Metabolism Clinical and Experimental 85 (2018) 250–258

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

Nighttime light exposure enhances Rev-erbα-targeting microRNAs and contributes to hepatic steatosis

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ARTICLE INFO

Article history:
Received 28 February 2018
Accepted 3 May 2018
Available online xxxx

Keywords:
Clock genes
De novo lipogenesis
Exposure to artificial light at night
Hepatic steatosis
microRNAs

ABSTRACT

Objective: The exposure to artificial light at night (ALAN) disrupts the biological rhythms and has been associated with the development of metabolic syndrome. MicroRNAs (miRNAs) display a critical role in fine-tuning the circadian system and energy metabolism. In this study, we aimed to assess whether altered miRNAs expression in the liver underlies metabolic disorders caused by disrupted biological rhythms.
Results: We found that C3H/HePas mice exposed to ALAN developed obesity, and hepatic steatosis, which was paralleled by decreased expression of *Rev-erbα* and up-regulation of its lipogenic targets ACL and FAS in liver. Furthermore, the expression of *Rev-erbα*-targeting miRNAs, miR-140-5p, 185-5p, 326-5p and 328-5p were increased in this group. Consistently, overexpression of these miRNAs in primary hepatocytes reduced Rev-erbα expression at the mRNA and protein levels. Importantly, overexpression of *Rev-erbα*-targeting miRNAs increased mRNA levels of *Acly* and *Fasn*.
Conclusion: Thus, altered miRNAs profile is an important mechanism underlying the disruption of the peripheral clock caused by exposure to ALAN, which could lead to hepatic steatosis.

1. Introduction

In mammals, physiological and behavioral processes display a robust circadian rhythm. These daily oscillations are orchestrated by the intrinsic biological clock in the suprachiasmatic nucleus (SCN) located in the hypothalamus [1,2]. This central clock is the master pacemaker that generates the rhythmic signals to synchronize the clock machinery in the peripheral tissues, via autonomic nervous system, hormones secretion and temporal patterns of food intake [3,4]. The SCN is mainly entrained by light and, through this photic information, the central clock maintains the oscillatory physiological and behavioral processes coupled to light/dark cycles [5,6].

At the molecular level, a complex circuitry of transcriptional/translational regulatory loops maintains the central and peripheral clocks. In the core loop, the circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like1 (BMAL1), heterodimerize and activates the expression of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. PER and CRY protein complexes, accumulate in the nucleus and inhibit CLOCK/BMAL1-mediated transcription [7]. Additionally, the BMAL1: CLOCK activates the transcription of the nuclear receptors *Rev-erbα* and *Rora*, which in turn represses and induces the *Bmal1* expression, respectively [8,9].

In peripheral tissues, such as liver, the circadian system is closely related to the oscillatory pattern of energy metabolism, since the clock genes regulate the rhythmic expression of genes involved in gluconeogenesis, fatty acid oxidation, and *de novo* lipogenesis (DNL) [10–13]. Accordingly, some studies have demonstrated that *Rev-erbα* displays a critical role in the interplay between the circadian system and metabolism, due to its direct regulatory action on the *Phosphoenolpyruvate carboxykinase 1* and *Glucose 6-phosphatase*, key gluconeogenic genes [14], and lipogenic genes, including *ATP-citrate lyase* (*Acly*) and *Fatty acid synthase* (*Fasn*) [15].

The circadian disruption caused by exposure to artificial light at night (ALAN), in shift-workers and short sleepers, has emerged as a

Abbreviations: 6-SML, Urinary 6-sulfatoxymelatonin; ACL, ATP-citrate lyase; ALAN, Artificial Light at Night; BMAL1, Brain and Muscle ARNT-like1; CLOCK, Circadian Locomotor Output Cycles Kaput; Cry, Cryptochrome; DNL, *de novo* lipogenesis; FAS, Fatty acid synthase; kITT, glucose decay constant; L/D, Light/Dark; L/L, Light/Light; miRNA, microRNA; Per, Period; Rev-erbα, Nuclear Receptor Subfamily 1 Group D Member; SCN, Suprachiasmatic nucleus; UTR, Untranslated Region; ZT, Zeitgeber time.

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https://doi.org/10.1016/j.metabol.2018.05.002
0026-0495/

new risk factor for the development of obesity, cardiovascular diseases and metabolic syndrome [16–18]. Besides, the widespread use of electric light in the modern societies has been also linked to impaired metabolic health in standard living humans [19]. The deleterious effects induced by exposure to ALAN have also been studied in animal models of shift-work [20]. Mice exposed to ALAN exhibited increased body weight gain, decreased insulin sensitivity, and altered timing of food intake [21,22]. Moreover, exposure to dim light at night also disrupts the biological rhythms including the expression of clock genes in peripheral tissues [23]. However, it remains unclear by which mechanisms the exposure to ALAN leads to disruption of peripheral clock, and how this misalignment could be involved in the metabolic disorders caused by nighttime light exposure.

MicroRNAs (miRNAs) are small non-coding RNAs comprising approximately 22 nucleotides in length. Mature miRNAs associated with components of the RNA-induced silencing complex (RISC) bind in the seed sequence located in the 3`UTR region of target mRNAs and mediate mRNA degradation or translational repression [24]. It has been shown that various miRNAs display a key role in the regulation of glucose and lipid metabolism, as well as insulin sensitivity [25,26]. Consequently, altered expression of miRNAs is related to development of obesity and type 2 diabetes [27,28].

