



Epigallocatechin-3-gallate prevents oxidative phosphorylation deficit and promotes mitochondrial biogenesis in human cells from subjects with Down's syndrome

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ABSTRACT

A critical role for mitochondrial dysfunction has been proposed in the pathogenesis of Down's syndrome (DS), a human multifactorial disorder caused by trisomy of chromosome 21, associated with mental retardation and early neurodegeneration. Previous studies from our group demonstrated in DS cells a decreased capacity of the mitochondrial ATP production system and overproduction of reactive oxygen species (ROS) in mitochondria. In this study we have tested the potential of epigallocatechin-3-gallate (EGCG) – a natural polyphenol component of green tea – to counteract the mitochondrial energy deficit found in DS cells. We found that EGCG, incubated with cultured lymphoblasts and fibroblasts from DS subjects, rescued mitochondrial complex I and ATP synthase catalytic activities, restored oxidative phosphorylation efficiency and counteracted oxidative stress. These effects were associated with EGCG-induced promotion of PKA activity, related to increased cellular levels of cAMP and PKA-dependent phosphorylation of the NDUF54 subunit of complex I. In addition, EGCG strongly promoted mitochondrial biogenesis in DS cells, as associated with increase in Sirt1-dependent PGC-1 α deacetylation, NRF-1 and T-FAM protein levels and mitochondrial DNA content.

In conclusion, this study shows that EGCG is a promoting effector of oxidative phosphorylation and mitochondrial biogenesis in DS cells, acting through modulation of the cAMP/PKA- and sirtuin-dependent pathways. EGCG treatment promises thus to be a therapeutic approach to counteract mitochondrial energy deficit and oxidative stress in DS.

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1. Introduction

Down's syndrome (DS) is a genetic disorder caused by trisomy of chromosome 21. Individuals with DS show an early aging associated with a decline in intellectual abilities, with a high tendency to develop neuropathological features associated with Alzheimer's disease [1,2]. As in several genetic disorders characterized by propensity to premature aging, oxidative stress is a phenotypic hallmark also in DS [3]. Although the molecular mechanisms involved in DS are largely

unknown, several studies in different animal models [2,4,5] as well as in humans [6,7], have revealed that mitochondrial dysfunction is critically associated with DS pathogenesis. The role of mitochondrial dysfunction in the composite cellular and molecular events leading to neurodegeneration and intellectual deficiency occurring in DS remains, however, to be clarified. A deficit in the system of mitochondrial energy production, due to the impairment of mitochondrial respiratory chain complex I, ATP synthase, ADP/ATP translocator and adenylate kinase activities, was recently found by our group in fibroblasts from DS subjects [8,9]. These alterations were associated with perturbation in post-translational cAMP/PKA-mediated processes, as well as with exceeding oxidative stress in DS [8,9]. Attempts to improve mitochondrial function and/or mass can be an attractive strategy to correct DS-associated clinical phenotypes which are linked to increased oxidative stress and energy deficit. In this respect, mitochondrial functions and dynamics are increasingly recognized as a major factor in supporting neurogenesis and in preventing neurodegeneration [10]. Although recent studies indicated that mitochondrial-targeted molecules, such as coenzyme Q(10), provide

Abbreviations: CREB, cAMP response element-binding; DCF, dichlorofluorescein; DS, Down's syndrome; EGCG, epigallocatechin-3-gallate; H₂O₂, hydrogen peroxide; NRF-1, nuclear respiratory factor 1; OXPHOS, mitochondrial oxidative phosphorylation; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator; ROS, reactive oxygen species; Sirt1, sirtuin 1; T-FAM, mitochondrial transcription factor A

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a protective effect on oxidative imbalance in DS (for Refs. see [5]), intervention trials using standard antioxidant supplements have failed to produce uniform therapeutic effects [11].

In this study we tested the capability of epigallocatechin-3-gallate (EGCG) – a member of a natural polyphenol family, found in great amount in green tea leaves – to restore mitochondrial energy deficit and counteract oxidative stress in DS cells. EGCG, which has been extensively studied for its anticarcinogenic (for Refs. see [12]) and anti-inflammatory [13] effects, is a mitochondrial-targeted molecule displaying a selective antiapoptotic effect against inducers of mitochondrial oxidative stress in a variety of neuronal cell types [14]. EGCG has been found to prevent mitochondrial deterioration in aged rat brain [15], reduce cerebral amyloidosis [16] and correct amyloid-induced mitochondrial dysfunction in a transgenic mice model of Alzheimer disease [17]. Although many modalities of action have been recently suggested for this catechine (reviewed in [18]), the molecular mechanism underlying its effects on mitochondria remains poorly understood. It has been reported that EGCG modulates key regulators of mitochondrial metabolism such as Sirt1 activity [19] and cAMP levels [20,21]. What makes EGCG an interesting candidate drug in DS treatment is also its activity as specific and safe inhibitor of the chromosome 21 encoded DYRK1A [22], a kinase protein involved in brain development and in the control of synaptic plasticity [23]; EGCG was found to rescue brain defect induced by DYRK1A overexpression [24].

The results of the present work show that EGCG rescues mitochondrial energy impairment, prevents overproduction of reactive oxygen species (ROS) and peroxidation of lipid membranes and increases mitochondrial biogenesis in cultured lymphoblasts and fibroblasts from DS patients.

2. Materials and methods

2.1. Cell cultures and materials

Five normal and six DS human phenotypically characterized fetal skin fibroblast cell lines, established from fetuses spontaneously aborted at a gestational age between 14 and 18 weeks, were obtained from the Galliera Genetic Bank, member of Telethon genetic Biobank Network. The Galliera Genetic Bank operates in agreement with ethical guidelines stated in the TGB Network Charter with informed consent obtained from the guardians. The fibroblast cell lines were cultured at 37 °C in humidified 5% CO₂/95% air in RPMI 1640 medium (GIBCO/BRL) supplemented with 15% heat-inactivated fetal bovine serum (GIBCO/BRL), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml) (GIBCO/BRL). Adherent fibroblasts were subjected to a 1:2 split every 6 days. In the experiments a comparable number of culture passages (5–15) were used. At these passages the growth rate of DS fibroblasts was comparable with that of normal cells since the cell energy status did not significantly change in DS as compared with normal cells [8].

Epstein–Barr virus-immortalized lymphoblastoid cells lines, established from six children age ranging from 3 to 10 years, phenotypically characterized as DS, and five age-matched controls were provided by the Department of Pediatrics, Federico II University of Naples with informed consent obtained from the patients and their guardians. Floating lymphoblastoid cell lines were grown in RPMI 1640 medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 200 units/ml penicillin, 200 µg/ml streptomycin and 160 mg/l gentamicin sulfate (GIBCO/BRL) at 37 °C in humidified 5% CO₂/95% air. Cells were subjected to a 1:2 split every 4 days.

Protein concentration was quantified by using the modified Bradford assay (BIO-RAD protein assay) from Bio-RAD according to the manufacturer's instructions. All reagents used were from Sigma-Aldrich unless otherwise specified.

