Original article

MOLECULAR METABOLISM

Role of the circadian clock gene *Per2* in adaptation to cold temperature

Sylvie Chappuis [1], Jürgen Alexander Ripperger [1], Anna Schnell [1], Gianpaolo Rando [2], Corinne Jud [1,4], Walter Wahli [2,3], Urs Albrecht [1,*]

ABSTRACT

Adaptive thermogenesis allows mammals to resist to cold. For instance, in brown adipose tissue (BAT) the facultative uncoupling of the proton gradient from ATP synthesis in mitochondria is used to generate systemic heat. However, this system necessitates an increase of the Uncoupling protein 1 (Ucp1) and its activation by free fatty acids. Here we show that mice without functional *Period2* (*Per2*) were cold sensitive because their adaptive thermogenesis system was less efficient. Upon cold-exposure, Heat shock factor 1 (HSF1) induced *Per2* in the BAT. Subsequently, PER2 as a co-activator of PPARα increased expression of *Ucp1*. PER2 also increased *Fatty acid binding protein 3* (*Fabp3*), a protein important to transport free fatty acids from the plasma to mitochondria to activate UCP1. Hence, in BAT PER2 is important for the coordination of the molecular response of mice exposed to cold by synchronizing UCP1 expression and its activation.

Keywords Brown adipose tissue; Humidity; Season

1. INTRODUCTION

In homeothermic animals such as mammals, body temperature is kept within a narrow range in spite of large environmental temperature fluctuations. An average body temperature is maintained by continuous correction of the body temperature by thermoregulatory centers located in the preoptic anterior hypothalamus arranging behavioral and autonomous body temperature responses [1]. This homeostatic regulation is achieved through feedback mechanisms that control heat loss and production, resulting in small variations of temperature around the average. In addition, the circadian clock regulates daily temperature fluctuations by acting on thermoregulatory centers in the brain. Body temperature rises during the activity phase and falls during the rest phase [2]. Thus, daily fluctuations in body temperature are the result of a continuous interplay between circadian and homeostatic mechanisms. Over a day and over a year the interplay between circadian and homeostatic mechanisms experiences changes with the factors light, temperature and humidity as major variables. Day-length appears to affect phase distribution of oscillating neurons in the SCN [3] and thyroid hormone (TH) availability in the hypothalamus by regulating TH deiodinases (Dio) [4]. Temperature increase can lead to the denaturation of proteins. The denatured polypeptides compete for binding to the heat-shock protein (HSP90) with heat-shock factor 1 (HSF1), thereby liberating HSF1 from the HSP90/HSF1 complex [5]. Subsequently, free HSF1 forms a homotrimer, which binds to heat shock elements (HSEs) in promoters of HSF1 target genes. Furthermore, HSF1 has circadian activity [6] and compounds that specifically inhibit its function block the ability of heat pulses to shift the phases of peripheral clocks [7]. To evoke such a shift requires changes in the oscillations of central clock genes in peripheral tissues. Interestingly, HSF1 is not only activated by a heat shock but also by a cold pulse [8]. A recent study identified the clock component *Per2* as a potential target gene of HSF1 [9]. The *Per2* promoter contains HSEs necessary to synchronize *Per2* rhythms to a heat shock pulse in vitro, linking the heat shock response to the circadian clock mechanism.

Adaptive thermogenesis, also referred to as facultative thermogenesis, is defined as heat production in response to environmental temperature (or diet) and serves the purpose of protecting the organism from cold (or regulating energy balance after changes in diet). Cold sensed by the brain leads to activation of sympathetic nerves resulting in the release of noradrenaline and binding of it to beta-adrenergic receptors on the cells of BAT. This leads to acute and chronic effects changing metabolism in BAT [10]. A portion of the acute response to cold is due to shivering, however, with adaptation shivering disappears quite rapidly in rodents [11] and other mechanisms in BAT become dominant. These mechanisms involve stimulation of lipolysis and increase in activity and

[1]Department of Biology, Unit of Biochemistry, University of Fribourg, Switzerland [2]Center for Integrative Genomics, University of Lausanne, Switzerland [3]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore

[4]Present address: Adolphe Merkle Institute, University of Fribourg, Switzerland.

