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Chronic consumption of dietary proanthocyanidins modulates peripheral clocks in healthy and obese rats

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Abstract

Circadian rhythm plays an important role in maintaining homeostasis, and its disruption increases the risk of developing metabolic syndrome. Circadian rhythm is maintained by a central clock in the hypothalamus that is entrained by light, but circadian clocks are also present in peripheral tissues. These peripheral clocks are trained by other cues, such as diet. The aim of this study was to determine whether proanthocyanidins, the most abundant polyphenols in the human diet, modulate the expression of clock and clock-controlled genes in the liver, gut and mesenteric white adipose tissue (mWAT) in healthy and obese rats. Grape seed proanthocyanidin extracts (GSPEs) were administered for 21 days at 5, 25 or 50 mg GSPE/kg body weight in healthy rats and 25 mg GSPE/kg body weight in rats with diet-induced obesity. In healthy animals, GSPE administration led to the overexpression of core clock genes in a positive dose-dependent manner. Moreover, the acetylated BMAL1 protein ratio increased with the same pattern in the liver and mWAT. With regards to clock-controlled genes, *Per2* was also overexpressed, whereas *Rev-erbα* and *RORα* were repressed in a negative dose-dependent manner. Diet-induced obesity always resulted in the overexpression of some core clock and clock-related genes, although the particular gene affected was tissue specific. GSPE administration counteracted disturbances in the clock genes in the liver and gut but was less effective in normalizing the clock gene disruption in WAT. In conclusion, proanthocyanidins have the capacity to modulate peripheral molecular clocks in both healthy and obese states.

Keywords: Flavonoids; *Bmal1*; *Clock*; *RORα*; *Per2*; *Rev-erbα*

1. Introduction

Proanthocyanidins are a class of polyphenolic compounds in vegetables, fruits, cacao, nuts and some beverages such as red wine and tea; therefore, their presence in the human diet is considerably high [1]. Importantly, proanthocyanidins are considered to be bioactive compounds for their physiological and cellular processes, and several studies using various *in vitro* and animal models have elucidated a varied range of health effects in relation to metabolism, such as effects on insulin resistance [2], obesity [3], inflammation [4], cardiovascular disease [5], hypertension [6], oxidative stress [7] and lipid abnormalities. In fact, the effect of proanthocyanidins on lipid metabolism via correcting dyslipidemia in obese rats and reducing triglyceridemia and lipogenesis is due to some well-studied mechanisms in liver, such as the repression of lipogenic genes, transcriptional activation of the nuclear receptor FXR or even the modulation of miRNAs [8–11].

However, it is well established that lipid and carbohydrate metabolism and the expression of their key genes exhibit circadian oscillation. The circadian rhythm of every organism is regulated by a central molecular clock localized in the hypothalamic suprachiasmatic nuclei (SCN). However, an equal core clock mechanism is also expressed in extra-SCN regions of the brain and nearly all peripheral tissues [12]. The molecular clock consists of a transcription–translation autoregulatory feedback loop that cycle with a periodicity of approximately 24 h. The positive limb of this loop is driven by the transcriptional activators circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT like protein 1 (BMAL1), which after their own heterodimerization activate the transcription of the period (Per) and cryptochrome (Cry) genes, and once they reach a critical concentration, the PER and CRY proteins translocate into the nucleus and inhibit the activity of the CLOCK/BMAL1 heterodimer, thus leading to a decrease in Per and Cry expression. In addition, the active CLOCK/BMAL1 heterodimer also promotes the transcription of retinoic acid-related orphan receptor alpha (Rorα) and nuclear receptor subfamily 1, group D (Nr1d1, also known as Rev-erbα), its own activator and repressor, respectively, generating another loop of regulation. Finally, the CLOCK/BMAL1 heterodimer enhances the transcription of metabolic genes or clock-controlled genes, e.g., nicotinamide phosphoribosyltransferase (Nampt) [13].

It has been well established that circadian rhythms play an important role in maintaining homeostasis and normal body function [14], and the disruption of circadian regulation affects normal

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physiological and biochemical functions, inducing diseases. Association studies have revealed that shift workers, night workers and sleep-deprived individuals have an increased risk for developing metabolic syndrome symptoms [15,16]. Interestingly, whereas light is the major synchronizer of the central clock, peripheral clocks are entrained by other cues such as rhythmic access to food [17], diet composition [18] and food biocompounds [19].Therefore, the aim of this study was to determine whether proanthocyanidins can entrain the peripheral clock to identify novel cellular mechanisms by which proanthocyanidins can modulate lipid metabolism and cell functionality. The capacity of proanthocyandins to entrain peripheral clocks was evaluated in normal (healthy rats) and disrupted (obese rats) circadian rhythm states.