2.2. EGCG treatment

EGCG – extract from green tea leaves with a purity >95% (Sigma-Aldrich) – was freshly prepared for each experiment at 20 mM concentration in PBS. Fibroblasts (once they reached 70% confluence) and lymphoblastoid cells (2–4 × 10⁶ cells in 10–20 ml of culture media) were both treated with 20 µM EGCG added to the culture medium for 24 h. Since it has been demonstrated that in culture condition EGCG could induce apoptosis and produce itself ROS in a dose-dependent manner [25], dose- and time-response studies were performed to verify that the treatment with 20 µM EGCG did not affect viability of both control and DS cells and did not induce release of hydrogen peroxide (H₂O₂) in the culture medium (see Supplementary Fig. S1). For time-dependent experiments, the incubation medium containing EGCG was changed every 24 h and freshly prepared EGCG was added for 72 h-time period.

2.3. Measurement of mitochondrial ATP production rate

The rate of ATP production by oxidative phosphorylation was determined in digitonin-permeabilized cells, essentially as previously described [8]. Briefly, aliquots of trypsinized fibroblasts or floating lymphoblastoid cells (0.5 mg protein), washed with PBS, were incubated at 37 °C in 2 ml of respiratory medium consisting of 210 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 5 mM KH₂PO₄/K₂HPO₄ (pH 7.4), 3 mM MgCl₂ in the presence of the ATP detecting system (ATP-ds) consisting of glucose (2.5 mM), hexokinase (HK) (2 e.u.), glucose 6-phosphate dehydrogenase (G6P-DH) (1 e.u.) and NADP⁺ (0.25 mM) in the presence of rotenone (3 µM) and succinate (5 mM) as energy source, plus 10 µM diadenosine pentaphosphate (Ap5A), used to specifically inhibit adenylate kinase [26]. After 5 min of incubation with 0.01% digitonin, the reduction of NADP⁺ in the extramitochondrial phase, which reveals ATP formation from externally added ADP (0.5 mM), was monitored as an increase in absorbance at 340 nm. Care was taken to use enough HK/G6P-DH coupled enzymes to ensure a non-limiting ADP-regenerating system for the measurement of ATP production.

2.4. Measurement of cellular ATP content

Fibroblasts were detached from plate by trypsin treatment, washed with PBS and cellular ATP was extracted by using the boiling water procedure as described in [27]. The amount of intracellular ATP was determined enzymatically in the extracts as described in [8].

2.5. Measurement of mitochondrial complex I and complex V activities

Measurements of complex I and complex V activities were carried out in mitochondrial membrane-enriched fractions from both cultured fibroblast and lymphoblastoid cells. Aliquots of trypsinized fibroblasts or floating lymphoblastoid cells were washed with ice-cold PBS, frozen in liquid nitrogen and kept at –80 °C until use. For isolation of mitochondrial membrane-enriched fractions, the cell pellets were thawed at 2–4 °C, suspended in 1 ml of 10 mM Tris–HCl (pH 7.5), supplemented with 1 mg/ml BSA, and exposed to ultrasound energy for 15 s at 0 °C. The ultrasound-treated cells were centrifuged (10 min at 600 g, 4 °C). The supernatant was collected and centrifuged again (10 min at 14,000 g, 4 °C) to obtain a mitochondrial pellet that was suspended in 0.1 ml of the respiratory medium. The activity of NADH:ubiquinone oxidoreductase (complex I) and ATPase-dependent ATP hydrolase (complex V) was measured in the same assay with sequential addition of reagents, essentially as described in [28].

2.6. ROS detection

ROS were visualized in live fibroblasts by using laser scanning confocal microscopy imaging. Cells were cultured at low density on

fibronectin-coated 35-mm glass-bottom dishes and incubated for 20 min at 37 °C with 3 μ M MitoSOX™ (Molecular Probes), a selective mitochondria-targeted probe specific for superoxide anion [29]. After washing with PBS, stained cells were examined under a Leica TCS SP5 II microscope (images collected using a 60 \times objective). The red fluorescence of MitoSOX was analyzed by exciting the sample with a HeNe laser 543 (λ_{ex} 543 nm).

Quantitative analysis of intracellular ROS was performed by means of an LS50 Perkin Elmer spectrofluorimeter using 2',7'-dichlorofluorescein diacetate (DCFH-DA), a non-fluorescent dye which is hydrolyzed in cells and reacts with multiple types of ROS, mainly H₂O₂, to give the fluorescent product, dichlorofluorescein (DCF) [30]. Cultured cells were incubated with 5 μ M DCFH-DA for 30 min in growth conditions, washed and suspended in PBS. Fluorescence emission was recorded at λ_{ex} 488 nm and λ_{em} 520 nm and normalized to the protein content to determine the relative ROS production.

To measure the pro-oxidant dose–response of EGCG, 1 h after the treatment with different concentrations of EGCG, culture media were collected and the amount of H₂O₂ in the culture medium was analyzed using Amplex® Red Hydrogen Peroxide/Peroxidase Assay kit (Molecular Probes) (see Supplementary Fig. S1).

2.7. Assessment of lipid peroxidation

Cis-parinaric acid fluorescence was used to determine the chemical process of lipid peroxidation of membranes. Cis-parinaric acid, a fatty acid that contains 4 conjugated double bonds that render it naturally fluorescent, is attacked during lipid peroxidation reactions. Accordingly, cis-parinaric acid fluorescence is quenched in lipid peroxidation reactions [31]. Fibroblasts (100 μ g protein) were suspended into 2 mL of PBS that contained cis-parinaric acid (5 μ M; Molecular Probes), and incubated in darkness at 37 °C for 30 min. Afterward, fluorescence at λ_{ex} 318 nm and λ_{em} 410 nm was recorded and normalized to the protein content to determine the chemical process of lipid peroxidation. The more lipid peroxidation that occurs, the less fluorescence is detected.

2.8. Measurement of MnSOD and Cu/ZnSOD activity

MnSOD and Cu/ZnSOD activities were determined by using a native-gel activity-stain [32]. Lymphoblastoid cells were collected by centrifugation and resuspended in PBS in the presence of the protease inhibitor phenylmethanesulfonylfluoride (PMSF). Total cellular protein extracts were prepared by sonicating cell suspensions on ice. Equal amounts of protein were separated by native gel-electrophoresis, and SOD activity assayed by incubating the gel with nitroblue tetrazolium. MnSOD was distinguished from cyanide-sensitive Cu/ZnSOD, by the addition of 2 mM cyanide. Band intensity relative to the in situ activity staining gels was calculated densitometrically using Quantity One-4.4.1 imaging software (Bio-Rad Laboratories). Immunoblot analysis of the same samples with antibody against actin was used to verify equal loading.

2.9. Measurements of cellular cAMP levels and PKA activity

Cellular cAMP levels were measured in cell extracts with cAMP ELISA Kit from Stressgen, following the manufacturer's instructions.

The activity of PKA was measured in cell extracts with the cAMP-dependent PKA Assay System (Stressgen), as recommended by the manufacturer.

