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RESEARCH ARTICLES

All-*trans* retinoic acid modifies the expression of clock and disease marker genes

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Abstract

Restricted feeding (RF), a regimen that restricts the duration of food availability with no calorie restriction, entrains the circadian clock in peripheral tissues. Restricted feeding leads to high-amplitude circadian rhythms, which have been shown to promote wellness and reduce disease and inflammatory markers. Retinoids, such as all-*trans* retinoic acid (ATRA), act as anti-inflammatory agents. Thus far, the effect of ATRA combined with RF on the ability to delay the occurrence of age-associated changes, such as cancer and inflammation, is not known. We measured circadian expression of clock genes, disease marker genes and inflammatory markers in the serum, liver and jejunum in mice fed *ad libitum* (AL) or RF supplemented with 15 or 250 μg/kg body/day ATRA for 16 weeks. Our results show that ATRA supplementation led to phase shifts and reduced amplitudes in clock genes. Under AL, ATRA reduced the average daily messenger RNA (mRNA) levels of some disease markers, such as liver *Afp* and jejunum *Afp*, *Alt* and *Gadd45*β and aspartate transaminase (AST) protein in the serum, but increased the expression level of liver *Crp* mRNA. Under RF, ATRA reduced the average daily levels of jejunum *Alt* and *Gadd45*β and AST protein in the serum, but increased liver *Afp*, *Alt*, *Gadd45*β and *Arginase* mRNA. Altogether, our findings suggest that ATRA strongly affects circadian oscillation and disease marker levels. Moreover, its impact is different depending on the feeding regimen (AL or RF).
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1. Introduction

Circadian rhythms in mammals are regulated by the master circadian clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus [1]. A critical feature of circadian timing is the ability of the clockwork to be reset by environmental light to the 24-h day, with the retinohypothalamic tract being the principal pathway through which entrainment information reaches the SCN [2]. Synchronization among SCN neurons leads to coordinated circadian outputs from the nuclei, ultimately regulating sleep–wake cycles and rhythms in physiology and behavior [3–5]. Similar clocks are found in peripheral tissues, such as the liver and digestive system [6,7]. The clockwork consists of the transcription factor CLOCK [8] that dimerizes with BMAL1 to activate transcription by binding to enhancer E-box sequences. Thus, CLOCK and BMAL1 constitute the positive limb of the clock [1]. Periods (*Per1*, *Per2*, *Per3)* and cryptochromes (*Cry1*, *Cry2*) are induced by the CLOCK:BMAL1 heterodimer; but once the proteins are produced, they inhibit transcription and serve as the negative feedback loop of the clock [1,9,10].

High-amplitude circadian rhythms have been previously associated with aging retardation and extended lifespan. Longevity was increased in older hamsters given fetal suprachiasmatic implants that restored higher-amplitude rhythms [11,12]. Disruption of circadian rhythms by shift work or sleep deprivation or by mutations in clock genes can lead to manifestations of the metabolic syndrome, as well as certain types of cancer, coronary heart diseases, depression and overall reduced life expectancy [13–22].

Nuclear receptors constitute a large superfamily of proteins that functions as ligand-inducible transcription factors and include retinoic acid receptors (RARα/β/γ, NR1B1–3), RXRs (RXRα/β/γ, NR2B1–3), REV-ERBs and RORs [23,24]. A large number of nuclear receptors, among which are RARα and RARγ, have been found to exhibit circadian oscillation [25]. While RARs bind all-*trans* retinoic acid (ATRA) and 9-*cis* retinoic acid (9-*cis*-RA), RXRs bind only 9-*cis*-RA [23]. Retinoids, a family of vitamin A analogs such as ATRA and 9-*cis*-RA, are used clinically for the treatment of a number of dermatologic, hematopoietic and cancerous diseases [26]. Retinoids have been demonstrated to impart significant anti-inflammatory effects in experimental trials, such as inhibition of various immune factors, including the activity of leukocytes, the release of proinflammatory cytokines and other mediators and the expression of transcription factors and toll-like receptors involved in immunomodulation [27].

