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Basal mTORC2 activity and expression of its components display diurnal variation in mouse perivascular adipose tissue

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ABSTRACT

In adipose tissue mTOR complex 2 (mTORC2) contributes to the regulation of glucose/lipid metabolism and inflammatory molecule expression. Both processes display diurnal variations during the course of the day. RICTOR and mSIN1 are unique and essential components of mTORC2, which is activated by growth factors including insulin.

To assess whether mTORC2 components display diurnal variations, we analyzed steady state mRNA expression levels of *Rictor*, *mSin1*, and *mTor* in various adipose tissues during a 24 h period. Diurnally regulated expression of *Rictor* was detected in brown adipose tissues displaying highest mRNA expression levels at the beginning of the 12 h light period (zeitgeber time 2, ZT2). Gene expression patterns of *mSin1* and *mTor* displayed a similar diurnal regulation as *Rictor* in PVAT while smaller changes were detected for these genes in aorta during the course of the day. Basal mTORC2 activity was measured by phosphorylation of protein kinase C (PKC) α at serine 657 was higher at ZT14 as compared with ZT2 in PVAT. In line, gene expression of inflammatory molecules *nitric oxide synthase 2* and *tumor necrosis factor α* was lower at ZT 14 compared to ZT2.

Our findings provide evidence for a diurnal regulation of expression of mTORC2 components and activity. Hence, mTORC2 is possibly an integral part of diurnally regulated signaling pathways in PVAT and possibly in other adipose tissues.

1. Introduction

The circadian system, an endogenous time-keeping mechanism, facilitates behavioral and physiologic adaptation to daily environmental cycles to maintain energy balance, thus ensuring survival [1]. Clock genes encode the circadian clock which is hierarchically organized: the light responsive master clock is located in the suprachiasmatic nucleus whereas peripheral clocks are present in nearly all cells of the body. While the master clock entrains and synchronizes the peripheral clocks with the day-night cycle, peripheral clocks are additionally entrained by feeding and behavioral signals to regulate tissue specific programs in an anticipatory manner [2,3].

Misalignment of the circadian system with the diurnal variation of resting/activity and feeding/fasting cycles results in metabolic dysfunction and increased morbidity [3]. Adipose tissue is a metabolically highly active organ and is actively involved in the integration of fasting/feeding periods into the whole organism [4]. Adipocyte-specific deficiency of mTORC2 by ablation of *Rictor* results in higher lean mass due to increased levels of insulin and insulin-like growth factor, enhanced glucose metabolism and insulin resistance in mice [5,6]. Using this mouse model, we have recently shown that clock gene expression in perivascular adipose tissue (PVAT) is altered in comparison with tissue derived from control mice [7]. Deletion of *Rictor* in adipocytes had no effect on aortic tissue consisting mainly of smooth muscle and endothelial

Abbreviations: mTOR, mammalian target of rapamycin; mTORC2, mTOR complex 2; ZT, zeitgeber time; PKC, protein kinase C; TNFα, tumor necrosis factor α; NOS2, inducible nitric oxide synthase 2; PVAT, perivascular adipose tissue; BAT, brown adipose tissue; EFAT, epididymal fat.

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cells. These alterations in gene expression were associated with changes in diurnal blood pressure regulation.

Mammalian target of rapamycin (mTOR) is a ubiquitously expressed serine/threonine kinase which is present in two multiprotein complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2) [8,9]. mTORC2 contains RICTOR and mSIN1 as unique components. The presence of RICTOR [10], the rapamycin-insensitive companion of mTOR, and mSIN1 [11] are *sine qua non* for mTORC2 to propagate phosphoinositid-3-kinase elicited signals originating from hormones and growth factors to regulate metabolic and other processes [12]. Here we hypothesized that molecules of the mTORC2 pathway in PVAT may be diurnally regulated to participate in anticipatory responses such as blood pressure regulation.

2. Materials and methods

2.1. Mice and tissue collection

In all experiments, male mice (18–23 weeks old) carrying a floxed *Rictor* gene were used as control mice (mice without the *Cre* transgene, $Rictor^{fl/fl}$, termed henceforth control). Adipocyte deletion of *Rictor* ($Rictor^{aP2KO}$) was accomplished by crossing $Rictor^{fl/fl}$ mice with C57BL/6J mice expressing CRE recombinase under the control of the adipocyte protein 2 (aP2, also known as fatty acid binding protein 4) gene promoter (purchased from JAX Laboratories, Bar Harbor, Maine, USA).

