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Disrupted daily light–dark cycle induces the expression of hepatic gluconeogenic regulatory genes and hyperglycemia with glucose intolerance in mice

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ABSTRACT

We elucidated associations between metabolic disorders and the environmental light–dark (LD) cycle that entrains the circadian clock located in the suprachiasmatic nucleus of mammals. Mice were fed with a high-fat/high-sucrose diet for eight weeks under a normal 12 h light–12 h dark cycle (LD 12:12) or an ultradian 3 h light–3 h dark cycle (LD 3:3) that might perturb the central clock. The circadian behavioral rhythms were gradually disturbed under LD 3:3. Hyperglycemia with glucose intolerance and increases in diabetic markers, glycated albumin and hemoglobin A1c, were significantly induced without affecting body weight gain and food consumption in LD 3:3. Expression levels of hepatic gluconeogenic regulatory genes such as *Pck1*, *G6pc*, *Hnf4a*, and *Foxo1/3/4* genes were increased under LD 3:3. Hypercholesterolemia with hepatic cholesterol accumulation was also induced in LD 3:3. Ultradian LD 3:3 cycles did not affect the adipose inflammation that is considered a major player in obesity-associated metabolic disorders. Our findings provide a link between metabolic disorders and environmental photoperiodic cycles in genetically normal animals.

1. Introduction

Shift work is implicated as a risk factor for chronic diseases such as cardiovascular disease, breast cancer, metabolic syndrome and diabetes [1]. Misaligned endogenous circadian rhythms with unpredictable daily photoperiodic cycles facilitated by artificial lighting might contribute to development of metabolic disorders in 24-h societies [2]. However, whether lighting stimuli directly or indirectly affect metabolic regulation via interaction with the endogenous circadian clock remains unknown.

Endogenous oscillators control many behavioral and physiological circadian rhythms such as those governing body temperature, blood pressure, immune functions, hormonal secretion and glucose and lipid metabolism. The central circadian clock is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in mammals [3]. Environmental light is the critical cue for daily resetting of the central clock in the SCN, and the phase and period of the pacemaker are entrained to environmental light–dark (LD) cycles [3]. According to the discrete (non-parametric) entrainment model [4], the circadian rhythm becomes synchronized to LD cycles by daily phase-resetting to adjust the endogenous (tau) to the Zeitgeber (T) period. The central circadian clock cannot entrain to environmental LD cycles when phase shifts caused by light pulses are smaller than the difference between tau and T.

Previously, we examined the effect of disrupted LD cycles that might perturb the central clock in the SCN of genetically intact mice with diet-induced obesity [5]. The behavioral patterns of the mice were disturbed under an ultradian 3 h light–3 h dark cycles (LD 3:3) due to light–induced direct suppression of the behavior (masking effect). Plasma glucose and hemoglobin A1c (HbA1c) levels were significantly increased under LD 3:3 compared with LD 12:12. In the present study, to elucidate the underlying mechanisms of the disrupted LD cycle-induced hyperglycemia, we examined the expression profiles of metabolic-related genes in peripheral tissues under LD 3:3. The circadian behavioral rhythms were gradually disturbed and hyperglycemia was induced under LD 3:3 as expected [5]. Glucose intolerance and increases in diabetic markers, glycated albumin and HbA1c, were significantly induced without affecting body weight gain and food consumptions in LD 3:3. Expression levels of hepatic gluconeogenic regulatory genes were increased under LD 3:3. Hypercholesterolemia with hepatic cholesterol accumulation was also induced in LD 3:3. Ultradian LD 3:3 cycle did not affect the adipose inflammation that is considered a major player in the obesity-associated metabolic disorders. Our findings provide a link between metabolic disorders and the environmental photoperiodic cycles in genetically normal animals.