2.10. Immunoblot analysis

Cells were lysed with 0.1% Triton in PBS in the presence of a protease inhibitor cocktail (Sigma-Aldrich). Cell lysate (0.05 mg protein) was resolved by a 10%–12% SDS-polyacrylamide gel (depending on the molecular weight) and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked by TBS-T (50 mM

Tris, 150 mM NaCl, 0.02% Tween 20, pH 7.5) containing 5% non-fat milk and probed with primary antibodies overnight at 4 °C (see Supplementary Table 1). Immunoblot analysis was performed, essentially as described in [33], using horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies and enhanced chemiluminescence Western blotting reagents (Amersham, Pharmacia Biotech). Membranes were also probed with anti- β -actin antibody as internal loading control and densitometry value of immunoreactive bands for each sample was normalized versus the corresponding densitometry value of β -actin.

2.11. Quantitative analysis of mtDNA content

Total genomic DNA was extracted from normal and DS cell lines using the NucleoSpin kit (Macherey–Nagel) and quantitative real-time PCR reactions were performed by using the Applied Biosystems™ 7900HT and the SYBR-Green PCR Master Mix (Qiagen). Primers for two mtDNA sequences, D-loop and cytochrome c oxidase II (COX II), and for the nuclear DNA sequence of β -actin were used, as described in [8]. Triplicate reactions were performed for each marker in a 96-well plate using a two-step amplification program consisting in an initial denaturation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 20 s and 61 °C for 30 s. Standard curves were generated from each experimental plate using a serial 5-fold dilutions of genomic DNA.

2.12. Immunoprecipitation of PGC-1 α

Lymphoblastoid cells (0.5 mg protein) were sonicated and 350 μ g of protein was incubated for 12 h at 4 °C in 700 μ l of RIPA buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl, 0.1% SDS, 1% Triton X-100, pH 7.4, in the presence of 2 μ g of antibody against PGC-1 α (Millipore). After 12 hour incubation, 40 mg of protein A-sepharose was added to the mixture and the immunocomplex was pelleted by centrifugation and washed with RIPA buffer supplemented with the protease inhibitor cocktail. To remove the protein A-sepharose-antibody complex, the pellet was suspended in 100 mM glycine, pH 2.5 and then centrifuged at 600 \times g for 5 min at 4 °C. The supernatant containing the released PGC-1 α protein was finally collected, separated by SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with antibody against PGC-1 α and Acetyl-Lysine.

2.13. Measurement of NAD⁺/NADH ratio

NAD⁺ and NADH levels were measured in untreated and EGCG-treated cell extracts prepared from 1 to 3 \times 10⁶ cells by using NAD⁺/NADH Assay Kit from Abcam, following the manufacturer's instructions. After NADH developer addition, OD_{450 nm} was read 1 h after incubation.

2.14. Transmission electron microscopy

24 h after EGCG treatment, DS and control fibroblasts were collected by centrifugation. Cell pellets were then washed in PBS and fixed as previously described [34]. Ultrathin sections were placed on Formvar carbon-coated copper grids, stained with uranyl acetate and lead citrate and observed under a Jeol 100 SX transmission electron microscope (Jeol, Ltd, Japan). The number of total and RER-surrounded mitochondria was quantified in 20 cells of similar size and nucleo/cytoplasm ratio. Two independent samples were analyzed for both DS patients and controls.

2.15. Statistical analysis

All data are expressed as mean \pm standard deviation (SD). Statistical evaluation of the differential analysis was performed by one way ANOVA and Student's *t*-test. The threshold for statistical significance was set at *P* < 0.01.

3. Results

3.1. EGCG restores oxidative phosphorylation deficit in human DS cells

In the light of our previous studies showing a deficit of mitochondrial ATP synthase and complex I activities in DS cells, ascribed to depression of post-translational cAMP/PKA-mediated phosphorylation [8,9], and since EGCG is reported to be an inhibitor of phosphodiesterases [20] as well as an activator of adenylate cyclase [21], we investigated whether EGCG was able to prevent mitochondrial energy impairment in DS cells.

First we checked the optimal conditions for EGCG treatment (see Supplementary Fig. S1) and found that exposure of both DS fibroblast and lymphoblastoid cells to 20 μ M EGCG for 24 h, almost completely restored the deficit of mitochondrial ATPase (complex V) activity (Fig. 1A and B) and completely prevented the decline of complex I activity (Fig. 1C and D). No significant difference in these activities was found between untreated and EGCG-treated control cells (not shown). EGCG concentrations lower than 20 μ M did not completely prevent this impairment and no changes in EGCG effects were observed extending cell exposure to 20 μ M EGCG for 72-h period (not shown).

To investigate whether EGCG could improve energy production in DS cells, we measured both ATP synthesized via mitochondrial oxidative phosphorylation (OXPHOS) and the total cellular levels of ATP (Fig. 2). Treatment of both fibroblast and lymphoblastoid DS cells with 20 μ M EGCG for 24 h, significantly prevented the impairment of mitochondrial ATP synthesis (Fig. 2A and B). EGCG concentrations lower than 20 μ M were not fully effective in the prevention of mitochondrial energy impairment in DS cells (Fig. 2C). Consistent with a

compensatory enhancement of glycolysis in DS cells [8], in a glucose growth medium the cellular level of ATP was only slightly reduced in DS compared with normal cells (Fig. 2D); conversely, ATP content was drastically decreased when DS cells were grown in a galactose medium in the absence of glucose, a condition in which ATP is essentially produced by mitochondrial OXPHOS [8]. EGCG treatment conferred to DS cells the capability to maintain ATP levels in a galactose medium in the absence of glucose (Fig. 2D).

3.2. Activation of cAMP/PKA pathway by EGCG

The possible involvement of cAMP/PKA pathway in the capability of EGCG to restore complex I deficit in DS cells was then investigated. Measurements of both cellular cAMP pool (Fig. 3A) and PKA activity (Fig. 3B), as a function of EGCG incubation time, was analyzed in DS cells. As in fibroblasts [9], also in lymphoblastoid cells both cAMP basal levels and PKA activity were found lower in DS cell than in control. 6 h after incubation, both cAMP levels and PKA activity increased by about 50% in EGCG-treated DS cells and both remained higher with respect to untreated DS cells, although with a decreasing trend of cAMP levels during incubation time. No significant difference was found between untreated and EGCG-treated control cells (not shown).

