ORIGINAL ARTICLE

Disrupted light-dark cycle induces obesity with hyperglycemia in genetically intact animals

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Abstract

BACKGROUND: The environmental light-dark (LD) cycle entrains the circadian clock located in the suprachiasmatic nucleus (SCN) of mammals. Recent studies of genetically impaired animals with clock gene mutations have revealed associations between metabolic disorders and the circadian clock. However, whether such disordered phenotypes are due to a loss of circadian clock function within specific metabolically relevant tissues, or the result of disrupted circadian behavioral activities governed by the SCN remains unknown.
OBJECTIVES: The present study examines the effect of disrupted LD cycles that might perturb the circadian clock in the SCN and peripheral organs on a high-fat/high-sucrose diet-induced obesity in genetically intact mice.
METHODS: The behavioral patterns of the mice were disturbed under an ultradian 3 h light-3 h dark cycle (LD 3:3) due to light-induced direct suppression of the behavior (masking effect).
RESULTS: Obesity with hyperglycemia was significantly enhanced and levels of hemoglobin A1c were significantly higher under LD 3:3 compared with LD 12:12.
CONCLUSIONS: These findings provide a link between metabolic disorders and the "environmental mutation" in genetically intact animals.

INTRODUCTION

Most organisms exhibit a variety of physiological and behavioral circadian rhythms controlled by endogenous oscillators. The central circadian clock in mammals is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Richter *et al.*, 2004). Environmental light is the critical cue for daily resetting of the central clock in the SCN. The phase and period of the pacemaker are entrained to an environmental light-dark (LD) cycle (Pittendrigh & Daan, 1976; Richter *et al.*, 2004). According to the discrete (non-parametric) entrainment model (Pittendrigh & Daan, 1976), synchronization of the circadian rhythm to an LD cycle is achieved by daily phase-resetting of the rhythm to adjust the endogenous period (tau) to the period of the Zeitgeber (T). When phase shifts caused by light pulses are smaller than the difference between tau and T, the circadian clock cannot entrain to environmental LD cycles. Under LD conditions, behavioral rhythms reflect both endogenous clock-dependent effects and direct light-induced suppression that is called "masking" (Mrosovsky *et al.*, 1999; Redlin & Mrosovsky, 1999).

Numerous studies at the molecular level have suggested that the circadian oscillator in the SCN is driven by self-sustained transcription/translation-based feedback loops consisting of the periodic expression of clock genes. Studies of clock genes in mammals have implied that oscillatory mechanisms function in various peripheral tissues such as the heart, lung, liver, kidney, and adipose tissues, and that such peripheral oscillators play important roles in regulating various physiological functions (Green *et al*., 2008).

Emerging evidence suggests that circadian regulation is intimately linked to metabolic homeostasis and that dysregulation of circadian rhythms can contribute to metabolic diseases such as obesity and diabetes (Green *et al*., 2008). Homozygous *Clock* mutant mice exhibit attenuated circadian feeding rhythms, hyperphagia, and obesity with hyperglycemia and dyslipidemia (Turek *et al*., 2005), although the phenotypes are strain-dependent (Kudo *et al*., 2007; Oishi *et al*., 2006). CLOCK protein regulates the circadian expression of sterol regulatory element binding protein-1 that transactivates several factors required for lipogenesis such as fatty acid synthase, acetyl-CoA carboxylase, and ATP-citrate lyase (Kudo *et al*., 2007; Oishi *et al*., 2006; Shimba *et al*., 2005). BMAL1 is involved in the gluconeogenesis and insulin resistance that evolves in response to a high-fat diet in mice (Rudic *et al*., 2004). However, the most compelling linkage between metabolic disorders and the circadian clock is demonstrated by the phenotypes of animals with clock gene mutations. Therefore, whether the phenotypes are due to a loss of circadian clock function within specific metabolically relevant tissues, or a result of disrupted circadian behavioral activities governed by the central clock in the SCN remains to be elucidated (Williams & Schwartz, 2005). To address this issue, we examined the effect of disordered LD cycles that might disrupt the central clock in the SCN in genetically intact mice with diet-induced obesity.

METHODS

Animals and treatments

Male Jcl:ICR mice (Clea Japan Inc., Tokyo, Japan) at 3 weeks of age were housed for 2 weeks under a 12 h light-12 h dark cycle (LD 12:12; lights on at 0:00 and lights off at 12:00), followed by a high-fat/high-sucrose diet (F2HFHSD; Oriental Yeast Co. Ltd., Tokyo, Japan) comprising 54.5% fat, 28.3% carbohydrates and 17.2% protein for 9 weeks under LD 12:12 or ultradian LD 3:3 cycles. A white fluorescent lamp served as a daytime light source.

