death *vs.* obese.) *3*) sex, 4) culture conditions (that were kept constant in our study, regardless of age); or 5) cell types (neuroblastoma *vs.* pancreatic cells *vs.* fibroblasts *vs.* adipocytes, and so forth).

This is the first time the relationship between circadian period and age has been investigated in human adipocytes. The inverse correlation between age and period of mature adipocytes was not displayed

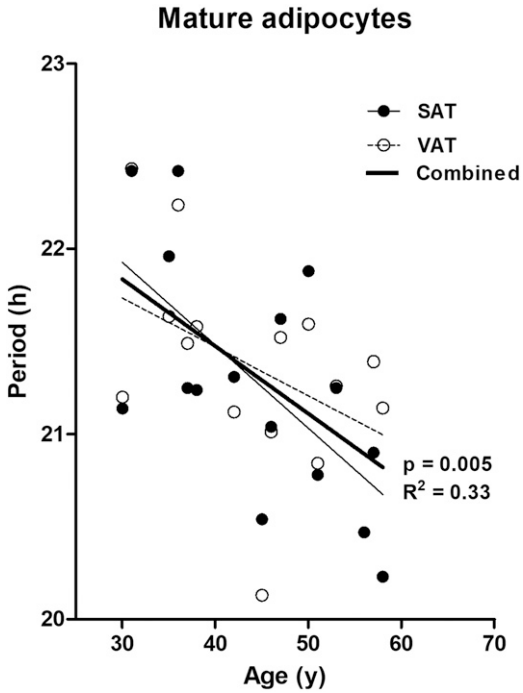

Figure 2. Correlation between age (yr) and period (h) of mature adipocytes. A significant and inverse correlation was found between age and the period of mature adipocytes [combination of subcutaneous (SAT) and visceral adipose tissue (VAT)]. $P = 0.005$. Separate analyses showed significant correlation for SAT, whereas correlation was not significant for VAT. $P = 0048$ and 0.196, respectively.

in previous experiments with other primary cells of peripheral tissues, such as skin fibroblasts. In that study, no age differences were found in the circadian period (19), but the study was performed in healthy (nonobese) volunteers and with a larger proportion of evening-type subjects (midpoint of sleep, ~5 AM) than in the current study which was performed in more morning-type subjects (midpoint of sleep, ~3:30 AM) all of which were severely obese.

Pagani *et al.* (19) found that the presence of human serum from older donors shortened the circadian period in skin fibroblasts compared with treatment with serum from young subjects, suggesting that an age-related circulating factor influences the cellular circadian period. The intrinsic period of the central circadian pacemaker appears not to change with age, as shown in a series of forced desynchrony experiments in which rhythms in healthy older subjects are not faster (or slower) than in young adults (18). Therefore, age-related changes in daily rhythms demonstrated in clinical and epidemiologic studies with consequences for both health and quality of life (31) could be the result either of changes in peripheral tissue oscillators, as demonstrated for AT in the current study, or of an alteration in the phase relations between the SCN and peripheral clocks. It has been hypothesized that each oscillating tissue differs with respect to age-related changes in period, affecting the normal phase relation between tissue clocks (12). In the current study, the period of AT was shortened, specifically in subcutaneous fat, suggesting that the effect of aging depends on AT depot location. This finding may be of particular clinical interest, given that this AT depot specifically expresses an endogenous circadian rhythm in insulin sensitivity. Further studies are needed to determine the relationship between the central clock in the SCN (*e.g.*, by measuring core body temperature or circulating melatonin concentrations) and the peripheral clocks, such as in the AT, by using forced desynchrony or constant routine protocols to assess whether aging is associated with a change in the relative timing of the circadian phase of central and peripheral clocks with essential metabolic functions (*i.e.*, internal misalignment). FJ

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AUTHOR CONTRIBUTIONS

F. A. J. L. Scheer, H. Oster, and M. Garaulet designed the experiment (project conception, development of overall research plan, and study oversight); I. Kolbe, M. P. Carrasco-Benso, and J. López-Mínguez conducted the experiments (hands-on execution of experiments and data collection); J. Luján collected samples; I. Kolbe and M. P. Carrasco-Benso analyzed data and performed statistical analysis; I. Kolbe, M. P. Carrasco-Benso, F. A. J. L. Scheer, H. Oster, and M. Garaulet wrote the manuscript; and H. Oster and M. Garaulet have primary responsibility for the final content.

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