It has been reported that cAMP/PKA pathway activation in cell cultures is associated with increase in the phosphorylation level of the NDUFS4 complex I subunit and complex I activity [35,36]. Analysis of the NDUFS4 phosphorylation level by a specific antibody for the phosphorylated C-terminus of NDUFS4 protein showed a reduced NDUFS4 phosphorylation level in DS cells with respect to normal cells (Fig. 3C and D). 6 h-EGCG treatment promoted the expression

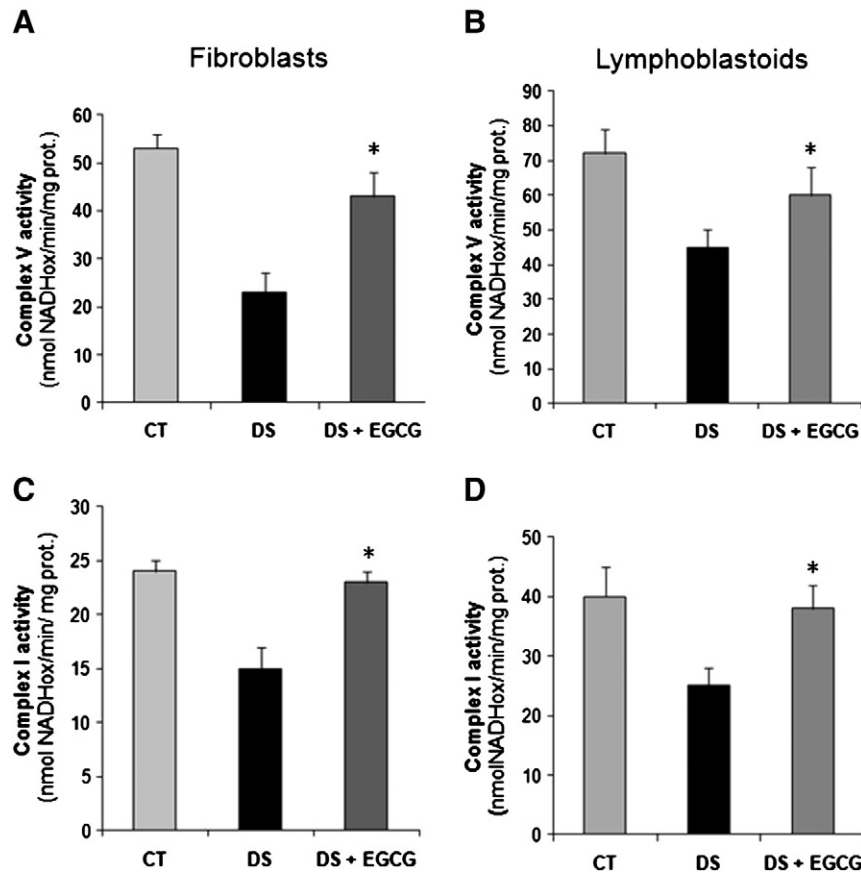


Fig. 1. Effect of EGCG on complex I and V activities in DS fibroblast and lymphoblastoid cells. (A and B) The activity of the ATPase-dependent ATP hydrolysis (complex V) and (C and D) the activity of the complex I of the mitochondrial respiratory chain were measured spectrophotometrically at 340 nm at 37 °C in 1 ml of respiratory medium in mitochondrial membrane-enriched fractions (0.1 mg protein) from fibroblasts and lymphoblastoids. Data are reported as the mean values (\pm S.E.) from three different experiments on six DS and five CT samples from different subjects. Significant differences between untreated and treated cells are indicated with asterisks (* = $P < 0.01$).

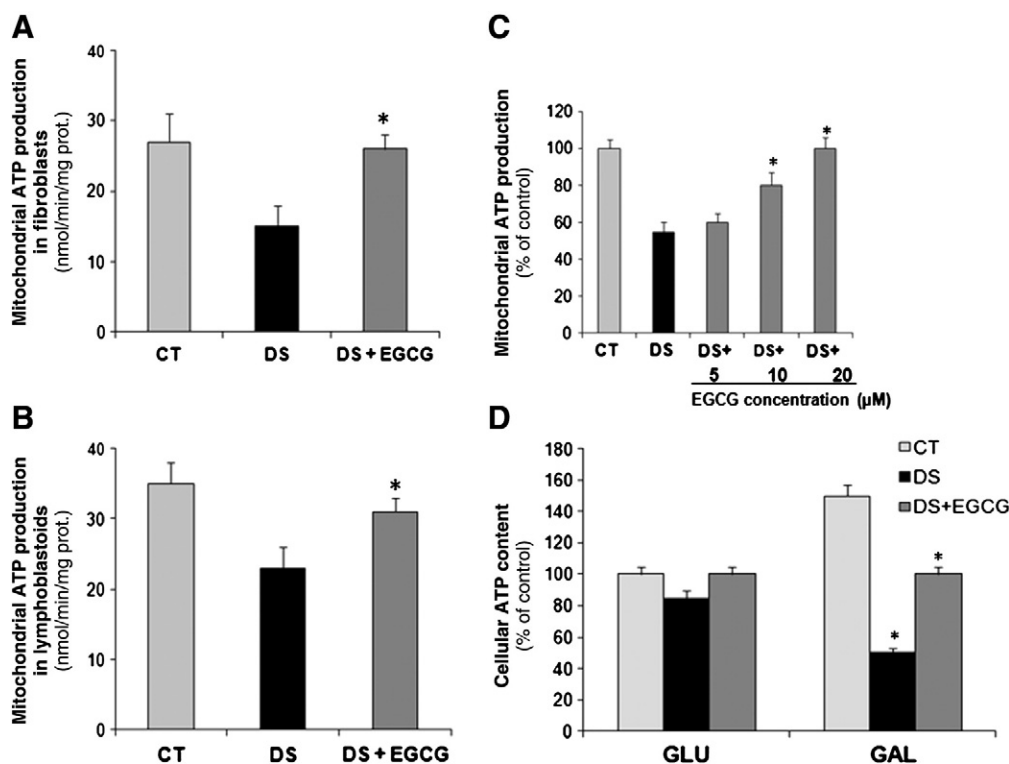


Fig. 2. Effect of EGCG on the rate of mitochondrial ATP synthesis and cellular ATP content in DS fibroblast and lymphoblastoid cells. (A and B) The rate of mitochondrial ATP synthesis was measured spectrophotometrically at 340 nm at 37 °C in 2 ml of respiratory medium in live digitonin-permeabilized control (CT) and DS (A) fibroblast and (B) lymphoblastoid cells. Where indicated, DS cells were incubated for 24 h with 20 μM EGCG (DS + EGCG). (C) EGCG dose-dependence effect on mitochondrial ATP synthesis rate in fibroblasts. (D) EGCG effect on the steady state levels of ATP in DS fibroblasts. Cells were cultured for 48 h in a medium without glucose and supplemented with either 10 mM glucose (GLU) or 10 mM galactose (GAL). Where indicated, cells were incubated for 24 h with 20 μM EGCG. Cellular ATP content was measured as detailed in the [Materials and methods](#) section and reported as a percentage of that in control cells grown in the presence of 10 mM glucose. Data are reported as the mean values (\pm S.E.) from three different experiments on six DS and five CT samples. Significant differences between untreated and treated cells are indicated with asterisks (* = $P < 0.01$).