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2. Methods

2.1. Animals and protocols

Three-week-old male ICR mice (Japan SLC Inc., Hamamatsu, Japan) were fed with a normal diet (CE-2; Clea Japan Inc., Tokyo, Japan) *ad libitum* for two weeks under a 12 h light–12 h dark cycle (LD 12:12; lights on, 0:00; lights off, 12:00), followed by a high-fat/high-sucrose diet (F2HFHSD; Oriental Yeast Co. Ltd., Tokyo, Japan) under LD 12:12 or ultradian LD 3:3 cycles for eight weeks. The light source was a white fluorescent lamp. The glucose tolerance test proceeded after seven weeks of LD 3:3. Tissues from sacrificed mice were dissected, rapidly frozen and stored in liquid nitrogen at 02:00 h after eight weeks of LD 3:3. Drinking behavior was continuously recorded using Chronobiology Kits (Stanford Software Systems, Stanford, CA) as described [5]. Animals were maintained and experiments proceeded under the approval of our institutional Animal Care and Use Committee (Permission #2011–020).

2.2. Measurement of blood hormones and metabolic parameters

Mouse blood collected in EDTA-coated tubes was immediately separated by centrifugation for 15 min at 5800*g*. Platelet-poor plasma was collected and stored at −80 °C. Plasma glucose, free fatty acids (FFA), triglyceride (TG), and total cholesterol levels were measured using kits (Wako Pure Chemical Industries Ltd., Osaka, Japan). Plasma insulin levels were measured using Mouse Insulin U-type ELISA kit (Shibayagi Co. Ltd., Shibukawa, Japan). Glycated albumin and HbA1c levels were measured enzymatically using Lucica® GA-L (Asahi Kasei Pharma Corp., Tokyo, Japan) and NORUDIA® HbA1c (Sekisui Medical Co. Ltd., Tokyo, Japan), respectively.

2.3. Glucose tolerance test

After seven weeks under LD 3:3, mice were fasted for four hours and then intraperitoneally administered with glucose (2 g/kg) in water. Blood glucose concentrations were determined from the tail vein using an Accu-check (Roche Diagnostics K.K., Tokyo, Japan) at 0, 15, 30, 60, 90, and 120 min. The incremental area under the curve (AUC) was calculated according to Wolever et al. [6].

2.4. Quantitative reverse transcription (RT)-PCR

Total RNA was extracted using RNAiso (Takara Bio Inc., Otsu, Japan). Single-stranded cDNA was synthesized using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara Bio Inc., Otsu, Japan). Real-time RT-PCR proceeded using SYBR® Premix Ex Taq™ II (Takara Bio Inc., Otsu, Japan) and a LightCycler™ (Roche Diagnostics, Mannheim, Germany). The reaction conditions were 95 °C for 10 s followed by 45 cycles of 95 °C for 5 s, 57 °C for 10 s and 72 °C for 10 s. Supplemental Table 1 shows the sequences of the primer pairs. The amount of target mRNA was normalized relative to that of *β-actin*.

2.5. Statistical analysis

All values are expressed as means ± SEM. Pairs of groups were compared using Welch's or Student's *t*-test. $P < 0.05$ indicated a statistically significant difference.

3. Results

Mice were completely entrained to the LD cycle under LD 12:12 (Fig. 1A). Under ultradian LD 3:3, the activity rhythm was free-running with a period of 25.7 ± 0.2 h at least for two weeks and gradually became arrhythmic after 2–4 weeks of LD 3:3.

