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Eicosapentaenoic acid promotes mitochondrial biogenesis and beige-like features in subcutaneous adipocytes from overweight subjects

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Abstract

Eicosapentaenoic acid (EPA), a n-3 long-chain polyunsaturated fatty acid, has been reported to have beneficial effects in obesity-associated metabolic disorders. The objective of the present study was to determine the effects of EPA on the regulation of genes involved in lipid metabolism, and the ability of EPA to induce mitochondrial biogenesis and beiging in subcutaneous adipocytes from overweight subjects. Fully differentiated human subcutaneous adipocytes from overweight females (BMI: 28.1–29.8 kg/m^2) were treated with EPA (100–200 μM) for 24 h. Changes in mRNA expression levels of genes involved in lipogenesis, fatty acid oxidation and mitochondrial biogenesis were determined by qRT-PCR. Mitochondrial content was evaluated using MitoTracker® Green stain. The effects on peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PGC-1α) and AMP-activated protein kinase (AMPK) were also characterized. EPA down-regulated lipogenic genes expression while up-regulated genes involved in fatty acid oxidation. Moreover, EPA-treated adipocytes showed increased mitochondrial content, accompanied by an up-regulation of nuclear respiratory factor-1, mitochondrial transcription factor A and cytochrome *c* oxidase IV mRNA expression. EPA also promoted the activation of master regulators of mitochondrial biogenesis such as sirtuin 1, PGC1-α and AMPK. In parallel, EPA induced the expression of genes that typify beige adipocytes such as fat determination factor PR domain containing 16, uncoupling protein 1 and cell death-inducing DFFA-like effector A, T-Box protein 1 and CD137. Our results suggest that EPA induces a remodeling of adipocyte metabolism preventing fat storage and promoting fatty acid oxidation, mitochondrial biogenesis and beige-like markers in human subcutaneous adipocytes from overweight subjects.
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Keywords: N-3 PUFAs; EPA; Lipogenesis; Fatty acid oxidation; Mitochondrial biogenesis; Browning

1. Introduction

Obesity is getting importance as a worldwide epidemic disease, which leads to the development of several co-morbidities such as insulin resistance, dyslipidemia and metabolic syndrome [1].

Obesity is characterized by altered lipid metabolism and mitochondrial activity, promoting accumulation of triglycerides in white adipose tissue, liver and muscle [2,3]. White adipocytes are poor in mitochondria and have a low oxidative capacity [4]. Moreover, a recent study has revealed defective mitochondrial biogenesis and oxidative metabolic pathways in subcutaneous adipose tissue during acquired obesity, preceding the metabolic disturbances in obesity [5]. Therefore, stimulation of mitochondrial biogenesis and oxidative capacity of white adipocytes by pharmacological and nutritional agents has been proposed as a potential target for obesity and related metabolic disorders [6].

During the last years a new type of adipose tissue has been described and named as beige adipose tissue [7]. Beige or “brite” (brown-in-white) adipocytes are multilocular and have thermogenic properties due to increased mitochondrial function and expression of inducible uncoupling protein 1 (UCP1). Interestingly, beige adipocytes have been identified in adult humans [7], making this cell type an attractive therapeutic target for the management of obesity and metabolic-related diseases [8]. In addition to the canonical activators of browning (cold exposure and β-3 adrenergic agonist), recent studies have described several bioactive molecules (cardiac natriuretic peptides, irisin, fibroblast growth factor 21, cardiotrophin-1) and

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food components (curcumin, resveratrol, quercetin) able to induce adipose tissue beiging [6,9–11].

The marine long-chain polyunsaturated omega-3 fatty acids (n-3 PUFAs), docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA), have been associated to beneficial effects on obesity and related-metabolic disorders. Several studies have evidenced that adipose tissue is one of the main target organs regulated by n-3 PUFAs and involved in the metabolic responses [12–14]. Thus, EPA and DHA have been shown to reduce inflammation [15–17], modulate adipokine secretion [18–21] and promote mitochondrial biogenesis and improve oxidative capacity of white adipocytes in animal models [22,23]. Nevertheless, the ability of n-3 PUFAs to induce UCP1 in adipose tissue and their thermogenic properties are still controversial [23,24]. Interestingly, it has been suggested that EPA is able to enhance energy dissipation in subcutaneous adipose tissue by promoting britening and inducing oxidative metabolism in mice [25]. However, there is no evidence about EPA ability to promote mitochondrial biogenesis and activate the beiging process in human subcutaneous adipocytes. For these reasons, this study aimed to characterize the actions of EPA on the modulation of genes/proteins involved in lipogenesis, fatty acid oxidation, mitochondrial biogenesis and adipocytes beiging in cultured subcutaneous primary adipocytes from overweight subjects.

