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Sirtuin 1 independent effects of resveratrol in INS-1E β-cells

Cihan S. Erdogan, Mathias Mørup-Lendal, Louise T. Dalgaard, Ole Vang*

Department of Science and Environment, Roskilde University, Roskilde, Denmark

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ABSTRACT

Resveratrol (Resv), a natural polyphenol, is suggested to have various health benefits including improved insulin sensitivity. Resv activates Sirtuin (Sirt1) in several species and tissues. Sirt1 is a protein deacetylase with an important role in ageing, metabolism and β-cell function. In insulinoma β-cells (INS-1E), Resv is previously shown to improve glucose-stimulated insulin secretion in a Sirt1-dependent mechanism and to protect against β-cell dedifferentiation in non-human primates, while inducing hypertrophy in myoblasts. Mammalian (mechanistic) Target of Rapamycin (mTOR), is a key regulator of cellular metabolism and regulates the cell size, β-cell survival and proliferation.

In order to understand the interaction of Sirt1 and mTOR cascade activity with Resv-induced changes in the INS-1E cell line, we generated stable Sirt1-down-regulated INS-1E cells, and analysed Sirt1-dependent effects of Resv with respect to mTOR cascade activity. Sirt1-knockdown (KD) had a significant increase in cell size compared to negative-control (NEG CTR) cells. Resveratrol treatment increased cell size in both cell types in a dose-dependent manner at 24 h (Resv conc: 15–60 μM), and decreased the cell number (Resv conc: 30–60 μM). Cell area was increased in NEG CTR cells (Resv conc: 60 μM) at 24 h and KD cells at 48 h (Resv conc: 15–60 μM). Rapamycin, a specific mTOR inhibitor, counteracted the Resv-induced cell enlargement (both cell diameter and area). Furthermore, Sirt1-downregulation by itself did not affect the mTOR cascade activities as measured by Western blotting for total and phosphorylated Akt and mTOR. Rapamycin decreased the mTORC1 activity, while increasing the pAkt levels. Resveratrol did not interfere with the mTOR activity or with Sirt1 expression. Altogether, this work indicates that Sirt1 is a negative regulator of cell size. Moreover, the effect of Resv on cell size increase is mTOR-cascade dependent.

1. Introduction

Mammalian sirtuins, which belong to the family of silent information regulator 2 (Sir2) proteins [1], are nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylases [2,3] and ADP-ribosyl transferases [4,5]. They are class III histone deacetylases that deacetylase both histone and non-histone proteins [6]. Reactions conducted by sirtuins occur by hydrolysis of their co-substrate NAD^+ and the cellular NAD^+/NADH ratio regulates their activity.

Sirt1 has important roles in cellular metabolism and ageing-associated conditions. Overexpression of Sir2 and its homologues cause increased longevity in lower organisms such as *S. cerevisiae* [7], *C. elegans* [8] and *D. melanogaster*, as well as in mice [9]. In skeletal muscle cells, Sirt1 is proposed to improve mitochondrial biogenesis and function through deacetylation and activation of PGC-1α [10]. In rat pancreatic β-cells, Sirt1 improves insulin secretion by downregulating UCP2 [11]. Also, Sirt1 knockout mice present with low levels of insulin and constitutively high levels of UCP2 [11].

Sirt1 is activated by the polyphenol resveratrol (3,5,4′-trihydroxy-trans-stilbene) (Resv), which is found in grapes, berries and many other plants [12,13]. Treatment with Resv improved health, increased insulin sensitivity and mitochondrial number in mice on high calorie diet and mimicked caloric restriction [14]. Low doses (20–30 mg/kg/day) of Resv have been proposed to activate AMP-activated protein kinase (AMPK), cause increased mitogenesis and NAD^+ levels via Sirt1 activation, whereas high doses (above 215 mg/kg/day) of Resv may activate AMPK in Sirt1-independent manner [15].

The aim of the current study was to characterize the Sirt1-dependent effects of Resv on cell size and growth of a pancreatic β-cell model. This is relevant, because Sirt1 as well as Resv

* Corresponding author. Department of Science and Environment, Roskilde University, Universitetsvej 1, DK-4000 Roskilde, Denmark.
E-mail address: ov@ruc.dk (O. Vang).

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Abbreviations

AMPK	AMP-activated protein kinase
mTOR	mammalian (mechanistic) Target of Rapamycin
NAD	Nicotinamide adenine dinucleotide
Rapa	Rapamycin
Resv	Resveratrol
SIR2	silent information regulator 2

improves β-cell insulin secretion [11,16]. Resv also protects against high energy diet-induced β-cell dedifferentiation in non-human primates [17], while in skeletal muscle Resv also induces hypertrophy [18]. Hypertrophy, cell growth and size are to a large extent determined by activity of the mTOR pathway in pancreatic β-cells [19,20] and we therefore examined β-cell size changes introduced by Sirt1, Resv as well as mTOR activity modulation.

2. Materials and methods

2.1. Cell culture and reagents

INS-1E cells are immortalized rat pancreatic β-cells, and were a kind gift from Claes Wollheim, University of Geneva, Switzerland [21]. Cells were mainly cultured in RPMI1640 with 25 mM Hepes and L-Glutamine (Lonza) or RPMI1640 with GlutaMAX™-I (used for culturing cells for RNA extraction) (Thermo-Fisher Scientific) supplemented with 10% heat inactivated foetal calf serum (Hyclone), 20 units/mL of penicillin/streptomycin (Sigma-Aldrich) and 50 μM β-mercaptoethanol at 37 °C under 5% CO_2 humidified atmosphere. Resveratrol (Resv, Sigma-Aldrich) and rapamycin (Rapa, Sigma-Aldrich) stocks with intended concentrations were prepared in DMSO. Plasticware was from Nunc (Thermo-Fisher Scientific).