Mice were kept in the institutional animal facilities (University of Zurich and University Hospital of Zurich, Switzerland) at 22 °C with a 12:12 h light/dark cycle. Light onset was at 7 am [zeitgeber time (ZT) 0] and light offset was at 7 pm (ZT 12). Animals had access to standard chow (4.5% calories from fat; Kliba Nafag, Kaiseraugust, Switzerland) and water *ad libitum*. All mice were genotyped for *Rictor* (standard PCR, forward primer 5′-3': TTATTAACTGTGTGTGGGTTG and reverse primer 5′-3′ CGTCTTAGTGTTGCTGTCTAG) and *Cre* recombinase (quantitative RT-PCR) using specific mouse primers listed in Table 1 and standard protocols (JAX Laboratories, Bar Harbor, Maine, USA). Before sacrifice, mice were weighed and anesthetized by intraperitoneal (i.p.) injection (xylazine: 20; ketamine: 100; and acepromazine: 3.0; in mg/kg body weight) and exsanguinated via cardiac puncture. All mouse experiments described here were approved by the Kantonales Veterinaeramt of Zurich, Switzerland (License numbers ZH44/2011 and ZH184/2014). Therewith, the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Edition, 2011).

2.2. RNA isolation, reverse transcription and quantitative real-time PCR

The method was performed essentially as described earlier [7]: RNA from PVAT, epididymal adipose tissue (EFAT) and brown adipose tissue (BAT) was extracted using RNeasy® lipid tissue kit and from thoracic aortas using RNeasy® fibrous tissue kit, according to the protocol of the manufacturer (Qiagen, Hombrechtikon, Switzerland) respectively. DNA was removed using an on-column DNase digestion method (RNase-free DNase, Qiagen). Before reverse transcription, RNA quantity and quality was determined using Nanodrop 2000 Spectrophotometer and Agilent 2100 Bioanalyzer. Reverse transcription of 50 ng RNA from PVAT, and thoracic aortas was performed using WT (Whole Transcript)-Ovation® Pico RNA Amplification System (Nugen, Bemmel, Netherlands) and from EFAT and BAT using iScript™ Reverse Transcription Supermix according manufactures recommendations (BioRad, Hercules, CA, USA). For quantitative RT-PCR, final cDNA concentration was adjusted to 10 ng in a 15 μl reaction volume. Each reaction was performed in duplicates using Bio-Rad CFX96 Real-Time System and iQ™ SYBR® Green Supermix (BioRad, Hercules, CA, USA) and specific mouse primers. Gene expression in PVAT, thoracic aortas, EFAT and BAT was normalized to the reference gene acidic ribosomal phosphoprotein (*Arbp*), using the comparative C(T) method [13]. If not otherwise noted, mouse primer pairs for selected genes were designed using Primer Blast (NCBI, USA) [14] and are listed in Table 1.

2.3. SDS-PAGE and Western blot analysis

Tissues were homogenized in tissue lysis buffer using Tissue Lyser (Qiagen, Hombrechtikon, Switzerland): 6 glass beads (3 mm) were used per 2 ml safe–lock reaction. Tissue lysis buffer constituted of 500 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 1% Triton X, 10% Glycerol, proteinase/phosphatase inhibitor (Roche, Switzerland). For further processing, tissue extracts were passed 4 times through a 20-G needle, centrifuged (10 min, 10 000 × g, 4 °C) and the supernatant transferred to a new 1.5 ml reaction tube. Protein concentration was determined using Quick Start™ Bradford Protein Assay according manufactures recommendations and extracts were diluted in sample buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8 and 5% β-mercaptoethanol) to equal concentrations. Samples were heated (95 °C, 5 min). A reducing SDS-PAGE was loaded with 20 μg total protein/well for subsequent Western blot analysis using a Criterion Precast Tris–HCl gel (Bio-Rad). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad), which were blocked by incubating for 1 h at room temperature in 5% bovine serum albumin (BSA, Gibco Europe) in Tris-buffered saline with Tween-20 (TBS-T, 50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 0.1% Tween-20 (Merck, Dietikon, Switzerland)) on an orbital shaker. Primary antibodies as listed in Table 2 were used for overnight incubation at 4 °C, diluted in TBS-T, supplemented with 5% BSA. Membranes were washed in TBS-T and appropriate HRP-conjugated secondary antibodies were incubated for 1 h at room temperature. After 4 washing steps using TBS-T blots were developed with chemiluminescence Western blotting detection reagent (SuperSignal West Pico or Femto, Thermo Fisher) and analyzed using a Chemi Doc XRS system with Quantity One 1-D Analysis software, Version 4.5.0 (Bio-Rad).

Table 1
Primer pairs used for quantitative real-time PCR.

Gene symbol	Forward primer (5′-3′)	Reverse primer (5′-3′)
mSin1	GGGACCCTCCGACCTCTCA	CTCACCTACCTAAGTCACACACC
mTor	GAAGCCGCGCGAACCT	GGACGCTCACGTTGCTAG AT
Nos2	GCACCGAGATTGGAGTTC	AGCACAGCCACATTGATC
Rictor	TGCGATATTGGCCATAGTGA	ACCCGGCTGCTCTTACTTCT
Tnfα	GCGGTGCCTATGTCTCAG	GCCATTTGGGAACTTCTCATC
Arbp	AGCTGAAGCAAAGGAAGAGTCGGA	ACTTGGTTGCTTTGGCGGGATTAG
Cre	GCGGTCTGGCAGTAAAAACTATC	ACCCGGCTGCTCTTACTTCT