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α-Lipoic acid treatment increases mitochondrial biogenesis and promotes beige adipose features in subcutaneous adipocytes from overweight/obese subjects

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ABSTRACT

α-Lipoic acid (α-Lip) is a natural occurring antioxidant with beneficial anti-obesity properties. The aim of this study was to investigate the putative effects of α-Lip on mitochondrial biogenesis and the acquirement of brown-like characteristics by subcutaneous adipocytes from overweight/obese subjects. Thus, fully differentiated human subcutaneous adipocytes were treated with α-Lip (100 and 250 μM) for 24 h for studies on mitochondrial content and morphology, mitochondrial DNA (mtDNA) copy number, fatty acid oxidation enzymes and brown/beige characteristic genes. The involvement of the Sirtuin1/Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (SIRT1/PGC-1α) pathway was also evaluated. Our results showed that α-Lip increased mitochondrial content in cultured human adipocytes as revealed by electron microscopy and by mitotracker green labeling. Moreover, an enhancement in mtDNA content was observed. This increase was accompanied by an up-regulation of SIRT1 protein levels, a decrease in PGC-1α acetylation and up-regulation of Nuclear respiratory factor 1 (*Nrf1*) and Mitochondrial transcription factor (*Tfam*) transcription factors. Enhanced oxygen consumption and fatty acid oxidation enzymes, Carnitine palmitoyl transferase 1 and Acyl-coenzyme A oxidase (CPT-1 and ACOX) were also observed. Mitochondria from α-Lip-treated adipocytes exhibited some morphological characteristics of brown mitochondria, and α-Lip also induced up-regulation of some brown/beige adipocytes markers such as cell death-inducing DFFA-like effector a (*Cidea*) and T-box 1 (*Tbx1*). Moreover, α-Lip up-regulated PR domain containing 16 (*Prdm16*) mRNA levels in treated adipocytes. Therefore, our study suggests the ability of α-Lip to promote mitochondrial biogenesis and brown-like remodeling in cultured white subcutaneous adipocytes from overweight/obese donors.

1. Introduction

Mitochondrial dysfunction in adipocytes has been associated with the development of obesity and type 2 diabetes [1]. In fact, the abundance of the mitochondrial copy number in adipocytes from obese is lower than in those from lean subjects [2]. This lower mitochondrial content is usually associated with reduced mitochondrial function and, therefore, decreased fatty acid β-oxidation leading to increased fatty acid accumulation, which contributes to the development of obesity-associated comorbidities such as insulin resistance and dyslipidemia [3,4]. For this reason, there is a high level of interest in developing therapeutic strategies aimed at modulating the regulatory

Abbreviations: α-Lip, α-Lipoic acid; LDH, lactate dehydrogenase; mtDNA, mitochondrial DNA; Sirt1, Sirtuin 1; PGC-1α, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; Nrf1, nuclear respiratory factor 1; Tfam, mitochondrial transcription factor; CPT-1, carnitine palmitoyl transferase 1; Acox, acyl-coenzyme A oxidase; CIDEA, cell death-inducing DFFA-like effector A; TBX1, T-box 1; Prdm16, PR domain containing 16; BMI, body mass index; TEM, transmission electron microscopy; EFTEM, energy filter transmission electron microscope; BCA, bicinchoninic acid assay; PVDF, polyvinylidene difluoride; CD36, Cluster of Differentiation 36; ASM, acid soluble metabolites; MTCO2, cytochrome c oxidase subunit II; BSA, bovine serum albumin; UCP1, uncoupling protein 1; PPARγ, peroxisome proliferator-activated receptor gamma; AMPK, AMP-activated protein kinase

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pathways that increase mitochondrial function and biogenesis in an attempt to prevent or treat these disorders related to mitochondrial dysfunction [5]. Mitochondrial biogenesis is a complex process requiring the coordinated expression and assembly of many proteins encoded by both nuclear and mitochondrial genomes [6,7]. SIRT1-mediated activation of PGC-1α is one of the pathways that are particularly important for mitochondrial biogenesis. Several studies have postulated that PGC-1α could be a target to prevent and reverse insulin resistance, obesity and diabetes [8,9]. This family of regulated coactivators plays an important role through their interactions with transcription factors such as NRF1, which coordinates the transcriptional control of nuclear and mitochondrial genomes and directly activates TFAM [10,11], a core component of the mitochondrial transcription machinery [12]. Moreover, PGC-1α is related to the switch from white to brown-like (brite or beige) inducible adipocytes [13]. Thus, PRDM16, a transcription factor that appears to control the fate of brown adipose tissue development, binds to PGC-1α and allows the activation of brown fat-specific genes triggering browning of adipocytes, which constitutes a novel strategy against obesity [14].