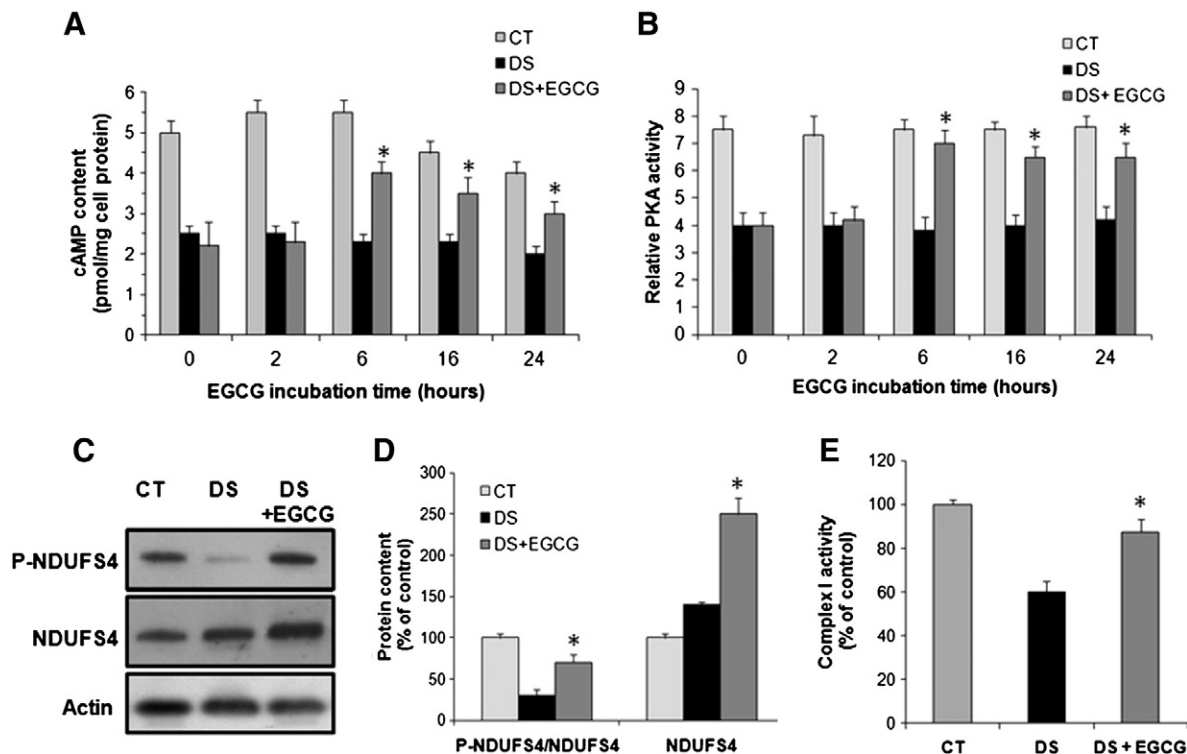


Fig. 3. EGCG modulates the cAMP/PKA signaling pathway. (A) Time-course measurement of cAMP cellular content and (B) PKA activity in cell lysate obtained from lymphoblastoid cells. Normal (CT) and DS cells were incubated with 20 μM EGCG and measurements made at the time-period indicated. (C) Representative immunoblotting and (D) densitometric analysis of protein level NDUFS4-complex I subunit (NDUFS4) and its phosphorylated form (P-NDUFS4) after 6 h of incubation with EGCG. The levels of NDUFS4 phosphorylation are calculated as ratio of P-NDUFS4/NDUFS4; (E) complex I activity measured at 6 h incubation. Values are reported as the means (\pm S.E.) of three independent experiments performed on five normal and six DS lymphoblastoid cell lines. Significant differences between untreated and treated cells are indicated with asterisks (* = $p < 0.01$).

of the NDUFS4 protein, stimulated its phosphorylation (Fig. 3C and D) and rescued complex I activity (Fig. 3E).

3.3. EGCG counteracts oxidative stress in human DS cells

To check the capability of EGCG to counteract oxidative stress, ROS production and lipid peroxidation, as well as the activities of certain antioxidant enzymes (MnSOD and Cu/ZnSOD), were measured in DS cells (Fig. 4). 24 h treatment with 20 μ M EGCG prevented superoxide anion overproduction by mitochondria, as revealed by confocal microscopy analysis carried out in DS fibroblasts by staining cells with the mitochondrial probe MitoSOX (Fig. 4A). EGCG also prevented the increase in H_2O_2 level in both DS fibroblast and lymphoblastoid cells, as observed by DCF fluorescence measurements (Fig. 4B and C).

When lipid peroxidation was assessed, a greater loss of *cis*-parinaric acid fluorescence (i.e. a higher membrane lipid peroxidation) was observed in DS fibroblasts with respect to normal cells (Fig. 4D). Consistent with the prevention of ROS accumulation by EGCG, lipid peroxidation was completely abolished by EGCG treatment.

Unlike *cis*-parinaric acid fluorescence, the activity of MnSOD and Cu/ZnSOD both higher in DS cells – particularly Cu/ZnSOD encoded

by chromosome 21 – did not disclose any change between untreated and EGCG-treated cells (Fig. 4E and F).

3.4. EGCG promotes mitochondrial biogenesis in human DS cells

To test whether the EGCG promoting effect on the activity of complexes I and V (see Fig. 2) was associated with changes in their content, the levels of some OXPHOS complex subunits were analyzed. A shared pattern of increase in the levels of subunits of all OXPHOS complexes was observed in DS fibroblasts and particularly in lymphoblastoid cells upon treatment with EGCG (Fig. 5A).

To verify if the general increase in the amount of OXPHOS proteins, found in DS cells treated with EGCG, was associated with an increased mitochondrial biogenesis, mtDNA content in DS and EGCG-treated DS cells compared with control cells was measured by real-time PCR. As shown in Fig. 5B and C, mtDNA, already higher in DS cells than in control, further increased (about 3-fold) in DS cells upon EGCG treatment. It can be noted that an increase of mtDNA content, albeit less extended (1.5-fold), was also found in EGCG-treated control cells.

To verify if the increase of both mitochondrial proteins and mtDNA promoted by EGCG was the consequence of the activation of key factors involved in mitochondrial biogenesis pathway, we measured in control,