In addition, retinoids affect peripheral clocks [23], as retinoic acid has been shown to up-regulate *Per1* and *Per2* expression in an E-box-dependent manner in mouse fibroblast NIH3T3 cells [28]. Similarly, retinoic acid could phase-shift *Per2* expression *in vivo* and in serum-induced smooth muscle cells *in vitro* [29]. However, when retinoic acid

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is administered to cells expressing the retinoic acid receptors RARα or RXRα, such as in vascular cells, the ligand–receptor complex competes with BMAL1 for binding to CLOCK or NPAS2, a CLOCK homolog [30,31]. These interactions negatively regulate CLOCK/NPAS2:BMAL1-mediated transcriptional activation of clock gene and clock-controlled gene expression, leading to reduced transcript levels [28,29].

Limiting the time and duration of food availability with no caloric reduction is termed restricted feeding (RF). Interestingly, diurnal RF in nocturnal animals shifts many physiological activities normally dictated by the SCN in peripheral tissues. Restricted feeding leads to robust circadian rhythms [32] and entrains rhythms of inflammation-related factors without promoting an acute-phase response [33]. It is assumed that RF affects the physiology of the animal not through the SCN but through a food-entrainable oscillator whose location has been elusive [17]. Since RF leads to high-amplitude circadian rhythms [32], which are associated with improved health [11,12], and ATRA leads to reduced inflammation [27], we investigated the effect of the combination of ATRA and RF on the expression of the circadian clock and inflammatory and disease markers.

2. Materials and methods

2.1. Animals, treatments and tissues

Nine-week-old male C57BL/6 mice were housed in a temperature- and humidity-controlled facility (23°C–24°C, 60% humidity). Mice were entrained to a light–dark cycle of 12 h light and 12 h darkness (LD) for 2 weeks with food available *ad libitum* (AL). After two weeks of AL feeding, mice were fed AL or restricted in feeding time (RF), and each group was divided into three subgroups: a control group and two groups supplemented with either 250 or 15 μg/kg/day ATRA for 16 weeks. Mice in each subgroup were housed together. The RF group was given food between ZT3 and ZT6 (ZT0 is the time of lights on). Daily food intake was measured, and body weight was monitored once weekly throughout the experiment. Average body weight and food consumption on the 8th and 16th week are shown in Table S1. After 4 months, mice were anesthetized with isoflurane and sacrificed around the circadian cycle on the first day of total darkness (DD) under dim red light to avoid the masking effects of light. Blood was drawn at two time points: CT6 and CT18 (CT0 is the time under DD conditions the lights used to turn on). These time points were selected as the midrest and midactivity points, respectively. Blood was left to clot at room temperature for 2 h, centrifuged for 10 min at 1500*g* and stored at −80°C until further analysis. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. The joint ethics committee (Institutional Animal Care and Use Committee) of the Hebrew University and Hadassah Medical Center approved this study.

2.2. Animal locomotor activity

Mice were housed individually in 17.5×28×13-cm plastic cages. After 14 days in 12:12 LD, mice were put in total darkness (DD) for 16 days. General activity was monitored using a system composed of infrared detectors (Intrusion detector model MH10; Crow group, Airport City, Israel) that were placed above each cage and connected to a computer [34]. Data were collected continuously using ADAMView software (Advantech, Milpitas, CA, USA) at 6-minute intervals.

2.3. RNA extraction and quantitative real-time polymerase chain reaction

For gene expression analyses, RNA was extracted from liver and jejunum using TRI Reagent (Sigma, Rehovot, Israel). Total RNA was DNase I-treated using RQ1 DNase (Promega, Madison, WI, USA) for 2 h at 37°C, as was previously described [6,35]. Two micrograms of DNase I-treated RNA were reverse transcribed using MMuLV reverse transcriptase (Promega) and random hexamers. One twentieth of the reaction was then subjected to quantitative real-time polymerase chain reaction (PCR) using primers spanning exon–exon boundaries and the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Primers for all genes were tested alongside the normalizing gene glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*). The primers used are shown in Table S2. Primers were designed with Primer Express v.2 (Applied Biosystems) and validated by a standard curve and dissociation curve of the product. The fold change in target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ relative quantification method (Applied Biosystems).