2. Materials and methods

2.1. Grape seed proanthocyanidin extract composition

Grape seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). The following GSPE composition used in this study was previously analyzed [20]: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g) and hexameric procyanidins (0.38 μmol/g).

2.2. Animals

All procedures involving the use and care of animals were reviewed and approved by The Animal Ethics Committee of the Universitat Rovira i Virgili (Permit number 4249 by Generalitat de Catalunya).

Forty-five male Wistar 6-week-old rats [Crl: WI (Han)] were purchased from Charles River (Barcelona, Spain) for both experiments.

Healthy rats: Rats were singly caged in animal quarters at 22°C with a 12-h light/dark cycle (light from 0800 to 2000 hours) and were fed *ad libitum* with a standard chow diet (STD; Panlab 04, Barcelona, Spain) and tap water. After 1 week of adaptation, the animals were randomly divided into four groups ($n=6$) and supplemented with 0 (control group), 5, 25 or 50 mg GSPE/kg body weight for 3 weeks. GSPE was dissolved in sugary milk (100 g: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, 1175 kJ) at appropriate concentrations such that the same volume of milk (750 μl) was always administered to the animals. Before supplementation, all of the rats were trained to voluntarily lick the milk, and all groups were administered the same volume of sugary milk for 3 weeks. Treatment was administered every day at 9:00 a.m.

After 3 weeks of supplementation, the rats were fasted overnight. At 9:00 a.m., the rats were orally gavaged with lard oil (2.5 ml/kg of body weight) with or without (control groups) an adequate dose of GSPE (5, 25 or 50 mg/kg body weight). After 3 h, the rats were sedated using a combination of ketamine (70 mg/kg body weight; Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg/kg body weight; Bayer, Barcelona, Spain). After anesthetization, the rats were exsanguinated from the abdominal aorta. Blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Liver, mesenteric white adipose tissue (mWAT) and the intestines were excised, immediately frozen in liquid nitrogen and then stored at −80°C until RNA and protein extraction. Before freezing, duodenal mucosa was extracted by scraping with a small glass plate.

Obese rats: Rats were housed in animal quarters at 22°C with a 12-h light/dark cycle (light from 0800 to 2000 h) and fed an STD *ad libitum* (Panlab). After 1 week, the rats were divided into three groups ($n=7$): the STD control group in which rats were fed STD *ad libitum*, and 2 other groups, which were fed an STD plus a cafeteria diet (CD) that comprised 23.4% lipids (0.05% cholesterol), 35.2% carbohydrates and 11.7% protein. The CD consisted of the following foods: cookies with foie-gras and cheese triangles, bacon, biscuits, carrots and sugary milk. After 10 weeks, rats feeding on the CD were trained to lick arabic gum (1 ml) (G9752; Sigma-Aldrich, Madrid, Spain), which was used as the vehicle, and they were randomly divided in two groups. One group was fed the CD plus 25 mg GSPE/kg bw dissolved in arabic gum for 3 more weeks. The second group was fed the CD plus the same volume of arabic gum (CD control group). All treatments were administered at the same time point (7 p.m.).

After 3 weeks of treatment, the rats were fasted overnight and killed at 9 a.m. by anesthetizing them with 50 mg/kg bw sodium pentobarbital (0804118; Fagron Iberica, Terrasa, Spain), and they were sacrificed by bleeding. Blood was collected using heparin (Deltalab) as an anticoagulant. The livers, mesenteric adipose tissue and intestines were excised, immediately frozen in liquid nitrogen and stored at −80°C until RNA could be extracted. The duodenal mucosa was equally extracted as described above.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the liver, mWAT and intestinal mucosa using the TRIzol reagent and RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocols. RNA was quantified by spectrophotometry (Nanodrop 1000 Spectrophotometer; Thermo Scientific) at λ=260 nm and tested for purity (by A260:280 ratio) and integrity (by denaturing gel electrophoresis). Complementary DNA was generated using the High-Capacity complementary DNA Reverse Transcription Kit from Applied Biosystems (4368814).

2.4. mRNA quantification by real-time qRT-PCR

A total of 10 ng of cDNA was subjected to quantitative RT-PCR amplification using SYBR Green PCR Master Mix from Bio-Rad (172-5200). The forward and reverse primers for the rat genes analyzed are listed in Table 1. Reactions were run with a quantitative real-time PCR system (Bio-Rad), and the thermal profile settings were 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 2 min. Finally, statistical data were converted and normalized to the linear form using the $2^{-}CT$ ($\Delta\Delta C_T$) calculation [21]. The relative expression level of the clock genes *rorα*, *rev-erbα*, *bmal1, clock, per2, nampt* and *hmgCoAR* was assessed for the liver, mesenteric adipose tissue and intestinal mucosa, which was normalized to the cyclophilin mRNA level.