Drinking behavior was continuously monitored by an infrared sensor and recorded using Chronobiology Kits (Stanford Software Systems, Stanford, CA) and the results are displayed as actograms. To evaluate the activity rhythms of individual animals, water consumption was monitored at 5 min intervals and the period was estimated using a chi-square periodogram (Sokolove & Bushell, 1978).

Food intake was measured for 7 days before the end of the study.

All animal experiments and care proceeded with the approval of our institutional Animal Care and Use Committee (Permission #2008-084).

Measurement of blood metabolic parameters

At the end of the study, blood samples were collected at 14:00. Hemoglobin A1c (HbA1c) was measured by the latex-aggregation method (Rapidia^{TM} auto HbA1c, Fujirebio, Tokyo, Japan). Mouse plasma samples were collected by centrifugation and stored at –80 °C. Plasma glucose, free fatty acids (FFA), triglyceride (TG), and total cholesterol (T-Cho) levels were measured using kits (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Statistical analysis

All values are expressed as means ± SEM. The statistical evaluation of the data was carried out by applying the two-way and one-way analysis of variance (ANOVA). As a post-hoc test, Welch's or Student's *t*-test was performed, and values of $p< 0.05$ were considered as statistically significant.

RESULTS

Mice were completely entrained to the LD cycle under LD 12:12 cycle (Figure 1). Under ultradian LD 3:3, the activity rhythm was free-running with a period of 25.5 ± 0.2 h, in addition to the light-induced direct suppression of the behavioral activity (masking). Drinking behavior gradually became arrhythmic after 4 weeks of LD 3:3.

The body weight (BW) gain for 9 weeks was enhanced under LD 3:3 compared with LD 12:12 (Figure 2).

The amount of food intake was increased under LD 3:3 compared with LD 12:12 (Table 1). Blood glucose and HbA1c levels were significantly higher in the LD 3:3, than those in the LD 12:12 cycle group, although plasma lipids levels did not significantly differ between them.

DISCUSSION

The present findings showed that a disturbance of the circadian behavior by an ultradian LD cycle induced obesity in genetically intact mice. Obesity with hyperglycemia and an increase in HbA1c was induced under LD 3:3 without affecting the lipid parameters.

Under ultradian LD 3:3, the activity rhythm was free-running with a long period for at least 1 month, in addition to the light-induced direct suppression of the behavioral activity (masking). Free-running period of the Jcl:ICR mice was 23.8 ± 0.1 h and 25.7 ± 0.2 h in constant darkness and constant light, respectively. Our findings suggest that the circadian clock in the SCN cannot entrain to the LD 3:3 and free-running as well as that under constant illumination.

We showed that drinking behavior (usually accompanied with the food intake) became completely