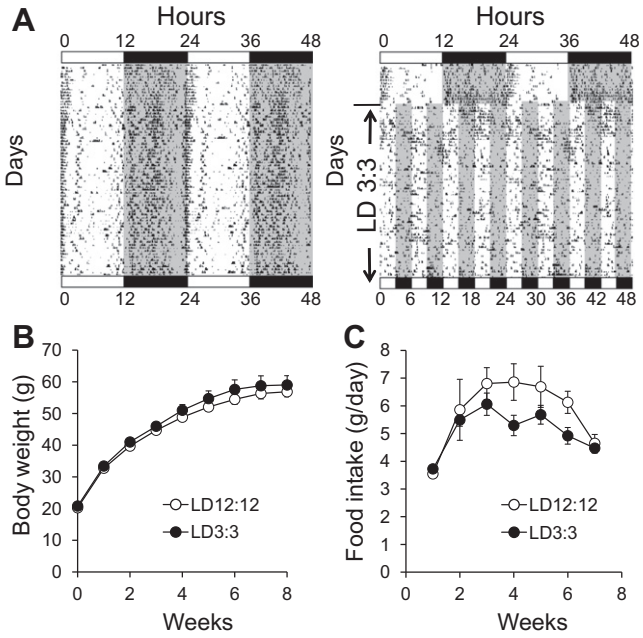

Fig. 1. Circadian behavioral activity, body weight gain, and food consumption of mice under LD 3:3. Representative double-plot actograms of drinking behavior (A). Mice were acclimated by feeding with a normal diet under LD 12:12 (lights on at 0 h) and then maintained on LD 12:12 or transferred to ultradian LD 3:3 cycles for eight weeks. Dark phase duration is shaded in gray. Horizontal unfilled and filled bars indicate day and night, respectively. Dots represent drinking behavior at 5-min intervals. Body weight gain (B) and food consumption (C) of mice under LD 12:12 (○) or LD 3:3 (●) cycles for eight weeks. Values are shown as means ± SEM.

Levels of blood glucose and glycated proteins such as glycated albumin and HbA1c were significantly higher under LD 3:3, than LD 12:12 group (Table 1), although all mice gained an identical amount of body weight (BW; Fig. 1B). The intraperitoneal glucose tolerance test showed a significantly increased AUC in LD 3:3 compared with LD 12:12 (Fig. 2). Plasma insulin levels were almost identical between the groups. The fasting insulin to glucose ratio, a marker of insulin sensitivity, was not affected by ultradian LD 3:3 cycles (Table 1). Hypercholesterolemia with hepatic cholesterol accumulation was significantly induced under LD 3:3, while plasma TG and FFA levels were similar between the groups (Table 1).

Table 1
Metabolic parameters of mice under disrupted light–dark cycles.

	LD 12:12	LD 3:3
Blood		
Glucose (mg/dL)	177.7 ± 9.6	219.1 ± 13.8*
Free fatty acids (mEq/L)	1.54 ± 0.13	1.67 ± 0.080
Triglyceride (mg/dL)	164.5 ± 11.5	201.3 ± 20.4
Total cholesterol (mg/dL)	171.6 ± 10.2	210.5 ± 8.3*
Insulin (ng/mL)	6.0 ± 1.3	6.4 ± 2.0
Glycated albumin (%)	2.0 ± 0.26	3.1 ± 0.24**
HbA1c (%)	3.2 ± 0.046	3.4 ± 0.067*
Ratio insulin:glucose	0.033 ± 0.0060	0.027 ± 0.0064
Liver		
Triglyceride (mg/g tissue)	19.0 ± 3.4	20.4 ± 0.92
Total cholesterol (mg/g tissue)	1.9 ± 0.10	2.5 ± 0.16*

Data are shown as means ± SEM ($n = 6$ and 7 in LD 12:12 and LD 3:3, respectively).
* $P < 0.05$.
** $P < 0.01$ significant differences compared with LD 12:12.

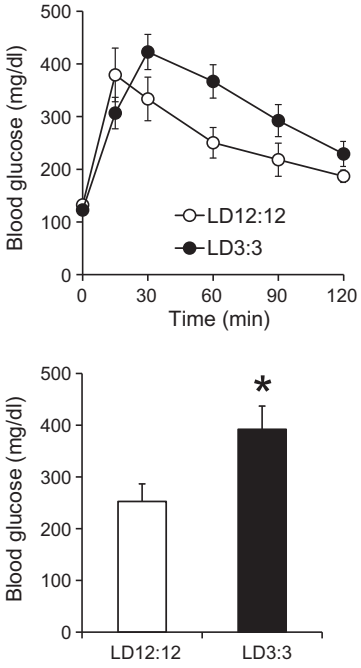

Fig. 2. Ultradian LD 3:3 cycles induce glucose intolerance in mice. Mice housed under LD 12:12 or LD 3:3 for seven weeks underwent glucose tolerance test (GTT) in fasting state. A: Blood glucose concentration curves during GTT. Open and filled circles (○,●), LD 12:12 and LD 3:3, respectively. B: Area under curve (AUC) for glucose during GTT. Values are means ± SEM. Between-group comparisons performed using *t*-test (*$P < 0.05$).