2. Material and methods

2.1. Chemicals and cell culture reagents

EPA was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved in ethanol (Sigma-Aldrich, St. Louis, MO, USA). All other reagents/chemicals were of analytical grade. Human subcutaneous pre-adipocytes and cell culture reagents including PM-1, DM-2 and AM-1 were obtained from Zen-Bio Inc. (Research Triangle Park, NC, USA). Moreover, MitoTracker Green FM fluorescent mitochondrial stain was obtained from Molecular Probes (Life Technologies Ltd., Paisley, UK).

2.2. Cell culture of human subcutaneous pre-adipocytes and treatments

Human subcutaneous pre-adipocytes cryopreserved from overweight females (BMI: 28.1–29.8 kg/m^2) were obtained from Zen-Bio Inc. and differentiated according to the manufacturer's procedures. As previously described [26], pre-adipocytes were cultured in 12 wells plates at 40.000 cells/cm^2 and incubated at 37°C in an humidity atmosphere of 5% CO_2 in presence of pre-adipocytes medium PM-1 ZenBio (DMEM/Ham's F-12 medium, HEPES pH=7.4, fetal bovine serum, penicillin, streptomycin, amphotericin B). Pre-adipocytes were feed every two days with 1 ml of PM-1 until confluence. After that, PM-1 medium was replaced with 1 ml of DM2 (ZenBio, PM-1 with biotin, pantothenate, human insulin, dexamethasone, isobutylmethylxanthine, PPARγ agonist) in order to induce differentiation to adipocytes (days 0–7). After 7 days of differentiation, 600 μl of DM2 medium were removed and 800 μl of AM1 medium (Zen-Bio) was added, which includes PM-1, biotin, pantothenate, human insulin and dexamethasone. Cells were additionally incubated in AM1 medium, which was replaced every two days. At day 14 post-differentiation, cells displayed typical mature adipocyte phenotypes characterized by the presence of multilocular small lipid droplets and were ready for EPA treatment. Cell media was replaced with 1 ml of new AM1 medium, and EPA was added to a final concentration of 100–200 μM and incubated for 24 h. Control adipocytes were treated with the same amount of vehicle (ethanol). After incubation period, cells were harvested and frozen at−80°C for further analysis. Cell viability was measured by lactate dehydrogenase (LDH) activity in the culture medium as an indicator of cell membrane integrity, and, consequently, as a measurement of cell necrosis/apoptosis [26]. No statistical differences between control and EPA-treated adipocytes were found, indicating that treatment did not alter cell viability (data not shown).

2.3. Analysis of mRNA levels

Total RNA was isolated from mature human subcutaneous adipocytes using TRIzol reagent (Invitrogen, CA, USA) according to manufacturer's procedures. RNA-concentrations and quality were measured using Nanodrop Spectrophotometer ND1000 (Thermo Scientific, DE, USA). RNA was then incubated with a RNAse-free kit DNAse (Ambion, Austin, TX, USA) for 30 min at 37°C. RNA was reverse transcribed to cDNA using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen). Fatty acid synthase (*FAS*), stearoyl-CoA desaturase-1 (*SCD-1*), diacylglycerol *O*-acyltransferase (*DGAT1–2*), carnitine palmitoyltransferase (*CPT-1*), acyl-coenzyme A oxidase (*ACOX1*), sirtuin 1 (*SIRT1*), nuclear respiratory factor-1 (*NRF-1*), mitochondrial transcription factor A (*TFAM*), cytochrome c oxidase IV *(COX IV)*, *UCP1*, PR domain containing 16 (*PRDM16*), cell death-inducing DFFA-like effector a *(CIDEA)*, *CD137*, T-box 1 transcription factor *(TBX1)* and Transmembrane Protein 26 *(TMEM26)* mRNA levels were determined using predesigned Taqman Assays-on-Demand and Taqman Universal Master Mix (Applied Biosystems, CA, USA). Amplification and detection of specific products were done using the ABI PRISM 7900HT Fast Sequence Detection System (Applied Biosystems) [26,27].

The levels of mRNA were normalized to *18S* as housekeeping obtained from Applied Biosystems. Samples were analyzed in duplicate. Ct values were generated by the ABI PRISM 7900HT (Applied Biosystems). Finally, the relative expression of the genes was calculated by the $2^{-\Delta\Delta Ct}$ method [28].

