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Epigallocatechin gallate counteracts oxidative stress in docosahexaenoxic acid-treated myocytes

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ABSTRACT

Skeletal muscle is a key organ of mammalian energy metabolism, and its mitochondria are multifunction organelles that are targets of dietary bioactive compounds. The goal of this work was to examine the regulation of mitochondrial dynamics, functionality and cell energy parameters using docosahexaenoic acid (DHA), epigallocatechin gallate (EGCG) and a combination of both in L6 myocytes. Compounds (at 25 μM) were incubated for 4 h. Cells cultured with DHA displayed less oxygen consumption with higher ADP/ATP ratio levels concomitant with downregulation of *Cox* and *Ant1* gene expression. The disruption of energetic homeostasis by DHA, increases intracellular reactive oxygen species (ROS) levels and decreases mitochondrial membrane potential. The defence mechanism to counteract the excess of ROS production was by the upregulation of *Ucp2*, *Ucp3* and *MnSod* gene expression. Moreover myocytes cultured with DHA had a higher mitochondrial mass with a higher proportion of large and elongated mitochondria, whereas the fission genes *Drp1* and *Fiss1* and the fusion gene *Mfn2* were downregulated. In myocytes co-incubated with DHA and EGCG, ROS levels and the adenosine diphosphate (ADP)/adenosine triphosphate (ATP) ratio were similar to untreated myocytes and the decrease of oxygen consumption, higher mitochondrial mass and the overexpression of *Ucp2* and *Ucp3* genes were similar to the DHA-treated cells with also a higher amount of mitochondrial deoxyribonucleic acid (DNA), and reduced *Drp1* and *Fiss1* gene expression levels. In conclusion the addition of EGCG to DHA returned the cells to the control conditions in terms of mitochondrial morphology, energy and redox status, which were unbalanced in the DHA-treated myocytes.

1. Introduction

Mitochondria are ubiquitous organelles in eukaryotic cells whose primary functions are to generate energy, regulate the cellular redox state and calcium homeostasis, and initiate cellular apoptosis [1]. In addition, mitochondria are the main intracellular source and immediate target of reactive oxygen species (ROS), which are continuously generated as byproducts of aerobic metabolism in mammalian cells. Thus, mitochondria play a pivotal role in the determination of the life and death of the mammalian cell [2]. The size, shape, and abundance of mitochondria vary dramatically in different cell types and may change under different energy demands and physiological environmental conditions [3]. In many cell types, especially muscle fibres, mitochondria form tubular structures or networks [4]. Increasing evidence, suggests that mitochondrial structure determines mitochondrial function [5] including substrate metabolism and mitochondrial bioenergetics. Mitochondria are highly dynamic organelles with constant fusion and fission events mediated by conserved cellular machineries. The frequencies of these fusion and fission events are balanced to maintain the overall morphology of the mitochondrial population [6] and to control mitochondrial energy metabolism, protecting cells from mitochondrial damage and maintain the overall architecture of these organelles [7,8].

Mitochondria are recognised as major targets of bioactive compounds, such as omega-3 polyunsaturated fatty acids (PUFAs) and flavonoids, which are found in healthy diets. The current data support a role for omega-3 PUFA supplementation, particularly docosahexaenoic acid (DHA), which is strongly associated with changes

Abbreviations: AFU, arbitrary fluorescence units; *Ant* 1, adenine nucleotide translocase 1; *Cox*, cytochrome c oxidase subunit V; *Cs*, citrate synthase; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; *Drp1*, dynamin-related protein 1; EGCG, epigallocatechin-3-gallate; ETC, electron transport chain; ETS, electron transport capacity; FBS, fetal bovine serum; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; *Fiss1*, mitochondrial fission 1 protein; Fluo-3 AM, fluo-3 acetoxymethyl ester; *Gapdh*, glyceraldehydes-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; *Mfn2*, mitofusin 2; MMP, mitochondrial membrane potential; *MnSod*, manganese superoxide dismutase; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; *Nd3*, NADH-dehydrogenase subunit 3; OXPHOS, oxidative phosphorylation; *Opa1*, optic atrophy 1; PUFAs, polyunsaturated fatty acids; Rhd123, Rhodamine 123; ROS, reactive oxygen species; ROX, residual oxygen consumption; *Ucp2*, uncoupling protein 2; *Ucp3*, uncoupling protein 3

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and the remodelling of mitochondrial phospholipid composition and organisational domains [9–14]. Although DHA is a likely target for oxidation [15–17], its health benefits are largely derived through DHA (22:6n − 3) rapidly in place of other fatty acids into biological membranes, particularly plasma and mitochondrial membranes and its cell signalling mechanisms. Incorporation of 22:6n − 3 influences membrane structure and function [18], increasing membrane permeability [19] fluidity and plasticity altering conformational states with their acyl chains, which are extremely flexible [13], and influences the physical properties of biological membranes, thereby altering protein function and fusion [13,20]. In addition to being susceptible to lipid peroxidation, DHA could decrease mitochondrial function simply as a result of the accumulation of oxidised products [21], altering the lipid bilayer and decreasing bioenergetic activities due to membrane perturbations [19]. In mitochondria, PUFAs play a role in several mitochondrial processes, including mitochondrial calcium homeostasis, gene expression, and respiratory function, and act as protonophores to reduce mitochondrial ROS production through uncoupling protein (UCP)-mediated decreases in mitochondrial membrane potential [17].