Table 2
Metabolism-related gene expression in liver under disrupted light–dark cycles.

	LD 12:12	LD 3:3
Foxo1	100.0 ± 17.6	242.8 ± 21.7**
Foxo3	100.0 ± 7.9	140.6 ± 12.4*
Foxo4	100.0 ± 9.6	146.6 ± 9.8**
Hnf4a	100.0 ± 7.0	123.2 ± 7.3*
Ppargc1a	100.0 ± 8.7	100.9 ± 10.1
Pck1	100.0 ± 17.9	192.2 ± 29.5*
G6pc	100.0 ± 10.5	145.3 ± 10.3*
Gck	100.0 ± 7.7	131.7 ± 7.4*
Pklr	100.0 ± 20.3	146.0 ± 15.4
Slc2a2	100.0 ± 10.5	149.5 ± 11.2**
Irs1	100.0 ± 6.4	114.6 ± 10.0
Irs2	100.0 ± 6.7	83.6 ± 11.9
Srebf1	100.0 ± 5.5	139.5 ± 13.5*
Srebf2	100.0 ± 4.7	87.4 ± 6.5
Nr1h3	100.0 ± 6.0	110.9 ± 4.0
Fasn	100.0 ± 17.0	125.5 ± 14.5
Acaca	100.0 ± 12.6	121.1 ± 11.7
Pdk4	100.0 ± 20.5	77.7 ± 11.4
Fgf21	100.0 ± 28.9	76.9 ± 11.4
Ppara	100.0 ± 7.3	135.9 ± 12.3*
Abcg5	100.0 ± 5.7	134.2 ± 11.2*
Abcg8	100.0 ± 5.3	126.3 ± 17.1
Hmgcs1	100.0 ± 6.7	82.7 ± 8.2
Hmgcr	100.0 ± 15.4	131.1 ± 23.5
Cyp7a1	100.0 ± 16.2	232.5 ± 60.8
Ldlr	100.0 ± 14.3	134.9 ± 9.1

Value of each gene under LD 12:12 is expressed as 100%. Data are shown as means ± SEM ($n = 6$ and 7 under LD 12:12 and LD 3:3, respectively).
* $P < 0.05$.
** $P < 0.01$ significant differences compared with LD 12:12.

To better understand the underlying mechanisms of metabolic disorders induced by ultradian LD cycles, we collected samples of liver, white adipose tissue and skeletal muscle from mice at the conclusion of the experiment. We used quantitative RT-PCR to measure the expression of genes involved in glucose homeostasis, because hyperglycemia with glucose intolerance was developed under LD 3:3. Hepatocyte nuclear factor 4α (HNF4α, encoded by *Hnf4a*) and forkhead box O (FoxO) transcription factors play important roles in glucose homeostasis through transcriptional regulation of glucose-6-phosphatase (G6Pase, encoded by *G6pc*) and phosphoenolpyruvate carboxykinase (PEPCK, encoded by *Pck1*), which are rate-limiting enzymes in gluconeogenesis. Levels of *Hnf4a*, *Foxo1*, *Foxo3*, *Foxo4*, *G6pc* and *Pck1* gene expression were significantly increased under LD 3:3 (Table 2). The mRNA expression of glucose transporter 2 (GLUT2, encoded by *Slc2a2*) and a glycolytic key enzyme glucokinase (encoded by *Gck*) were also significantly increased under LD 3:3. Insulin signaling in the liver seemed largely unaffected because mRNA expression levels of IRS1 and IRS2 were similar between LD 12:12 and LD 3:3.

The mRNA expression of the insulin-sensitive transcription factor, sterol regulatory element binding protein 1c (SREBP1c, encoded by *Srebf1*), was significantly increased, whereas its downstream targets, fatty acid synthase (FAS, encoded by *Fasn*) and acetyl-CoA carboxylase (ACC1, encoded by *Acaca*) were only slightly affected under LD 3:3. The expression of genes involved in fatty acid oxidation such as pyruvate dehydrogenase kinase 4 (PDK4, encoded by *Pdk4*) and fibroblast growth factor 21 (FGF21, encoded by *Fgf21*) were unaffected, although the expression of their transcriptional regulator peroxisome proliferator-activated receptor α (PPARα, encoded by *Ppara*) was up-regulated under LD 3:3. These results suggested that ultradian LD 3:3 cycles little affected fatty acid regulation in the liver.