2.4. Mitochondrial content

The mitochondrial content of the mature subcutaneous adipocytes was assessed using MitoTracker Green FM fluorescent mitochondrial stain (Molecular Probes, Life Technologies). In order to determine mitochondrial content, 48,000 cells *per* well were seeded in 12-wells plates and 2–3 wells *per* condition were used in three independent experiments. Mitochondria were labeled with a final concentration of dye of 100 nM for 30 min before visualization. In order to determine the fluorescence intensity, a Polarstar Galaxy fluorimeter (BMG labtech, Ortenberg, Germany) set up to 490 nm excitation and 516 nm emission wavelengths was used [9].

2.5. SIRT1 activity

The activity of SIRT1 was determined in nuclear protein fractions isolated from mature adipocytes with or without EPA treatment, using a fluorometric SIRT1 activity assay kit as described by the manufacturer (Abcam, Cambridge, UK). Nuclei isolation and extraction was carried out following the supplier's instructions. The activity of SIRT1 was measured by mixing simultaneously fluorescence-labeled acetylated peptide (substrate), trichostatin A, NAD, lysylendopeptidase and the samples or the recombinant SIRT1 (as positive control) at room temperature. Fluorescence intensity (Excitation: 340 nm/Emission: 460 nm) was read for 48 min, at 2 min intervals, to determine the enzyme activity. The fluorescence signal generated was in proportion to the amount of deacetylation of the lysine by SIRT1.

2.6. Western blot analysis

Human subcutaneous adipocytes lysates were obtained by the addition of a buffer containing 2 mM Tris HCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail 1 (Sigma-Aldrich), 2 mM orthovanadate, and 1 mM PMSF. Samples were centrifuged and protein concentrations were determined by the BCA method according to the supplier's instructions (Pierce-Thermo Scientific, Rockford, IL). Briefly, equivalent amounts of total protein (30–40 μg) were electrophoretically separated by 12% SDS-PAGE in the presence of a reducing agent (2-mercaptoethanol). Proteins were electroblotted from the gel to polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were stained with Ponceau S as control for total protein loaded, and then blocked and probed with specific primary antibodies diluted at 1:1000 against phospho-AMPK, total AMPK (rabbit, Cell Signaling Technology, Danvers, MA) and β-actin (mouse, Sigma-Aldrich) with overnight stirring at 4°C. Moreover, PPARγ and SREBP-1c were measured in nuclear extracts and probed with specific antibodies against PPARγ (1:1000, rabbit, Cusabio, College Park, MD, USA) and SREBP-1c (1:200, mouse, Santa Cruz Biotechnology, Santa Cruz, CA, USA). To determine total AMPK or β-actin protein expression levels, stripping (ReBlot Plus Mild Solution, Millipore, Temecula, CA, USA) of the membranes was carried out for 15 min. Then the membranes were blocked and probed overnight with specific total AMPK or β-actin antibody at 4°C. After that, infrared fluorescent secondary anti-rabbit (Cell Signaling Technology) or anti-mouse (LI-COR Biosciences, Lincoln, NE, USA) antibodies were used (diluted 1:15,000) and incubated for one hour at room temperature. Fluorescence signal was quantitated using an Odyssey Sa infrared imaging system (LI-COR).

2.7. Immunoprecipitation

In order to determine the acetylation of PGC-1α, 2 μg of anti-PGC-1α (Santa Cruz Biotechnology) antibody was added to 200 μg of protein extracts (1 μg/μl) and incubated stirring for 2 h at 4°C. After that, 20 μl of protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added and incubated at 4°C with shaking overnight. The A/G PLUS-Agarose-beads were pelleted by centrifugation at 12,000 *g* for 1 min at 4°C and washed four times with PBS at 4°C. Finally, protein was released from the beads by treatment at 95°C for 7 min in 2x sample buffer (Invitrogen, Carlsbad, CA, USA). Equivalent amounts of total protein were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked and probed with specific antibodies against anti-acetylated Lysine diluted at 1:100 (rabbit, Cell Signaling Technology) and anti-PGC-1α diluted at 1:100 (rabbit, Santa Cruz Biotechnology) and detected as defined before [26].

2.8. Statistical analysis

Statistical analyses were performed using the program GraphPad Prism 5.0 (GraphPad Software Inc., *CA*, USA). Data were expressed as mean ± S.E. The differences

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