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Caffeine alters circadian rhythms and expression of disease and metabolic markers

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ABSTRACT

The circadian clock regulates many aspects of physiology, energy metabolism, and sleep. Restricted feeding (RF), a regimen that restricts the duration of food availability entrains the circadian clock. Caffeine has been shown to affect both metabolism and sleep. However, its effect on clock gene and clock-controlled gene expression has not been studied. Here, we tested the effect of caffeine on circadian rhythms and the expression of disease and metabolic markers in the serum, liver, and jejunum of mice supplemented with caffeine under *ad libitum* (AL) feeding or RF for 16 weeks. Caffeine significantly affected circadian oscillation and the daily levels of disease and metabolic markers. Under AL, caffeine reduced the average daily mRNA levels of certain disease and inflammatory markers, such as liver alpha fetoprotein (*Afp*), C-reactive protein (*Crp*), jejunum alanine aminotransferase (*Alt*), growth arrest and DNA damage 45β (*Gadd45β*), Interleukin 1α (*Il-1α*), *Il-1β* mRNA and serum plasminogen activator inhibitor 1 (PAI-1). Under RF, caffeine reduced the average daily levels of *Alt*, *Gadd45β*, *Il-1α* and *Il-1β* mRNA in the jejunum, but not in the liver. In addition, caffeine supplementation led to decreased expression of catabolic factors under RF. In conclusion, caffeine affects circadian gene expression and metabolism possibly leading to beneficial effects mainly under AL feeding.

1. Introduction

The suprachiasmatic nuclei (SCN) of the anterior hypothalamus is the central oscillator that controls the approximately 24-h cycle (circadian rhythms) of behavior and physiology in mammal (Reppert and Weaver, 2002). Circadian oscillators are also found in various peripheral tissues, such as the liver and digestive system (Lee et al., 2001; Froy and Chapnik, 2007; Pan and Hussain, 2009). The clockwork consists of a primary loop that is composed of the helix-loop-helix transcription factors CLOCK and BMAL1, which mediate transcription of the *Period* (*Per1*, *Per2*, *Per3*) and *Cryptochrome* (*Cry1*, *Cry2*) genes through E-box enhancer elements. In turn, PER and CRY proteins repress CLOCK/BMAL1-mediated gene trans-activation, thereby shutting down their own transcription (Reppert and Weaver, 2002). Light is the most potent synchronizer for the SCN, yet food consumption, timed meals, and some nutrients also entrain the circadian clock (Schibler et al., 2003; Froy, 2007).

Limiting the time and duration of food availability has been shown to affect the circadian clock and life span (Duffy et al., 1990a,b; Schibler et al., 2003; Froy, 2010). Interestingly, diurnal restricted feeding (RF) in nocturnal animals shifts many physiological activities in peripheral tissues normally dictated by the SCN. Recently, we found that long term RF led to robust circadian rhythms, altered metabolism, and to the lowering of the inflammatory state and disease proneness (Sherman et al., in press). Robust circadian rhythms have been previously associated with aging retardation and extended life span, as longevity was increased in older hamsters given fetal suprachiasmatic implants that restored higher amplitude rhythms (Hurd and Ralph, 1998; Hofman and Swaab, 2006). Conversely, disruption of circadian rhythms by shift work, 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 disease, depression, and overall reduced life expectancy (Davis and Mirick, 2006; Filipski et al., 2006; Froy, 2007).

In addition to RF, caffeine, the world's most popular psychoactive compound, has been shown to shift circadian rhythms. Caffeine administered systemically at the mid-sleep period induced arousal without shifts, and dose-dependently attenuated shifts to a 3-h sleep deprivation procedure in Syrian hamsters (Antle et al., 2001). It has been shown that when ingested at night caffeine suppressed melatonin levels in humans, causing reduction in sleep duration (Wright et al., 1997). Caffeine can also alter physiological rhythms as it delays the phase of circadian activity through ryanodine

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receptors in mouse brain slices containing the SCN (Ding et al., 1998). However, to the best of our knowledge, its effect on clock gene expression has not been tested.

The circadian clock regulates the expression and/or activity of enzymes and hormones involved in metabolism. In turn, key metabolic enzymes and transcription activators interact with and affect the core clock mechanism (La Fleur et al., 1999; Pan and Hussain, 2007, 2009; Green et al., 2008; Froy, 2010). In addition, caffeine has been shown to affect metabolism (Magkos and Kavouras, 2005). As resetting the biological clock by timed meals or nutrients is of general interest, we set out to test the effect of caffeine on circadian gene expression. In addition, as RF leads to high amplitude circadian rhythms and decreased expression of disease markers, we investigated the combination of RF and caffeine, on the expression of the circadian clock, disease, inflammatory, and metabolic markers.