2.2. Generation and verification of stable Sirt1 knockdown INS-1E cell lines

Sirt1-knockdown and negative control INS-1E lines were generated using retroviral transduction with plasmids pRetroSuper control and pRetroSuper Sirt1 (a kind gift from Laura Bordone [11]). In brief; retroviral particles were prepared by transient transfection of pRetroSuper plasmids into Phoenix Eco HEK-293 cells and medium containing retroviral particles were harvested 48 h post-transfection, centrifuged and filtered through 40 μM filters. Subsequently, viral particles were mixed with sequabrene (5 mg/mL, Sigma-Aldrich) and added to INS-1E cultures (passage 92). 48 hrs after infection, selection was applied (puromycin 0.75 μg/mL) and pools of clones were established. In total, 3 Negative Control (NEG CTR.) and 3 Sirt1 knockdown (KD) clone pools were established. Cells were kept under puromycin selection for 7 days, expanded and frozen in aliquots.

RNA from selected INS-1E pools were extracted using Trireagent (Sigma-Aldrich) using manufacturers' protocol, quantified using a ND1000 spectrophotometer and reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen) according to manufacturers' protocol for hexamer primed cDNA. Steady state Sirt1 mRNA levels were measured by Q-PCR using primers 5′-TGCCATCATGAAGCCAGAGA-3′ and 5′-GAGAAGACCCAATAACAATGAGGA-3′ for Sirt1 and 5′-GTTCTGCTCCAACCTTTGCCT-3′ and 5′-TGTGTAGCTGCCATCTGCACTT-3′ for TFIIB as reference transcript with QuantiTect Sybr (Qiagen) and standard curve determination of cDNA quantities on an Agilent MX3005P.

2.3. Cell size, number and impedance measurements

INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 1×10^5 cells/well in 24-well plates. After o/n attachment cells were treated with Resv, Rapa or combinations of Resv and Rapa for 24–48 h with indicated concentrations. Cells were detached by trypsination (0.05% trypsin with 0.02% EDTA for 20 min) and cell number and cell diameter were measured on Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter). Cell size measurements were then analysed according to best fit with Gaussian distribution log-transformed counts to obtain the mean cell diameter. Microscopy images were obtained for each indicated time point and analysed with ImageJ software.

For impedance measurements, INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 6×10^4 cells/well in E-plate L-8 (Acea Biosciences, Inc.). As the first step, basal readings were obtained with 300 μL cell culture medium for 10×1 min. Then, 200 μL of cell suspension with desired cell density was added into the wells. During the attachment period for 24 h, cell index measurements were obtained every 10 min for 4 h and then every 30 min. After the attachment period, medium was gently removed cells were treated with Resv, Rapa or combinations of Resv and Rapa. Cell index measurements were obtained every 10 min for 4 h and then every 30 min.

2.4. Cell cycle analysis

INS-1E cells (NEG CTR and Sirt1 KD) were seeded at a density of 1×10^6 cells/well in 6-well plates. After o/n attachment period, cells were treated either with vehicle (DMSO) or Resv (30 μM) for 6–24 h. Cells were collected by trypsination and washed with cold PBS. The cell number and cell diameter were measured on Z2 Coulter® Particle Count and Size Analyzer (Beckman Coulter). 500,000 cells/sample were collected in 200 μL cold PBS. After fixation with ice-cold ethanol (70% v/v) for 30 min on ice, samples were preserved at −20 °C until staining and flow cytometry analysis.

For flow cytometry analysis of cell cycle phase distribution, the fixed sample was centrifuged and supernatant discarded. After washing with PBS, cells were resuspended in 400 μL PBS and treated with RNAse A (100 μg/mL) and propidium iodide (40 μg/mL) at room temperature in the dark for 30min. The supernatant was discarded by centrifugation and sample was collected in 500 μL PBS, and analysed by flow cytometry (BD FACS Calibur). The obtained results were analysed by using ModFit LT 4.1 (Verity Software House).

2.5. Antibodies and western blotting

All primary antibodies were used at 1:1000 dilution in 5% skim milk-TBS-T (Sigma-Aldrich). Anti-phospho-mTOR (Ser2448) antibody (2971), anti-mTOR antibody (2972), anti-phospho-Akt (Ser473) antibody (4060), anti-Akt (pan) antibody (4685), anti-β-Actin antibody (4967), anti-phospho-S6 ribosomal protein (Ser235/236) antibody (2211) were obtained from Cell Signalling Technology. Anti-Sirt1 antibody (07–131) was purchased from Merck Millipore, anti-GAPDH (ab8245) was purchased from Abcam, and anti-Deptor antibody (SAB4200241) was purchased from Sigma-Aldrich. HRP conjugated anti-rabbit IgG (P0217) and anti-mouse IgG (P0260) was purchased from Dako (Agilent Technologies Denmark), and used at 1:2000 dilution in 5% skim milk.

For immunoblotting from total lysate, 2×10^6 cells were seeded in 6 cm dishes. Cells were lysed in lysis buffer (1% Triton x-100, 0.5% NP-40, 75 mM NaCl, 50 mM Tris at pH = 7.4, 10% glycerol) supplemented with phosphatase inhibitor mix (Halt Phosphatase