Cholesterol synthesis in the liver is controlled by a series of genes regulated by the transcription factor, sterol regulatory element binding protein 2 (SREBP2, encoded by *Srebf2*). The mRNA expression of *Srebf2* and its transcription target genes, HMG-CoA synthase (HMGCS, encoded by *Hmgcs1*) and HMG-CoA reductase (HMGCR, encoded by *Hmgcr*) were not affected under LD 3:3 despite enhanced cholesterol accumulation (Table 1). In contrast, the mRNA expression levels of the low-density lipoprotein (LDL) receptors (encoded by *Ldlr*) that clear cholesterol including LDL particles from the bloodstream, and of cholesterol 7-α-hydroxylase (CYP7A1, encoded by *Cyp7a1*), which is the rate-limiting enzyme for the conversion of cholesterol into bile acids, tended to increase under LD 3:3, although the differences did not reach statistical significance ($p = 0.057$ and $p = 0.074$, respectively). Hepatic mRNA expression levels of the ATP binding cassette (ABC) proteins ABCG5 (encoded by *Abcg5*) and ABCG8 (encoded by *Abcg8*) that form complexes and promote cholesterol excretion into bile were increased under LD 3:3. The mRNA expression of liver X receptor α (LXRα, encoded by *Nrlh3*) that is involved in the transcriptional regulation of *Srebf1*, *Cyp7a1*, *Abcg5* and *Abcg8* remained unaltered in LD 3:3.

Skeletal muscle is involved in the regulation of energy balance and glucose homeostasis throughout the body [7] and FoxO1 plays an important role in these functions by regulating the transcription of energy metabolism- and atrophy-related genes [8,9]. Levels of *Foxo1* mRNA expression were significantly increased in skeletal muscle under LD 3:3 (Supplemental Table 2). On the other hand, the mRNA expression of the atrophy-related ubiquitin ligases, atrogin-1 (encoded by *Fbxo32*) and MuRF1 (encoded by *Trim63*), and of PDK4, an enzyme that shuts down glucose oxidation by blocking the activity of pyruvate dehydrogenase, were little affected under LD 3:3, although FOXO1 transcriptionally regulates the expression of these genes.

Chronic low-grade inflammation in adipose tissues is associated with metabolic disorders such as insulin resistance [10,11]. Expression levels of macrophage-specific F4/80 (encoded by *Emr1*) were unaffected by LD 3:3 (Supplemental Table 3), suggesting that macrophage accumulation is not induced by ultradian LD cycles. The

mRNA expression of proinflammatory factors such as interleukin-6 (IL-6, encoded by *Il6*), tumor necrosis factor-a (TNF-α, encoded by *Tnf*), monocyte chemoattractant protein-1 (MCP-1, encoded by *Ccl2*), and plasminogen activator inhibitor-1 (PAI-1, encoded by *Serpine1*), and of adipose tissue-specific metabolic hormones, such as leptin and adiponectin, did not significantly differ in epididymal white adipose tissues between LD 12:12 and LD 3:3.

4. Discussion

The present findings showed that disrupting circadian behavior using ultradian LD 3:3 cycle to which mice cannot entrain induced glucose intolerance and hyperglycemia with increases in diabetic markers, glycated albumin and HbA1c, were significantly induced without affecting body weight gain and food consumptions. The hepatic expression of gluconeogenic genes such as *Foxo1*, *Hnf4a*, *G6pc* and *Pck1* was induced, while adipose inflammation, which is generally associated with the metabolic disorders, was absent in behaviorally arrhythmic mice under LD 3:3. These observations suggest that the ultradian LD cycles disturb glucose homeostasis via the up-regulation of hepatic gluconeogenic gene expressions.