2. Materials and methods

2.1. Animals, treatments, and tissues

12 week-old male C57BL/6 mice ($n = 144$) were entrained to a light–dark cycle (12 h light:12 h darkness, LD) for two weeks with food *ad libitum* (AL). Subsequently, mice were fed AL or restricted in feeding time (restricted feeding, RF) and each group ($n = 72$) was divided into 3 subgroups ($n = 24$ each): a control group and two groups supplemented with 3.5 mg/kg/day or 7 mg/kg/day caffeine for 16 weeks. The RF group was given food between ZT3 and ZT6 (ZT0 – lights on). Average body weight and food consumption on the 8th and 16th week are shown in Table S1. After 4 months, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100/7.5 mg/kg) and liver and jejunum were removed every 3 h ($n = 3$ per time point) around the circadian cycle in total darkness (DD) under dim red light to avoid masking effects by light. Blood was drawn at 2 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 mid-rest and mid-activity points, respectively. Mice were humanely killed by cervical dislocation under anesthesia at the end of the experiment. The joint ethics committee (IACUC) of the Hebrew University and Hadassah Medical Center approved this study.

2.2. Animal locomotor activity

Mice were housed individually in 17.5 cm × 28 cm × 13 cm plastic cages in LD for 14 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 (Gutman et al., 2007). Data were collected continuously using ADAMView software (Advantech, Milpitas, CA), at 6-min intervals.

2.3. RNA extraction and quantitative real-time PCR

RNA was extracted from tissue or cells using TRI Reagent (Sigma, Rehovot, Israel). RNA were reverse-transcribed and subjected to quantitative real-time PCR using primers spanning exon–exon boundaries and the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Primers for all genes (Table S2) were tested alongside the normalizing gene Glyceraldehyde 3 phosphate dehydrogenase (*Gapdh*). Primers were designed with Primer Express v.2 (Applied Biosystems, Foster City, CA) 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, Foster City, CA).

2.4. Western blot analysis

Tissue samples (~200 mg) were homogenized in lysis buffer and run onto a 10% SDS-polyacrylamide gel, as was described (Sherman et al., in press). Blots were incubated with goat anti-mouse ALT polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-mouse AMPK/pAMPK polyclonal antibody (Cell Signaling Technology, Beverly, MA), goat anti-mouse ARGINASE (Everest Biotech, Oxfordshire, UK), and anti-mouse actin (MP Biomedicals, Solon, OH). Membranes were reacted with horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnologies, Santa Cruz, CA) and anti-mouse (Jackson ImmunoResearch, West Groove, PA) IgG. The immune reaction was detected by enhanced chemiluminescence (Santa Cruz Biotechnologies, Santa Cruz, CA). Finally, bands were quantified by scanning and densitometry and expressed as arbitrary units.

2.5. Serum protein analyses

Serum protein levels of CRP, PAI-1, IL-6, and IL-10 were determined by MILLIPLEX™ Multiplex-Luminex Panel assay (Millipore, Billerica MA). Assays were performed according to the manufacturers' instructions.

2.6. Enzymatic colorimetric tests

Serum triglycerides, cholesterol, and ALT and AST protein levels were determined by Cobas® kits (Burgess Hill, UK) and analyzed in Roche/Hitachi analyzer (Roche Diagnostics, Indianapolis, IN) according to the manufacturers' instructions.

2.7. Cell culture and treatments

Human kidney 293 cells (HEK-293) were maintained at 37 °C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) (Sigma, Rehovot, Israel) and 10% heat-inactivated fetal bovine serum. Cells were grown to 80% confluence and synchronized with 40 μM dexamethasone for 2 h. Subsequently, medium was replaced with DMEM containing 2.5 mM caffeine for 6 h and then cells were harvested every 4 h and total RNA was extracted.

2.8. Promoter isolation

5 mg of mouse liver was lysed in proteinase K buffer and incubated at 50 °C for 5 h. The mixture was centrifuged and template genomic DNA was reacted with primers designed according to the 1035 bp promoter of mouse *Pai-1* (Genebank NT_039314) (mPai-1 F 5′-gcggtaccagccaatcctggaactttcccag-3′, mPai-1-R 5′-gcaagcttccctcatttgtcttcccctctttg-3′). The reaction was performed for 40 cycles (94 °C 1.5 min, 60 °C 1 min, 72 °C 2 min). Gel-purified *Pai-1* promoter was ligated into pGL3-Basic (Promega, Madison, WI) upstream of the luciferase gene.

2.9. Transfections and luciferase reporter assay

HEK-293 cells were transfected using jetPEI reagent (Polyplus Transfection, New York, NY) according to the manufacturer's instructions. 42 h after transfection, cells were treated with 0.5, 1, 2, or 5 mM caffeine in DMEM for 6 h and harvested in Reporter Lysis Buffer (Promega, Madison, WI). Luciferase activity was assayed with the Dual-Luciferase Reporter Assay System (Promega,