2.5. Western blot analyses

Protein was extracted from liver and white adipose tissue using radioimmunoprecipitation assay (RIPA) lysis buffer (15 mM Tris–HCl, 165 mM NaCl, 0.5% Na deoxycholate, 1% Triton X-100 and 0.1% SDS), containing a protease inhibitor cocktail (1:1000; Sigma-Aldrich) and 1 mM phenylmethanesulfonyl fluoride solution. The total protein levels of the lysates were determined using the BCA method from Thermo Scientific (23227). Then, the samples were placed in sample buffer [0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, 0.05% bromophenol blue]. After boiling for 5 min, 50 μg of protein was loaded and separated in a 10% SDS-polyacrylamide gel. The samples were then transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories) using a transblot apparatus (Bio-Rad) and blocked at room temperature for 1 h with 5% (wt/vol) nonfat milk in TTBS buffer [Tris-buffered saline (TBS) plus 0.5% (vol/vol) Tween-20]. The membranes were incubated overnight at 4°C with primary monoclonal antibodies directed against Nampt (Imgenex), Bmal1 (LS-Bio), acetyl-Bmal1 (Millipore) and anti-β-actin (Sigma-Aldrich) at a 1:1000 dilution in blocking solution. After washing with TTBS, the blots were incubated with a peroxidase-conjugated monoclonal antirabbit secondary antibody (Sigma-Aldrich) at a 1:10000 dilution at room temperature for 1.5 h. The blots were then washed thoroughly in TTBS followed by TBS. Immunoreactive proteins were visualized with an enhanced chemiluminescence substrate kit (ECL plus; Amersham Biosciences, GE Healthcare) according to the manufacturer's instructions. Images were obtained with a GBOX Chemi XL 1.4 image system (Syngene, pais). Band quantification was performed with ImageJ software (NIH, Bethesda, MD, USA). The results were expressed as relative intensity (Nampt/b-actin, Bmal1/b-actin and acetil-Bmal1/b-actin) and are relative to the loading control group.

2.6. Statistical analysis

The results are presented as the mean plus the associated standard error (S.E.). The data were analyzed using one-way analysis of variance (ANOVA) to determine significant differences using SPSS statistical software (version 17.0 for Windows; SPSS, Inc.). P values <.05 were considered statistically significant.

Table 1
Rat-specific primer sequences.

Gene	Primer sequence
rorα	Fw: 5'-GAAGGCTGCAAGGGCTTTTTCAGGA-3′
	Rv: 5′-CCAAACTTGACAGCATCTCGA-3′
rev-erbα	Fw: 5′-CTGCTCGGTGCCTAGAATCC-3′
	Rv: 5′-GTCTTCACCAGCTGGAAAGCG-3′
bmal1	Fw: 5′-GTAGATCAGAGGGCGACGGCTA-3′
	Rv: 5′-CTTGTCTGTAAAACTTGCCTGTGAC-3′
clock	Fw: 5′-TGGGGTCTATGCTTCCTGGT-3′
	Rv: 5′-GTAGGTTTCCAGTCCTGTCG-3′
per2	Fw: 5′-CGGACCTGGCTTCAGTTCAT-3′
	Rv: 5′-AGGATCCAAGAACGGCACAG-3′
nampt	Fw: 5′-CTCTTCACAAGAGACTGCCG
	Rv: 5′-TTCATGGTCTTTCCCCCACG-3′
hmgcr	Fw: 5′-GAAACCCTCATGGAGACGCA-3′
	Rv: 5′-ACCTCTGCTGAGTCACAAGC-3′
ppia	Fw: 5′-CTTCGAGCTGTTTGCAGACAA-3′
	Rv: 5′-AAGTCACCACCCTGGCACATG-3′

Rorα, RAR-related orphan receptor A; Rev-erbα (also known as Nr1d1), nuclear receptor subfamily 1, group D, member 1; Bmal1 (also known as ARNTL), aryl hydrocarbon receptor nuclear translocator-like; Clock, circadian locomotor output cycles kaput; Per2, period circadian clock 2; Nampt, nicotinamide phosphoribosyltransferase; Hmgcr, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; Ppia, cyclophilin A. Fw, forward primer sequence; Rv, reverse primer sequence.