2.4. Western blot analysis

Liver and jejunum samples (~200 mg) were homogenized in 1-ml lysis buffer (pH 7.8, 20 mM Tris, 145 mM NaCl, 5% glycerol, 1% Triton X-100, 50 nM phenylmethylsulfonyl fluoride, 50 μM sodium fluoride, 10 μM sodium orthovanadate, 50 ng/ml aprotinin, 100 ng/ml leupeptin and 0.8 μg/ml trypsin inhibitor [Sigma]), as was described [36]. Samples were run onto a sodium dodecyl sulfate polyacrylamide gel (10% for ARGINASE and alanine aminotransferase [ALT]). After electrophoresis, proteins were semidry transferred onto nitrocellulose membranes. Blots were incubated with goat antimouse ALT polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) or goat antimouse ARGINASE (Everest Biotech, Oxfordshire, UK). Antimouse actin (MP Biomedicals, Solon, OH, USA) was used to detect actin, the loading control. Reacted membranes were washed and reacted with horseradish-peroxidase-conjugated antigoat (Santa Cruz Biotechnologies) and antimouse (Jackson ImmunoResearch, West Groove, PA, USA) IgG. The immune reaction was detected by enhanced chemiluminescence (Santa Cruz Biotechnologies). Finally, bands were quantified by densitometry and expressed as arbitrary units.

2.5. Serum protein analyses

Serum protein levels of C-reactive protein (CRP), plasminogen activator inhibitor 1 (PAI-1), interleukin-6 (IL-6) and interleukin-10 (IL-10) were determined by MILLIPLEX Multiplex-Luminex panel assay (Millipore, Billerica, MA, USA). Assays were performed according to the manufacturers' instructions.

2.6. Enzymatic colorimetric tests

Serum ALT and aspartate transaminase (AST) protein levels were determined by Cobas kits (Roche Diagnostics Limited, Burgess Hill, UK) and analyzed in Roche/Hitachi analyzer (Roche Diagnostics, Indianapolis, IN, USA). Assays were performed according to the manufacturers' instructions.

2.7. Statistical analyses

All results are expressed as means±S.E. Tukey honestly significant difference (HSD) was performed for the evaluation of significant differences in average daily expression levels of disease and inflammation genes. A one-way analysis of variance (ANOVA) (time of day) was performed to analyze circadian pattern of clock genes with several time points. For all analyses, the significance level was set at $P<.05$. Statistical analysis was performed with JMP software (version 5.1; SAS Institute Inc., Cary, NC, USA). Further analysis of circadian rhythmicity was performed using Acro software (version 3.5; Circadian Rhythm Laboratory, University of South Carolina, Walterboro, SC, USA).

3. Results

To examine the impact of ATRA in combination with timed meals on circadian rhythms, mice received food enriched with retinoic acid (15 or 250 μg/kg/day) for 16 weeks either AL or restricted to 3 h (RF). A 16-week period of time allows circadian rhythms and aging-related biomarkers to change and stabilize [37,38]. The expression levels of several clock genes and disease and inflammation markers were analyzed in the serum, liver and jejunum. We used several time points throughout the circadian cycle to measure oscillation as well as average daily levels for better assessment of total protein and messenger RNA (mRNA) levels.

3.1. Effect of ATRA on body weight and food intake

The AL and RF groups supplemented with 15 or 250 μg/kg/day ATRA gained weight throughout the experiment (Fig. 1A). Nevertheless, AL feeding supplemented with 250 μg/kg/day ATRA and all the RF groups showed reduction in the final mean body weight (Fig. 1B). To test the reduction in body weight, we corrected food consumption to body mass on the 8th and 16th week (Table S1). At the end of the experiment, the average food intake to total body mass ratio of all groups ranged between 93% and 102% compared to the AL group (Table S1). As weight was gained throughout the experiment and calorie restriction is defined as 60%–75% food intake of AL [39], these results indicate that the RF-fed mice were not calorically restricted.

3.2. Effect of ATRA on locomotor activity

ATRA supplementation did not affect the pattern of circadian locomotor activity (Fig. 1C) or the 24-h activity of AL-fed mice held under LD conditions (Fig. 1D). Expectedly, the RF groups exhibited food anticipatory activity during the light period and increased activity during feeding time (Fig. 1C). Nevertheless, their

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