Mitochondria can also be regarded as important intracellular targets of agents that protect from the undesirable action of ROS, such as polyphenols, which prevent against many pathological states involving oxidative cell damage [22]. Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol isolated from green tea and is widely studied because it promotes cardiovascular and metabolic health by acting as a potent antioxidant that may have therapeutic applications in the treatment of many disorders. EGCG has antioxidant properties [23–25] with powerful radical scavengers. These antioxidant activities are due to the presence of phenolic groups that are sensitive to oxidation and are increased by the presence of the trihydroxyl structure in the D ring of EGCG [26]. Therefore, the presence of antioxidants jointly with omega-3 PUFAs would prevent possible oxidative deterioration. Despite the beneficial response of EGCG and DHA in muscle cells, here, we tested the hypothesis that EGCG has protective effects on the DHA oxidation, associated to the changes of physical properties of biological membranes and consequent changes in energy metabolism due to DHA intake. Taking into account that skeletal muscle tissue is a major determinant of whole-body energy metabolism, the aim of this study was to examine how EGCG and DHA, alone or in combination, affect cell energy homeostasis, mitochondrial functionality and morphology, oxidative phosphorylation, ROS generation and calcium homeostasis in L6 myocytes.

2. Materials and methods

2.1. Chemicals

(−) Epigallocatechin-3-gallate (EGCG) from green tea, cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), fatty acid-free BSA, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), DMSO, Bradford reagent, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), oligomycin, rotenone, antimycin A, rhodamine 123 (Rhd123), ethanol, succinate and Fluo-3 acetoxymethyl ester (Fluo-3 AM) were obtained from Sigma-Aldrich (Madrid, Spain), and the MitoTracker FM was obtained from Molecular Probes (Eugene, Oregon, USA). Dulbecco's modified Eagle's medium (DMEM), glutamine, fetal bovine serum (FBS), penicillin and streptomycin were obtained from BioWhittaker (Verviers, Belgium).

2.2. Cell culture

L6 myocyte cells (kindly supplied from Dr. Manuel Portero-Otín) were routinely cultivated in DMEM supplemented with 2 mM glutamine, 10% FBS, 1% penicillin (126.6 U/mL) and 1% streptomycin (0.126 mg/mL) at 37 °C in an atmosphere of 5% CO_2. The cells were grown to approximately 80% confluence and then induced to differentiate into myotubes in DMEM supplemented with 2% FBS. After 7 days, myotube differentiation was complete, and the experimental procedure was initiated. L6 cells were serum-starved for 4 h before the assay. All experiments were performed in triplicate in 3 independent experiments.

2.3. Cell treatment

To study the effects of EGCG, DHA and the combination of both compounds on mitochondrial function, metabolism and morphology, L6 myocyte cells were treated with the vehicle control, 25 μM EGCG, 25 μM DHA, or 25 μM EGCG + 25 μM DHA. Both compounds were dissolved in ethanol and added to the culture media. The medium used during the treatment was serum-free DMEM containing 2% BSA. The experiments were performed in triplicate and with 3 different passages. The final concentration of ethanol in the media was 0.05%, a nontoxic percentage. After 4 h, the cells were used for the different analyses.

2.4. Cell death

Cell death was assessed by lactate dehydrogenase (LDH) leakage into the culture medium. Following the 24 h of exposure to EGCG and DHA (25 μM), the culture medium was aspirated and centrifuged at 3000 rpm for 5 min to obtain a cell-free supernatant; on the other hand cells were lysed in cold buffer (25 mM HEPES pH 7.0, 0.1% Triton X-100). The next steps were performed according to the manufacturer's instructions for the LDH kit (QCA; Amposta, Spain). The % of LDH leakage was normalised to control group and calculated as follows:

$$\%\text{LDH leakage} = ((\text{mUmedium})/(\text{mU medium} * \text{mU cells})) * 100.$$

2.5. Oxygen consumption assay in intact cells

In vivo measurements of mitochondrial oxygen consumption with intact cells were performed using a high-resolution oxygraph (Oroboros Instruments, Innsbruck, Austria) to quantify the respiration states. L6 myocytes were treated in 6-well plates and removed from culture dishes through trypsinisation (0.05% trypsin–EDTA). After 5 min of centrifugation at 200 *g* (room temperature), the cells were resuspended in warmed respiration medium (DMEM without fetal bovine serum) and transferred to the corresponding respiration chamber at a concentration of 4–6 × 10^6 cells/mL. Analyses of respiration rates were performed in 2 mL of respiration medium at 37 °C with stirring at 750 rpm. When the oxygen concentration was stabilised, basal respiration was recorded (Routine state) to control the levels of respiration and phosphorylation in a physiologically coupled state, which was supported by exogenous substrates in the culture media. Following stabilisation of the Routine state, ATP synthesis was inhibited with 2 μg/mL oligomycin, and the nonphosphorylating or resting state (Leak state) was recorded. Subsequently, 10–12 μM FCCP was added to stimulate respiration maximally at a level flow, measuring the electron transport system (ETS) capacity in the noncoupled state (ETS state). In sum, respiration was blocked with 2.5 μM rotenone and 2.5 μM antimycin, representing the residual oxygen consumption (ROX) state that remains after electron transport chain (ETC) inhibition. The results are expressed as oxygen flow per number of cells (pmol oxygen/10^6 cells * s). All results were corrected using the ROX state capacity. Oxygen consumption was calculated using DataGraph Software from Oroboros Instruments (Innsbruck, Austria).

2.6. Determination of ADP/ATP ratio

The total ADP/ATP ratio in the muscle cells was determined after 4 h of treatment using the ApoSENSOR™ ADP/ATP ratio assay kit (Biovision, Mountain View, CA, USA) following the manufacturer's instructions.