*Correspondence to: University of Fribourg, Department of Biology, Unit of Biochemistry, Chemin du Musée 5, 1700 Fribourg, Switzerland. Tel.: +41 26 300 8636; fax: +41 26 300 9735.
Email: urs.albrecht@unifr.ch (U. Albrecht)

Abbreviations: Adr3, beta-adrenergic receptor 3; BAT, brown adipose tissue; BMAL1, brain and muscle ARNT-like factor; ChIP, chromatin immunoprecipitation; CLOCK, circadian locomotor output cycles kaput; FABP3, fatty acid binding protein 3; FFA, free fatty acids; HSE, heat shock element; HSF1, heat shock factor 1; luc, luciferase; NPAS2, neuronal PAS-domain containing protein 2; Per2, Period2; PGC-1, PPAR-coactivator -1; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR element; RXR, retinoid X receptor; SCN, suprachiasmatic nuclei; TAG, triglycerides; UCP1, uncoupling protein 1; WAT, white adipose tissue; WT, wild-type; ZT, zeitgeber time

Received April 25, 2013 • Revision received May 2, 2013 • Accepted May 3, 2013 • Available online 10 May 2013

http://dx.doi.org/10.1016/j.molmet.2013.05.002

production of UCP1, a mitochondrial inner-membrane protein that uncouples proton entry from ATP synthesis to generate heat [12].
Since we observed that mice mutant in the clock gene *Per2* may be less cold resistant in a natural wild environment [13] we set out to investigate adaptive thermogenesis in *Per2* mutant mice in the laboratory. We present evidence that *Per2* is a target of HSF1 in vivo and is involved in the regulation of adaptive thermogenesis via modulation of *Ucp1* activity and transcription. Surprisingly, the mode of this regulation in animals appears to be strongly dependent on external conditions such as humidity and temperature that probably reflect seasonal changes.

2. MATERIALS AND METHODS

2.1. Animals

Animal care and handling was performed according to the Swiss Federal law for animal protection authorized by the Office Veterinaire Cantonal de Fribourg (FR13_010). Wild-type and $Per2^{Brdm1}$ littermate mice were housed under a 12 h light/12 h dark cycle. $Per2^{Brdm1}$ mice harbor an in frame deletion of exon 10 of the *Per2* gene (part of PAS domain) yielding an unstable protein. The mice were bred on a 129S5/C57BL/6-$Tyr^{c\text{-}Brd}$ mixed background [17]. Water and standard rodent chow was provided ad libitum. Cold exposure experiments were performed with animals held under specific conditions in the animal facility: room temperature average was 21 °C and the humidity average was 30%. For temperature and humidity profiles see Figure S4.

2.2. Cold exposure of mice

Mice were 10–12 weeks old at the time of experimentation. Mice were exposed to 4 °C for 2, 4, 6, and 8 h. Before the cold exposure, mice experienced a specific food regimen. The food was removed at ZT14 two days before cold exposure. Then the food was given at ZT12 and removed again at ZT15 one day before cold exposure. On the day of cold exposure mice were transferred at ZT2 to a cold room at 4 °C without food. Skin temperature was measured with an infrared thermometer (ScanTemp 440) directed on a depilated area on the back of the mouse.

2.3. RNA extraction, reverse transcription, and real-time PCR detection

Total RNA was extracted from a piece of interscapular BAT (20–25 mg) using RNA-Bee (AMS Biotechnology). RNA was treated with RNase-free DNase I (Roche), precipitated in 4 M LiCl and purified further by phenol: chloroform extraction and ethanol precipitation. ssDNA complementary to the RNA starting from hybridized random hexamer primers was synthesized with SuperScript II (Invitrogen). SYBR green fluorescence-based real-time PCR was performed using the primers described in Table S2. All RNA samples were normalized to *βActin* mRNA accumulation.