5-(1,2-dithiolan-3-yl)-pentanoic acid or α-Lipoic acid (α-Lip) is an antioxidant compound [15] with promising anti-obesity properties both in rodents and humans [16,17]. In addition to the body lowering actions of α-Lip, beneficial effects on insulin sensitivity, glucose and lipid metabolism have been described in humans [17,18]. Several studies have revealed that white adipose tissue is a target for α-Lip therapeutic actions, by regulating key metabolic pathways such as lipolysis [19] and the secretion of important adipokines that controls body weight and insulin sensitivity [20–23]. Experiments in rodents and murine cells have suggested that the beneficial actions of α-Lip could be also related to its ability to promote mitochondrial biogenesis in different metabolic tissues such as liver [24]. However, there is no information available regarding the effects of α-Lip on mitochondrial biogenesis in human adipocytes under obesity conditions. Thus, we aimed to evaluate the effects of α-Lip on mitochondrial biogenesis and on the induction of brown-like features in human subcutaneous adipocytes obtained from overweight/obese subjects. The molecular mechanisms underlying these effects were also investigated in the present study.

2. Material and methods

2.1. Cell culture and differentiation of human subcutaneous preadipocytes

Commercially available cryopreserved human subcutaneous preadipocytes from non-diabetic overweight–obese female donors (BMI: 26.85–33.37 kg/m^2) were purchased from Zen-Bio Inc. (Research Triangle Park, NC) and differentiated according to the manufacturer's instructions. Fourteen days after the induction of differentiation, cells contained large lipid droplets and were considered mature adipocytes.

2.2. Treatments and cell viability assay

Before treatment, cell medium was removed and replaced with 1 ml of fresh AM1 (subcutaneous adipocyte medium, Zen-Bio Inc). α-Lip (Sigma; St. Louis, MO) was dissolved in ethanol. 1000× stocks were prepared and 1 μl/ml of media was added. Adipocytes were exposed to α-Lip for 24 h. Cell viability was measured using the determination of lactate dehydrogenase (LDH) activity in the culture medium as an indicator of cell membrane integrity, and, therefore, as a measurement of cell necrosis/apoptosis [25]. No statistical differences between control and α-Lip treated adipocytes were found, indicating that treatment did not alter cell viability.

2.3. Transmission electron microscopy (TEM)

Mitochondrial content of differentiated human subcutaneous adipocytes was examined by TEM using ultra-thin sectioning and negative staining respectively. The cultured adipocytes were prefixed in 4% glutaraldehide in cacodylate buffer 0.1 M during 1 h at 4 °C and postfixed in 1% osmium tetroxide during 1 h at 4 °C. After this time cells were detached by using a cell scrapper and included in 2% agarose. Then samples were embedded in Epoxy resin of low viscosity (SERVA Electrophoresis GmbH, Heidelberg, Germany) and sectioned to a thickness of 60–70 nm with a Leica ultracut R Ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Ultra-thin sections were examined with an energy filter transmission electron microscope (EFTEM) Libra 120 (Zeiss GmbH, Oberkochen, Germany). Images were obtained by using the software iTEM 5.1 (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

2.4. Analysis of mRNA levels

Total RNA was extracted from fully differentiated human subcutaneous adipocytes by using TRIzol® reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. RNA concentrations and quality were measured by Nanodrop Spectrophotometer ND1000 (Thermo Scientific, Wilminton, DE). RNA was then incubated with the DNA-free kit DNAse (Ambion, Austin, TX) for 30 min at 37 °C. RNA (1 μg) was reverse-transcribed to cDNA using the Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase (Invitrogen). For the real-time quantitative polymerase chain reaction analysis, 4.5 μl of 1/5, 1/50 or 1/10,000 dilution of cDNA per reaction was used in a final reaction volume of 10 μl.

Tfam, *Nrf1*, *Prdm16*, *Cidea*, *Ctp-1* and *Acox* mRNA levels were determined using predesigned Taqman® Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems, Foster City, CA). The reaction conditions were stated according to the manufacturer's instructions. Amplification and detection of specific products were performed using the ABI PRISM 7900HT Fast System Sequence Detection System (Applied Biosystems).

All mRNA levels were normalized by the housekeeping gene *18s* obtained from Applied Biosystems. Samples were analyzed in duplicate. Ct values (the cycle where the emitted fluorescence signal is significantly above background levels and is inversely proportional to the initial template copy number) were generated by the ABI software. Finally, the relative expression level of each gene was calculated as $2^{-\Delta\Delta Ct}$ [26].

2.5. Analysis of mitochondrial DNA content

The amount of mtDNA was quantified by real-time PCR as described previously [27]. Briefly, the relative amount of mtDNA was quantified by comparison of a mitochondrial target, the cytochrome c oxidase subunit II (MTCO2) with a nuclear target (18S). Quantitative real-time PCR was performed using the Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA) as described above. For quantification, a ratio between mtDNA (MTCO2) and 18S was calculated, and used as mtDNA content.

2.6. Analysis of mitochondrial content by mitotracker green staining

Mitochondria were labeled using the mitochondria-specific dye mitotracker green (Molecular Probes, Life Technologies Ltd, Paisley, UK) according to the manufacturer's protocol. The final dye concentration was 100 nmol/l and the incubation time was 30 min prior to visualization. Fluorescent microscopy was performed on living cells with a Leica DM IL-EL 6000 (Leica Microsystems GmbH) inverted microscope. For fluorescence intensity quantification a Polarstar Galaxy fluorimeter (BMG labtech) set up to 554 nm excitation and 576 nm emission wavelengths was used.