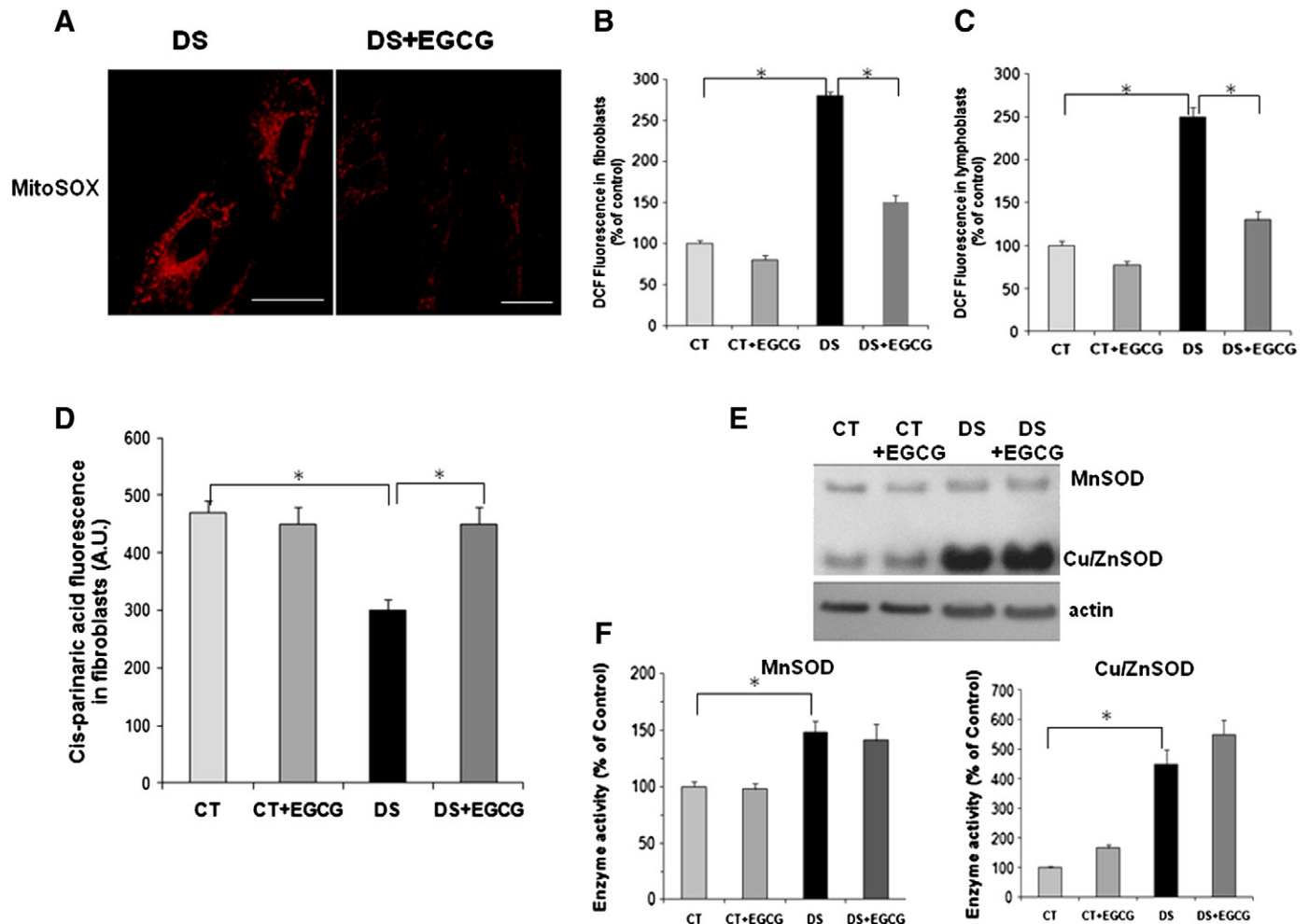


Fig. 4. Control of oxidative stress in human DS cells by EGCG. (A) Imaging by confocal microscopy of superoxide anion generation in DS fibroblasts. Cells were treated as described above and stained with 3 μ M MitoSOX while fluorescence of MitoSOX was visualized in live fibroblasts by laser scanning confocal microscopy. Images are superimpositions of 10 confocal z-planes and represent typical examples. Bars, 50 μ m. (B and C) Quantification of intracellular ROS was reported as DCF fluorescence arbitrary units (A.U.) measured by fluorimetric analysis in (B) fibroblasts and (C) lymphoblastoid cells. (D) Lipid peroxidation analysis in fibroblasts. Quantification of lipid peroxidation was reported as *cis*-parinaric acid fluorescence arbitrary units (A.U.) measured by fluorimetric analysis in fibroblasts. (E and F) Representative "in situ" SOD activity assay, determined by histochemical staining of a native-gel (E). Bar graph shows quantification of SOD positive bands by densitometric analysis. Data are expressed as percentage of CTRL values (F). Where indicated, control and DS cells were incubated for 24 h with 20 μ M EGCG (CT + EGCG and DS + EGCG, respectively). All data are reported as means (\pm SEM) of experiments performed in triplicate on five normal and six DS cell lines. Significant differences between untreated and treated cells are indicated with asterisks (* = $P < 0.01$).

DS and EGCG-treated lymphoblastoid cells the level of two transcription factors considered essential for the expression of OXPHOS genes in mammals: the mitochondrial transcription factor A (T-FAM), critical for the regulation of mitochondrial gene transcription and DNA replication, and the nuclear respiratory factor 1 (NRF-1), which is a positive regulator of T-FAM expression [37]. As shown in Fig. 5D and E, the level of both NRF-1 and T-FAM proteins, slightly higher in DS cells as compared with controls, further increased in EGCG-treated DS cells, indicating that an activation of mitochondrial biogenesis pathway by EGCG occurred. A slight but significant increase of the protein levels of these transcription factors was also found in EGCG-treated control cells.

We checked the morphology of mitochondria in EGCG-treated cells by Transmission Electron Microscopy (Fig. 6). Ultrastructural inspection of DS and control fibroblasts revealed that mitochondria, more abundant in EGCG-treated cells (see Table in Fig. 6), did not present morphological alterations or changes in size with respect to untreated cells. As a distinctive feature, DS and control fibroblasts showed an abundant rough endoplasmic reticulum (RER) characterized by expanded cisternae with a

roundish shape, studded with ribosomes. Interestingly, following EGCG treatment, both DS and control cells showed an almost full contraction of RER cisternae, which form large roundish shaped vesicles became stretched-out tubules with a significant reduction of organelle lumen. Interestingly, in EGCG treated cells, about 40% of total mitochondria established a strict connection with RER tubule, which appeared to surround mitochondria, a phenomenon only rarely observed in untreated fibroblasts.

3.5. EGCG promotes Sirt1 activity and PGC1 α activation in human DS cells

To investigate the molecular mechanism by which EGCG promotes mitochondrial biogenesis, we measured the protein level of the peroxisome proliferator-activated receptor- γ coactivator (PGC)-1 α , the master regulator of mitochondrial biogenesis [38,39], of the cAMP response element-binding (CREB) and phospho-CREB (P-CREB), which promotes the expression of PGC-1 α and of the NRF-1 transcription