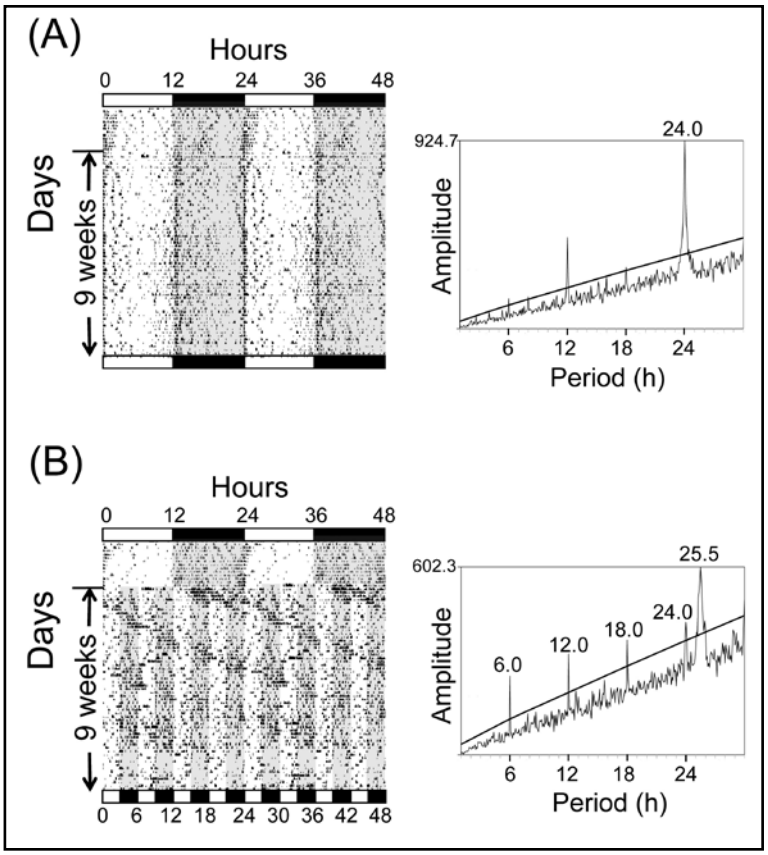

Figure 1. Circadian behavioral activity of mice is disrupted under LD 3:3.
Mice were housed under LD 12:12 (lights on at 0 h) and then maintained on LD 12:12 (**A**) or transferred to ultradian LD 3:3 (**B**) cycle for 9 weeks. Left: Representative double-plot actograms of drinking behavior. Dark phase duration is shaded in gray. Horizontal open and solid bars indicate day and night, respectively. Dots represent drinking behavior at 5 min intervals. Right: Chi-square periodgrams during first 4 weeks under LD 12:12 or LD 3:3 cycles. Solid lines denote 0.01 level of significance.

Table 1. Food consumption and plasma metabolic parameters in mice under LD 3:3.

	LD 12:12 (n=6)	LD 3:3 (n=7)
Food intake (g/day)	3.42 ± 0.34	4.90 ± 0.42*
Glucose (mg/dl)	229.9 ± 11.3	370.6 ± 49.2*
Free fatty acids (mEq/dl)	1.65 ± 0.08	1.47 ± 0.03
Triglyceride (mg/dl)	188.3 ± 20.5	163.6 ± 15.3
Total cholesterol (mg/dl)	260.0 ± 60.1	289.6 ± 76.1
HbA1c (%)	4.77 ± 0.15	5.54 ± 0.27*

Data are shown as means ± SEM. Significant differences compared with values under LD 12:12 are indicated as *p< 0.05.

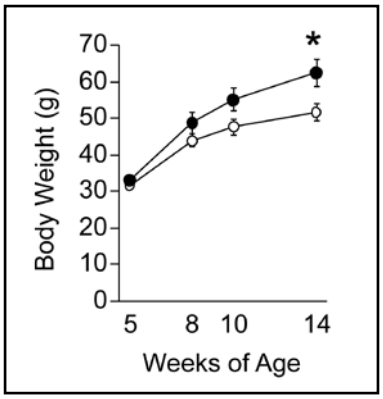

Figure 2. Diet-induced obesity is enhanced in mice under LD 3:3.
Mice were housed under LD 12:12 (open circles) or LD 3:3 (closed circles) cycles for 9 weeks. Values are means ± SEM (n=6–7). Between-group comparisons were performed using t-test (*p< 0.05).

arrhythmic under LD 3:3 for 9 weeks, suggesting that circadian changes in physiology such as food intake, energy expenditure, hormone secretion, and neural activity are impaired under this environmental condition. Animal experiments and epidemiological findings have demonstrated that a flattening in metabolic activities inputs constant signals to the brain and results in metabolic disorders (Kreier *et al.*, 2003). In the present study, the enhancement of BW gain under LD 3:3 might be caused by abnormal feeding rhythm, because the circadian phase of food consumption differentially affects a high-fat diet-induced BW gain (Arble *et al.*, 2009). The ultradian LD cycle seemed to directly affect glucose metabolism via the neural input of light to the SCN from the retina and/or indirectly by disrupting the rhythmic feeding behavior. Surgical blinding or SCN lesions abolish the hyperglycemia induced by glucopenia brought about by an intracranial injection of 2-deoxy-D-glucose (Nagai *et al.*, 1996). Electrical and chemical activation of the SCN also results in increased plasma glucose concentrations (Ruiter *et al.*, 2006). The peripheral administration of α- and β-adrenergic

receptor antagonists prevents SCN-dependent hyperglycemia, suggesting that the SCN and the environmental light information are involved in blood glucose regulation through the autonomic nervous system (Nagai *et al*., 1996; Ruiter *et al*., 2006). The SCN directly controls basal glucose levels independently of its influence on feeding activity, because a fasting or a scheduled feeding regimen has little effect on the circadian glucose fluctuation that is completely abolished in animals with a lesion of the SCN (La Fleur *et al*., 1999). Therefore, impaired SCN functions under ultradian LD cycles seemed to cause the hyperglycemia identified in the present study.

On the other hand, circadian misalignment under ultradian LD cycles has been previously shown to disrupt clock gene coordination in peripheral organs as well (Froy & Miskin, 2007). Therefore, impaired clock gene functions in peripheral organs might be involved in the disruptions of insulin secretion/ sensitivity and overall hormonal milieu such as insulin, glucagon, leptin, and glucocorticoids.

Our observations suggest that alignment between behavioral cycles and the environmental circadian LD cycle is important for metabolic control especially for glucose metabolism in mammals. Environmental LD cycles as well as circadian clock genes might be involved in the metabolic control exerted by the biological clock in the SCN.

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