The underlying mechanisms of the impaired glucose metabolism induced by ultradian LD cycles are unknown, but might involve direct physiological effects caused by frequent exposure to light, or indirect consequences of perturbed sleep and feeding times. Ultradian LD cycles might directly increase hepatic glucose production via the sympathetic input of light from the SCN to the liver. The hypothalamic SCN plays an important role in the regulation of glucose metabolism through autonomic nerve control [12–14]. The SCN directly controls basal glucose levels independently of its influence on feeding activity, because a fasting or scheduled feeding regimen has little effect on the circadian glucose fluctuation that is completely abolished in animals with SCN lesions [12–14]. Environmental light stimuli elicit SCN-dependent sympathetic neural activities in the liver, pancreas, spleen and kidney [12–14]. Electrical stimulation of the SCN increases plasma glucose levels that can be prevented by the peripheral administration of α- and β-adrenergic receptor antagonists, suggesting that increased glucose output is sympathoadrenally mediated [13,14]. Plasma glucose concentrations increase either by stimulating or inactivating excitatory glutamate and inhibitory GABA pathways in the paraventricular nucleus, which is an important target area of SCN output [12,14]. Therefore, unusually frequent illumination might affect SCN functions under ultradian LD cycles and cause hyperglycemia.

The ultradian LD cycle might alternatively affect glucose metabolism indirectly by disrupting rhythmic feeding behavior. Drinking behavior (usually accompanied by food intake) became completely arrhythmic under LD 3:3, suggesting that circadian changes in energy expenditure, hormone secretion and neural activity are impaired under LD 3:3. The circadian timing of food intake affects body weight and metabolism in experimental animals [15–18] and humans [19–21]. Phase-delayed eating habits such as skipping breakfast, or patients with night-eating syndrome, suggest an association with increased body mass index (BMI) [19–21]. Metabolic disorders have been associated with frequent feeding and reduced daily feeding rhythms in humans [19,21] and other animals [18,22]. Time-restricted feeding apparently prevents metabolic disorders being induced by a high-fat diet in mice [16,18]. The circadian timing of food intake affects plasma endocrine factors such as melatonin, leptin and insulin [23,24]. Flattening metabolic activities inputs constant signals to the brain and results in metabolic disorders [25]. In the present study, the enhanced hyperglycemia with glucose intolerance under LD3:3 seemed partly due to disrupted feeding rhythms.

Glucose levels are maintained within a relatively tight physiological range in the blood through regulating glucose production mainly in the liver. Circulating glycated albumin and HbA1c levels were significantly elevated under ultradian LD 3:3 cycles in the present study, suggesting that hyperglycemia was continuously induced throughout the experiment. Hepatic glucose production is a combination of gluconeogenesis and glycogenolysis. Although hepatic glucose production is considered high in patients with diabetes, the physiology of this abnormality remains dubious [26]. The molecular mechanisms responsible for appropriate metabolic adaptations by the liver to both hypoglycemia and hyperglycemia are largely controlled at the level of gene transcription. The transcription factors FoxO1 and FoxO3 play a critical role in the regulation of hepatic glucose production in response to hormonal signals and several genes encoding hepatic metabolic enzymes, such as *Pck1*, *G6pc*, *Slc2a2*, and *Gck*, contain FoxO binding sites [27]. We found that the hepatic mRNA expression of gluconeogenic genes such as *Pck1* and *G6pc* as well as *Hnf4a* and *Foxo* genes such as *Foxo1*, *Foxo3* and *Foxo4* were significantly increased under ultradian LD 3:3 cycles. FoxO1, FoxO3 and FoxO4 proteins synergistically promote glucose production in the liver of double and triple liver-specific knockout mice [28]. Increased *Foxo* gene expression might contribute to gluconeogenic gene expression under ultradian LD 3:3 cycles, although the regulation of FoxO1 activity is largely insulin-dependent, wherein phosphorylation of FoxO1 via the phosphatidyl-inositol 3-kinase (PI3K)-Akt pathway causes its nuclear exclusion and inactivation [27]. FoxO1 transcriptionally activates the expression of *Hnf4a* that encodes a liver-enriched transcription factor [27], and this might also contribute to enhanced gluconeogenesis via the transcriptional activation of *Pck1* [29]. GLUT2 is a facilitative glucose transporter and hypoinsulinemia or glycemia upregulate its mRNA expression. Increased expression of *Slc2a2* (encoding GLUT2) induced by FoxOs might contribute to hyperglycemia under ultradian LD 3:3 cycles by increasing glucose efflux from hepatocytes. On the other hand, the mRNA expression of genes encoding IRS-1 and IRS-2 was essentially normal, and the plasma insulin-to-glucose ratio was unaffected under LD 3:3, suggesting that ultradian LD cycles do not alter insulin sensitivity. Skeletal muscle regulates energy metabolism by contributing to >30% of the resting metabolic rate and 80% of whole body glucose uptake [7]. FoxO1 overexpression in skeletal muscle results in impaired glycemic control with severe muscular atrophy due to promoting the expression of muscle-specific ubiquitin ligases such as atrogin-1 and MuRF1 [9]. Here, LD 3:3 cycles essentially did not affect the mRNA expression of these ubiquitin ligases, although the mRNA expression of *Foxo1* was significantly increased in skeletal muscle as well as in the liver, suggesting that skeletal muscle atrophy is not involved in glucose intolerance induced by ultradian LD cycles. Enhanced gluconeogenesis resulting from the increased expression of gluconeogenic genes such as the *Foxo* genes, *Hnf4a*, *Pck1*, and *G6pc* in the liver seems to contribute to glucose intolerance under LD 3:3 cycles.