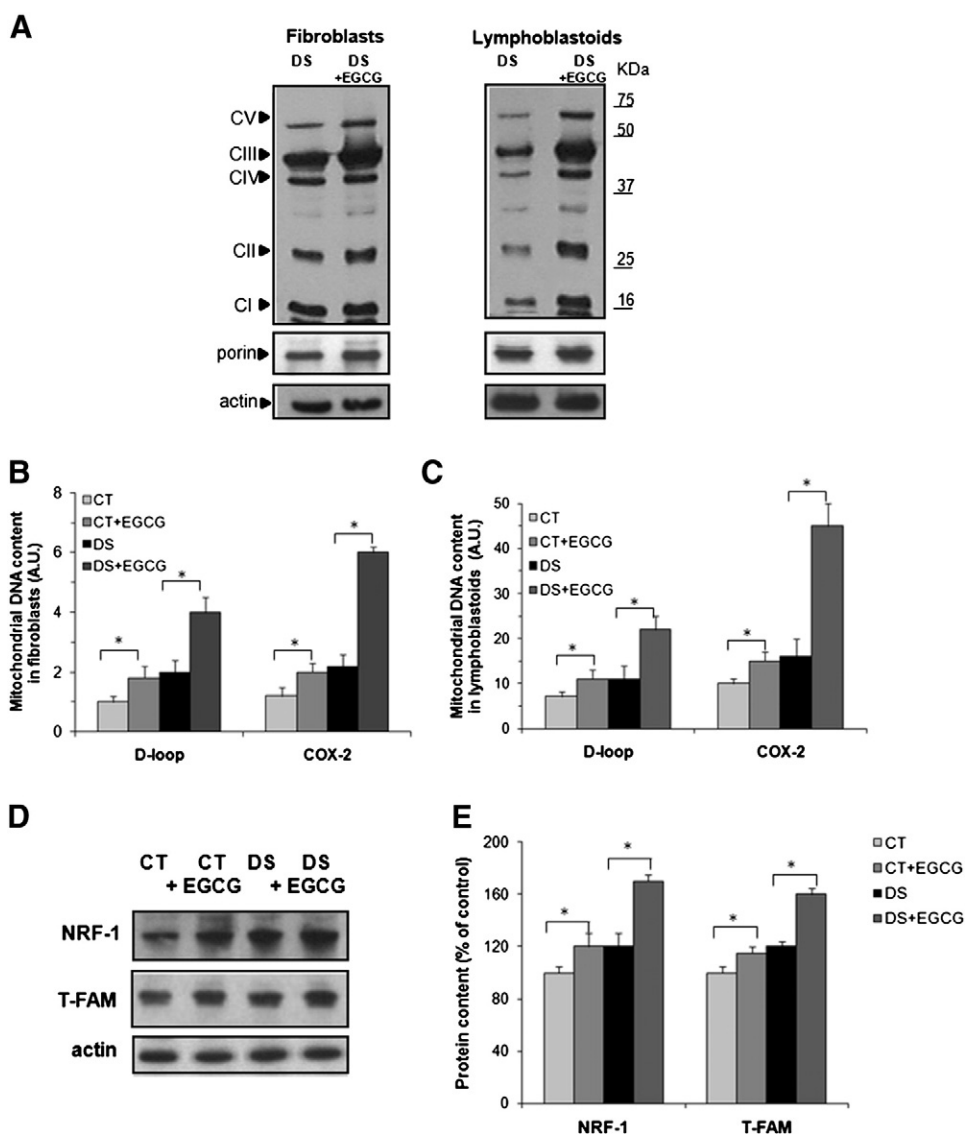


Fig. 5. EGCG promotes mitochondrial biogenesis in human DS cells. (A) Immunoblotting analysis of 20-kDa subunit of complex I (CI), 30-kDa subunit of complex II (CII), core 2 protein of complex III (CIII), COX I of complex IV (CIV) and α subunit of F_1 ATPase (CV) in untreated and treated normal (CT) and DS cells from both fibroblast and lymphoblastoid cell extracts (0.05 mg protein); protein levels of porin and β -actin as mitochondrial and cytosolic protein markers, respectively, were also analyzed. (B and C) mtDNA content was assayed using real-time PCR. Two mtDNA markers (D-loop and COX-2) were used. Values are the mean \pm S.E. of three independent experiments on five normal (CT) and six DS cell lines from both (B) fibroblast and (C) lymphoblastoid cell lines. (D) Representative immunoblotting and (E) densitometric analysis of protein levels of the nuclear respiratory factor 1 (NRF-1) and of the mitochondrial transcription factor A (T-FAM) measured in lymphoblastoid cell extracts (0.05 mg protein) using the respective antibodies. Where indicated, normal and DS cells were treated for 24 h with 20 μ M EGCG (CT + EGCG and DS + EGCG, respectively). Significant differences between groups are indicated with asterisks (* = $P < 0.01$).

factors [40,41], and of DYRK1A. DYRK1A is overproduced in DS subjects and can directly phosphorylate and activate CREB [42]. The amount of DYRK1A and P-CREB proteins was found significantly higher in DS as compared with control cells (Fig. 7A and B). Although DYRK1A protein level was not modified by EGCG treatment of DS cells, a significant decreased phosphorylation of CREB was found in the EGCG-treated DS cells in spite of no changes in CREB level. Interestingly, the level of PGC-1 α protein appeared to be lowered by EGCG treatment of DS cells. These findings indicate that activation of the mitochondrial biogenesis by EGCG does not occur through phosphorylation-dependent activation of CREB or promotion of PGC-1 α protein expression.

PGC-1 α activity can, on the other hand, be modulated by post-translational modifications. A key player in this respect is the NAD-dependent deacetylase Sirt1 [38]. In spite of no significant changes in Sirt1 and Sirt3 protein levels in untreated and EGCG-treated DS cells with respect to control cells (Fig. 7C and D), a significant decrease of acetylated histone 3 (AC-H3), a known downstream target of Sirt1 [43] was found in DS cells (approximately 2-fold with respect to normal cells) (Fig. 7E and F). Further decrease of AC-H3 was observed in EGCG-treated DS cells (4-fold with respect to untreated DS cells), an indirect evidence of increased Sirt1 deacetylase activity. A significant decrease of histone acetylation was also found in EGCG-treated control cells.

Consistently, analysis of the PGC-1 α acetylation level showed that PGC-1 α acetylation was lower (about 2-fold) in the PGC-1 α immunoprecipitated from DS cells as compared with normal cells (Fig. 7G and H). EGCG treatment resulted in further decrease in PGC-1 α acetylation both in control and in DS cells (about 3-fold with respect to untreated DS cells) (Fig. 7H).

The dependence of sirtuin-mediated deacetylation on NAD⁺ links this enzymatic activity to the energy status of the cell via the cellular NAD⁺:NADH ratio. Compatible with the activation of sirtuin-dependent deacetylation activity, a significant increase in NAD⁺ levels, as well as of the NAD⁺:NADH ratio, was observed in DS cells with respect to normal cells. NAD⁺ levels and NAD⁺:NADH ratio were further enhanced in EGCG-treated DS cells (Table 1).

4. Discussion

In the present study we tested whether and how EGCG can counteract mitochondrial energy deficit and oxidative stress in DS cells. Our investigation was based on the critical role of mitochondrial dysfunction in the pathogenesis of DS [6–9] and the distinctive features of EGCG as: i) a mitochondria-targeted drug [14,15], ii) a powerful antioxidant and modulator of cell signaling pathways (for Refs. see [18]), iii) an inhibitor of the chromosome 21-encoded DYRK1A, able to rescue brain defects induced by DYRK1A overexpression [22,24], iv) a molecule which can pass through both the blood–brain and feto-maternal placental barriers without side effects in humans [44–46]. EGCG was used in this study at a concentration of 20 μ M, which we directly verified to be safe for the cells being neither pro-oxidant nor cytotoxic even after prolonged exposure.

Cells with chromosome 21 trisomy, obtained from tissues of patients at different development stages, fetal fibroblasts and lymphoblastoids established from children, as well as fibroblasts from adults with DS, all show comparable mitochondrial alterations [see also 8,9]. This allows us to argue that dysfunction of mitochondrial bioenergetics in these cells is an inherent feature of DS and that designing drugs that can modulate mitochondrial functions is an attractive strategy for therapeutic gain [47].

It is shown here that the deficit of complex I and ATP synthase activities in DS cells results in depression of the rate of mitochondrial ATP synthesis, as well as in a marked depression of the cellular ATP content in a galactose cultivation medium, a condition in which ATP is essentially produced by mitochondrial OXPHOS. The present results show for the first time that EGCG treatment renews the capacity of DS cells to produce energy by mitochondrial OXPHOS by restoring the impaired activities of complex I and ATP synthase and the overall rate of mitochondrial ATP synthesis.