2.4. Western blot analysis

Tissue (about 20 mg) was homogenized in 400 μl of BAT lysis buffer (25 mM Tris–HCl pH7.5, 1 mM EDTA pH8.0, 1% Triton-X-100, 0.5% Na-deoxycholate, 1 × protease inhibitor (Roche)) twice for 30 s, 5000 rpm using a Precellys®24 machine at 4 °C. The fat layer was removed by sequential centrifugations for 10 min at 3300 rpm and three times for 10 min at 13,000 rpm. Protein concentration was determined using the BCA assay according to the manufacturer (Pierce). Samples were denatured for 15 min at 60 °C to prevent lipid aggregation. For the detection of UCP1, 30 μg of protein was separated on 12.5% SDS-PAGE and transferred to BA83 membrane. For FABP3, 60 μg of protein was separated on 15% SDS-PAGE. Primary antibodies were rabbit Anti-UCP1 1:5'000 (Abcam ab23841), rabbit Anti-Cardiac FABP/FABP3 1:250 (Abcam ab102075) and rabbit Anti-actin 1:250 (Sigma 5060). Quantification was performed using the Quantity One analysis software (BioRad). β-actin was used for normalization and relative protein levels were calculated by defining maximal protein levels as 1.

2.5. Chromatin immunoprecipitation (ChIP)

Interscapular BAT from 5 individual animals were combined per time point, homogenized in 1% formaldehyde/0.3 M sucrose, and kept for 5 min at 21 °C. Nuclei and soluble chromatin fragments were obtained by ultracentrifugation through 1.8 M sucrose cushions and sonication according to [14]. Chromatin was precipitated with antibodies raised against PER2 [15], PPARα (Abcam), HSF1 (Fisher Scientific) and BMAL1 [14]. Co-immunoprecipitated DNA was quantified with TaqMan real-time PCR using the primers and probes described in Table S2.

2.6. Luciferase reporter assays and transfections

A 2'982 bp fragment of the mouse *Ucp1* promoter region or a 1'038 bp fragment of the mouse *Fabp3* promoter region was cloned into the pGL3 basic vector (Promega) containing the firefly luciferase reporter gene. Full-length mouse cDNAs encoding *mPparα* (NM_011144.6), *mRxrα* (NM_011305.3), *mPer2* (NM_011066.3), *mBmal1* (NM_007489), *mClock* (NM_007715), *mNpas2* (BC109166), and bacterial β-galactosidase were cloned into pSCT1. Transfection experiments were performed according to [16].

2.7. Oil-red-O staining

Interscapular BAT tissue was dissected and fixed in 10% formaldehyde in 1 × PBS, incubated in 20% sucrose in 1 × PBS, and frozen in isopentane. Specimens were sectioned to 7 μm thickness and treated with Oil-red-O (Sigma-Aldrich) to stain neutral lipids, and were counter-stained with Mayer's hematoxylin solution (Sigma-Aldrich).

2.8. Measurement of free fatty acids in BAT

Interscapular BAT pieces (5 mg) were homogenized in 1% Triton X-100 in pure chloroform. After centrifugation, the organic phase was evaporated and dried lipids were dissolved in Fatty Acid Assay Buffer (Abcam). Free fatty acids were determined using the Free Fatty Acid Quantification Kit (Abcam).

2.9. Measurement of triglycerides in BAT

Interscapular BAT pieces (5 mg) were homogenized in 5% Triton X-100 in water. Triglycerides were solubilized by heating the samples at 100 °C for 5 min, and centrifuged to remove insoluble material. Triglycerides were determined using the Triglyceride Quantification Kit (Abcam).

2.10. Measurement of plasma metabolites

Blood was taken by retro-orbital bleeding from anesthetized mice, and plasma was separated by centrifugation and frozen. Plasma free fatty acids were determined using the Free Fatty Acid Quantification Kit (Abcam). Plasma triglycerides were determined using the Triglycerides GPO-PAP (Roche).

2.11. Statistical analysis

Statistical analysis of all experiments was performed using Prism4 software (GraphPad Software, Inc.). Significant differences between groups were determined using *t*-test, one- or two-way ANOVA, followed

