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Clock-genes and mitochondrial respiratory activity: Evidence of a reciprocal interplay

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ABSTRACT

In the past few years mounting evidences have highlighted the tight correlation between circadian rhythms and metabolism. Although at the organismal level the central timekeeper is constituted by the hypothalamic suprachiasmatic nuclei practically all the peripheral tissues are equipped with autonomous oscillators made up by common molecular clockworks represented by circuits of gene expression that are organized in interconnected positive and negative feed-back loops. In this study we exploited a well-established *in vitro* synchronization model to investigate specifically the linkage between clock gene expression and the mitochondrial oxidative phosphorylation (OxPhos). Here we show that synchronized cells exhibit an autonomous ultradian mitochondrial respiratory activity which is abrogated by silencing the master clock gene *ARNTL/BMAL1*. Surprisingly, pharmacological inhibition of the mitochondrial OxPhos system resulted in dramatic deregulation of the rhythmic clock-gene expression and a similar result was attained with mtDNA depleted cells (Rho0). Our findings provide a novel level of complexity in the interlocked feedback loop controlling the interplay between cellular bioenergetics and the molecular clockwork. This article is part of a Special Issue entitled 'EBEC 2016: 19th European Bioenergetics Conference, Riva del Garda, Italy, July 2-6, 2016', edited by Prof. Paolo Bernardi

1. Introduction

In mammals many physiological processes display rhythmicity endogenously generated by a circadian clock thought to represent an adaptive response which allows organisms to anticipate environmental oscillating changes (light/darkness, feeding/fasting, daily temperature oscillations, *etc.*) or internal endocrine secretion [1]. The main pacemaker controlling the diurnal rhythms is represented by the suprachiasmic nuclei (SCN), which are located in the hypothalamus and receive photic inputs from the retina through the retinohypothalamic tract. The molecular basis of the pacemaker activity of the SCN relays on a relatively low number of clock genes, which act by transcriptional-translational auto-regulatory feedback loops further modulable by epigenetic modifications, miRNA, post-transcriptional and post-translational modifications [2].

The positive limb of the loop is operated by the transcriptional activators CLOCK and *BMAL1* and their target genes Period (*PER1,2,3*) and Cryptochrome (*CRY1,2*), which encode circadian proteins accumulating and forming a repressor complex that interacts with CLOCK-*BMAL1* heterodimers to inhibit their transcriptional activity. The orphan nuclear

Abbreviations: CLOCK, circadian locomotor output cycles kaput; BMAL, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; SNC, suprachiasmic nuclei; PER, period; CRY, cryptochrome; ROR-alpha, RAR-related orphan receptor alpha; NAM, nicotinamide; NAD, nicotinamide adenine dinucleotide; NAMPT, nicotinamide phosphoribosyltransferase; PGC-1α, proliferator-activated receptor gamma coactivator 1-alpha; SIRT, sirtuin (silent mating type information regulation 2 homolog); OxPhos, oxidative phosphorylation; DMEM, Dulbecco's Modified Eagle's Medium; PBS, phosphate-buffered saline; OCR_{RR}, resting oxygen consumption rate; $OCR_{O/L}$, oxygen consumption rate in the presence of oligomycin; OCR_U, uncoupled oxygen consumption rate; FCCP, carbonilcyanide p-triflouromethoxyphenylhydrazone; $\Delta\Psi_m$, mitochondrial membrane electrical potential; Rho0, cells depleted of mitochondrial DNA.

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hormone receptors REV-ERBα and RORα operate a feedback loop controlling negatively and positively *BMAL1* transcription, respectively [3].

Accumulating evidences support the notion that the cells of virtually all the peripheral tissues are endowed with autonomous self-sustained molecular oscillators constituted by almost the same clock gene machinery operating in the SCN. The function of the SCN, therefore, is to entrain the phases of the peripheral oscillators [4]. Noticeably, recent evidences show that about half of all protein coding genes in mammals display circadian transcription rhythms largely in an organ-specific manner [5].

The amplitude of oscillation of many clock genes is influenced by SIRT1, a type III histone/protein deacetylase [6], whose activity depends on the oscillating levels of nicotinamide (NAM) adenine dinucleotide (NAD), synthesized in the salvage pathway by the committing nicotinamide phosphoribosyltransferase (NAMPT), whose expression is circadian rhythmic [7,8].

Importantly, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a master transcriptional coactivator positively controlling mitochondrial biogenesis and redox homeostasis [9] proved to be a target of SIRT1 thereby providing support for a reciprocal interaction between metabolism and circadian clocks [10,11]. Consistently, recent studies have addressed the role that mitochondrial bioenergetics and dynamics play in cell metabolism in relation to circadian rhythmicity [8,12–14], highlighting the interplay between mitochondrial oxidative phosphorylation (OxPhos) and the functioning of the biological clock and fostering further in-depth analysis.

Aim of the present study was to investigate the mutual impact of circadian clock gene oscillation and the mitochondrial respiratory activity taking advantage of a well-established *in vitro* model of synchronized cultured human cells.