Increasing evidence indicates that miRNAs may exhibit daily oscillatory pattern of expression, contributing to the circadian expression of molecular clocks and metabolic genes in peripheral tissues [29,30]. Importantly, rhythmic expression of miRNAs can be induced by photic entrainment cues within the SCN [31] and in mammary tissue [32]. In line with this notion, circadian disruption induced by changing the photoperiod exposure alters the expression of multiples miRNAs in mammary tissue, which are involved in the development of breast cancer [32]. Despite the close relation between miRNAs, circadian system, and energy metabolism, it is still unknown whether disruption in circadian rhythms, leading to development of metabolic disorders, could be triggered by altered expression of miRNAs.

2. Methods

2.1. Animal Model

Weaned male C3H/HePas mice, were obtained from a breeding colony at UNICAMP (University of Campinas, Campinas, Brazil). The mice were acclimated in a specific cabinet during four weeks, in 12:12 h light/dark cycles and were randomly assigned to either a Light/Dark (L/D) group, whose mice were maintained on standard 12:12 light/dark cycles (lights on at 6 h/ lights off at 18 h), and the Light/Light (L/L) group, whose mice were exposed to ALAN (12:12 light/light condition). Both groups were housed at a temperature of 22 °C, with free access to regular chow diet and water. After eight weeks, mice at 4 months old were euthanized in a chamber with CO_2, at 8 h and 20 h (Zeitgeber Time - ZT 2 and 14 for the L/D group). Blood was collected and centrifuged and the plasma samples were stored at −80 °C. Liver samples were also collected, snapped frozen, and stored at −80 °C. All experiments were carried out in accordance with the protocols approved by the Institutional Ethical Committee at UNICAMP (ethical no. 3246-1).

2.2. Insulin Tolerance Test (ipITT) and Food Intake

After fasting for two hours, ipITTs were performed at 8 h and 20 h. Baseline glycaemia was measured by a glucometer (Accu-Chek Advantage, Roche, Mannheim, Germany), and the mice received, intraperitoneally, 1.0 U/Kg body weight of human recombinant insulin (Humulin, Indianapolis, USA). Blood glucose was monitored at three, six, nine, 12, and 15 min after insulin administration. Next, the constant of glucose decay (kITT) was calculated as previously described [33]. For food intake, the mice were individually placed in chambers and, after 24 h of adaptation, the food consumption was measured at 6 h and 18 h.

2.3. Biochemical Plasma and Blood Markers

Blood glucose concentrations were measured from the tail tip of the mice using a glucose analyzer (Accu-Chek Performa, Roche Diagnostic, Switzerland). Plasma cholesterol and triglycerides (TG) levels were measured using colorimetric standard commercial kits, according to the manufacturer's instructions (Roche/Hitachi®; Indianopolis, USA, and Wako®; Richmond, USA, respectively). Plasma was also used for insulin measurement by radioimmunoassay [34]. All biochemical parameters were measured in fed state at 8 h and 20 h.

2.4. Locomotor Activity

The mice were individually placed in chambers containing an optical beam sensor system and allowed for adaptation during 24 h. Subsequently, the locomotor activity was measured during 48 h using the PheCOM system (Pan Lab/Harvard Instruments, Barcelona, Spain).

2.5. Urinary 6-Sulfatoxymelatonin (6-SML)

Mice from both groups were placed in a special chamber and, after 24 h of adaptation, the urine was collected between 18 h and 6 h. Then, the 6-SML was quantified using an ELISA kit according to the manufacturer's instructions (IBL, Mannedorf, Switzerland).

2.6. Histological Analysis of Liver and Adipose Tissue

After euthanasia, liver and perigonadal adipose tissue fragments were collected and fixed in phosphate-buffered saline (PBS) containing 4% formaldehyde for 24 h at room temperature. Samples were washed 3× with PBS and maintained in 70% etOH at 4 °C. Tissues were embedded in paraffin, sectioned and stained with hematoxylin-eosin. Adipocyte diameter was determined using the ImageJ software (National Institute of Health, MD, USA) by averaging counts from approximately 70 cells (±20 cells)/section, 3 sections per sample.

2.7. Hepatic Lipid Content

Liver samples were also collected to extract hepatic lipids through the Folch's method [35]. The extract was evaporated and diluted in isopropanol. Then, the measurement of triglycerides and cholesterol was performed according to the manufacturer's instruction (Roche, Mannheim, Germany).

2.8. Analysis of mRNA and miRNAs Expression

For the real-time PCR analysis, total RNA from liver was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, USA). The SYBR-green master mix (Applied Biosystems) was used in the qPCR reactions. The relative expression of mRNAs was determined after normalization with *36B4*, using the $2^{-\Delta\Delta Ct}$ method. For the miRNAs analysis, the total RNA was reverse transcribed using the miRScript II kit (Qiagen, Hilden, Germany), and the qPCR reactions were performed using the miScript Sybr Green PCR kit (Qiagen, Hilden, Germany). The relative expression was normalized to U6 using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are in the Supplementary Table 1.

2.9. Primary Hepatocyte Culture

Mice were anesthetized with pentobarbital (75 mg/kg, i.p.). Hank's buffer containing collagenase IV (0.5 mg/mL) was perfused via the portal vein and, after the liver was removed, the cells were dispersed and filtrated. Hepatocyte suspensions were washed with a new Hank's buffer and, after centrifugation, the Percoll solution was added to reduce the amount of non-parenchymal cells. Cell viability was determined