Obesity-associated chronic adipose inflammation is a key contributor to metabolic disorders such as diabetes and the metabolic syndrome [30]. Chronic immobilization stress induces adipose inflammation with insulin resistance in mice as well as obesity-associated metabolic disorders [31]. Macrophage accumulation in adipose tissue is a critical mechanism that links the immune and metabolic systems [30]. However, we could not find a significant effect of ultradian LD cycles on macrophage accumulation or the expression of proinflammatory cytokines in epididymal white adipose tissues. The endocrine functions of adipose tissue also seemed to remain intact since the mRNA expression levels of leptin and adiponectin were not affected. Thus, glucose intolerance induced by ultradian LD 3:3 cycles might be independent of adipose inflammation, unlike metabolic disorders in general.

In addition to impaired glucose homeostasis, hypercholesterolemia with hepatic cholesterol accumulation was significantly induced under LD 3:3. The ligand-activated nuclear receptors, LXRs, act as cholesterol sensors via activation by oxysterols and regulate the expression of genes involved in cholesterol homeostasis in the liver [32,33]. Here, mRNA expression levels of several LXR target genes such as *Srebf1*, *Abcg5*, *Cyp7a1* were increased under LD 3:3, suggesting the activation of LXR-dependent transcription in the liver although the mRNA expression of *Nr1h3* remained unaffected. The increased expression of hepatic *Abcg5/8* and *Cyp7a1* suggest increased cholesterol excretion as bile acids by the liver under ultradian LD cycles. However, the expression of cholesterol synthetic genes such as *Hmgcs1* and *Hmgcr* was not affected under LD 3:3. Therefore, the underlying mechanisms of hypercholesterolemia with hepatic cholesterol accumulation remain unclear. Disrupted feeding rhythms under LD 3:3 might be involved in the impaired cholesterol metabolism, because an attenuated circadian feeding rhythm (feeding with a high-cholesterol diet every six hours) results in hypercholesterolemia without affecting the expression of key genes associated with cholesterol synthesis [34]. Unusually frequent illumination under LD 3:3 might directly affect cholesterol metabolism via the central nervous system [35].

Animal experiments and epidemiological findings have demonstrated that flattening metabolic activity inputs constant signals to the brain and results in metabolic disorders [25]. Our findings suggest that alignment between behavioral and environmental circadian LD cycles is important for metabolic control, especially for glucose and cholesterol metabolism. Increasing evidence indicates that the molecular machinery of the peripheral clock regulates the rhythmic expression of genes encoding enzymes and transporters involved in glucose homeostasis [20,36]. Therefore, unusual frequent light/dark stimulation under LD 3:3 might affect glucose metabolism by disturbing peripheral clocks. Further studies are needed to clarify relationships between energy metabolism and environmental LD cycles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.076.

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