2. Materials and methods

2.1. Cell cultures, "in vitro" synchronization protocol and Rho0 generation

Human hepatoma-derived cell line (HepG2) was obtained from the European Collection of Cell Cultures (ECACC Salisbury, UK); neonatal normal human dermal fibroblasts (NHDF-neo) were from Camb-rex# CC-2509. Cell cultures were maintained at 37 °C in the presence of 5% CO_2 in DMEM (low-glucose Gibco) supplemented with 10 mM Hepes, 10% inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. For live-cell bioluminescence recording, HepG2 cells were maintained in phenol red-free DMEM supplemented with 250 μM D-Luciferin (PJK). The serum shock induced synchronization was performed in HepG2 and NHDF-neo as in [15]. Briefly: approximately 3×10 [6] cells/dish were plated the day before the experiments. At the day of the experiments, culture medium was exchanged with serum-rich DMEM, containing 50% FBS, for 2 h and then the medium was replaced with serum-free DMEM. The cells were harvested and assayed at the different time points indicated in the text/figures. Cell cultures were typically utilized at a passage number below 18–20 and at a confluence of 80–85%. Rho0 cells were generated from NHDF-neo by cellular exposure to ethidium bromide at low concentration (100 ng/ml) for 40 days and kept in culture in DMEM supplemented with 10% fetal bovine serum, 1 mM pyruvate and 50 mg/ml uridine [16]. Quantification of the mtDNA was carried by PCR amplification of the mtDNA gene ND6.

2.2. Quantitative RT-PCR

Total RNA from HepG2 at different time points was extracted using the RNeasy® Mini Kit (Qiagen S.p.a. Milan, Italy) and subsequently digested by DNase I. cDNA was synthesized from 100 ng total RNA with Quantifast RT-PCR kit (Qiagen). For real-time PCR, we used the following SYBR Green QuantiTect Primers purchased from Qiagen: *ARNTL/BMAL1* (QT00068250); *NR1D1* (RevErbα) (QT00000413). Reactions were set up in 96-well plates using a 7700 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Expression levels of the target gene were normalized using the housekeeping control gene TATA binding protein (TBP, QT00000721). mRNA amount of each target gene relative to TBP was calculated through the comparative Ct method (i.e. the $2^{(-\Delta\Delta Ct)}$ method).

2.3. BMAL1-specific siRNA transfection in HepG2 cells

BMAL1-specific siRNA was purchased from Sigma-Aldrich (Mission Pre-designed siRNA2D). HepG2 cells were seeded on 60-mm dishes and at 30–50% confluence were transiently transfected with the *BMAL1*-specific siRNA diluted in Opti-MEM using Lipofectamine® 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol. After 6 h of incubation at 37 °C, the transfection medium was replaced with complete medium containing 10% FBS and The experiments were conducted 12 and 24 h later.

2.4. Respirometric measurements

Cultured cells were gently detached from the dish by tripsinisation, washed in PBS, harvested by centrifugation at $500 \times g$ for 5 min and immediately assessed for O_2 consumption with a high resolution oxymeter (Oxygraph-2 k, Oroboros Instruments). About $8–10 \times 10$ [6] viable cells/ml were assayed in DMEM at 37 °C; after attainment of a stationary endogenous substrate-sustained resting oxygen consumption rate (OCR_{RR}), 2 μg/ml of the ATP-synthase inhibitor oligomycin was added ($OCR_{O/L}$) followed by addition of 0.2 μM of the uncoupler carbonilcyanide p-triflouromethoxyphenylhydrazone (FCCP) (OCR_U). The rates of oxygen consumption were corrected for 2 μM antimycin A plus 2 μM rotenone-insensitive respiration and normalized to the initial cell number or mg protein.

2.5. Immunoblotting

Total protein extract from 10 [7] HepG2 cells was subjected to SDS-PAGE (12% acrylamide) and electroblotted by standard procedures. For *BMAL1* detection a rabbit polyclonal Ab (18,986-Abcam, dil. 1:1000) was used and a HRP-conjugated anti-rabbit IgG as secondary Ab (Thermo Scientific, dil. 1:20,000). Bands were visualized by chemiluminescence densitometric analysis (Versadoc Imaging System) and densitometric analysis of digitalized images carried out by Image J (http://imagej.nih.gov/ij/) and nomalized to anti-β-actin (1:10,000 mouse Ab from Sigma).

2.6. Real Time bioluminescence measuring

Lentiviral elements containing a *BMAL1*-promoter-driven luciferase (BLH) were generated as previously described [17]. HEK293T cells were seeded in 175 cm^2 culture flasks and co-transfected with 12.5 μg packaging plasmid psPAX, 7.5 μg envelope plasmid pMD2G and 17.5 μg *BMAL1*-promoter (BLH)-luciferase expression plasmid using the CalPhos mammalian transfection kit (Clontech) according to the manufacturer's instruction. To harvest the lentiviral particles, the supernatant was centrifuged at $4100 \times g$ for 15 min to remove cell debris and passed through a 45 μm filter (Sarstedt). The lentiviral particles were stored at −80 °C. For lentiviral transduction, 2×10 [5] HepG2 cells were seeded in 6-well plates in 1 ml medium and 1 ml of lentiviral particles was added; 8 μg/ml protamine sulfate (Sigma) was used to enhance transduction efficiency. The next day, the medium was replaced with selection medium (complete growth medium containing 100 μg/ml hygromycin B to obtain stable transduced cells and incubated at 37 °C with 5% CO_2 atmosphere. For bioluminescence measurement, 1.2×10 [6] transfected HepG2 cells were plated in 35 mm dishes (Thermo Scientific) one day prior to measurements. Cells were synchronized by serum shock as described above. Next, cells were washed once with

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