There is general consensus that a disturbance in the balance of ROS might play a crucial role in DS pathogenesis; a misbalance between the chromosome 21-encoded Cu/ZnSOD and glutathione peroxidase activity is believed to underlie the increased oxidative stress in DS [48]. We previously indicated mitochondria as the major cellular site responsible for the

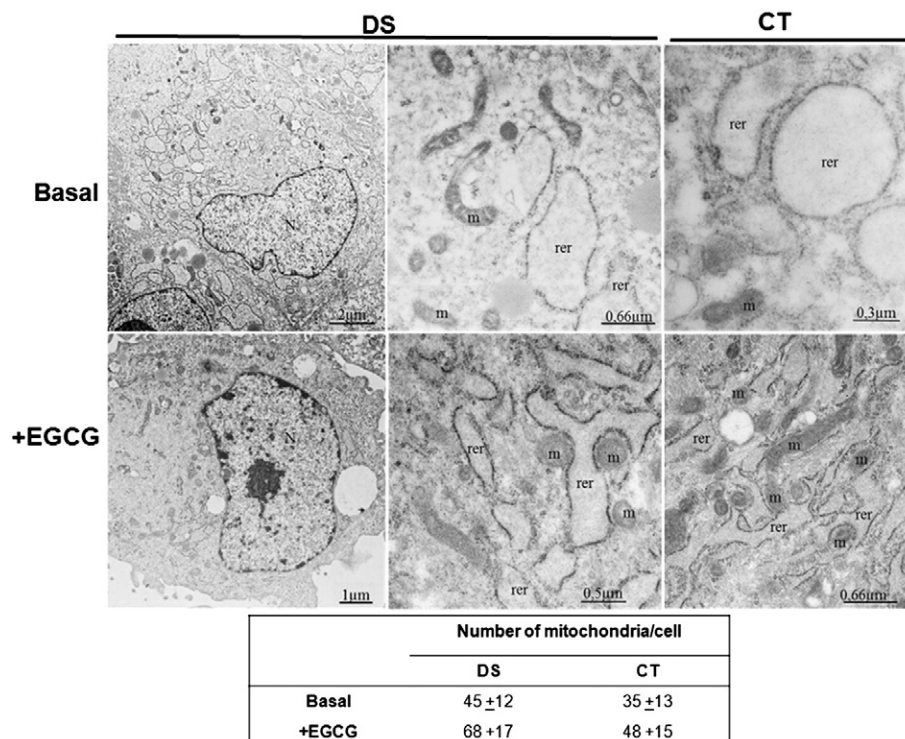


Fig. 6. Ultrastructural analysis of human DS and control fibroblast cells following EGCG treatment. Rough endoplasmic reticulum: rer; mitochondrion: m; nucleus: N.

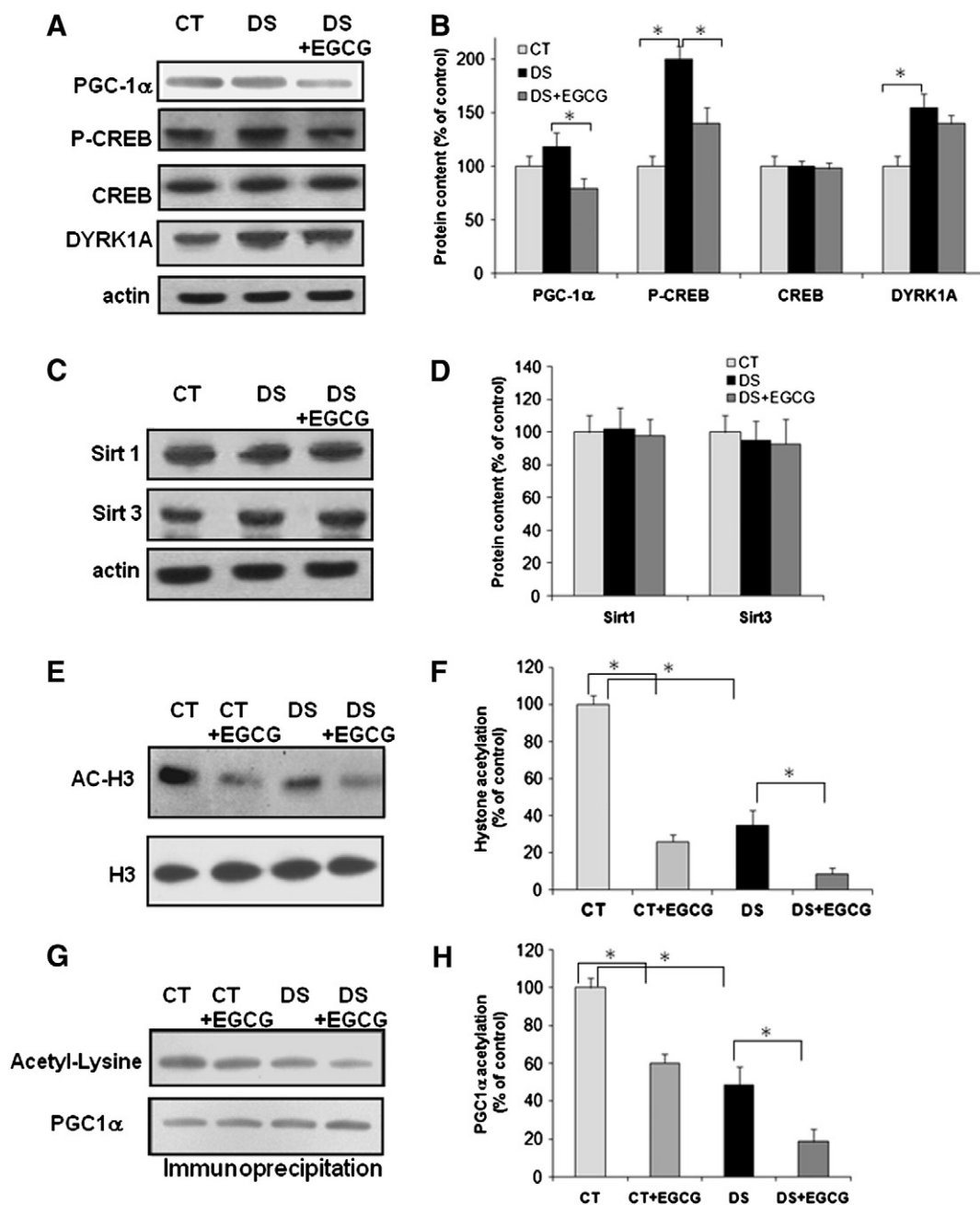


Fig. 7. EGCG modulates the Sir1/PGC1 α signaling pathway. (A) Representative immunoblotting and (B) densitometric analysis of protein levels of PGC-1 α , P-CREB, CREB and DYRK1A, measured in CT, DS and DS + EGCG lymphoblastoid cell extracts (0.05 mg protein) using the respective antibodies. (C) Representative immunoblotting and (D) densitometric analysis of protein levels of Sirt1 and Sirt3. (E) Representative immunoblotting and (F) densitometric analysis of acetylated histone 3 (AC-H3) and nonacetylated histone 3 (H3) measured in CT and DS untreated and EGCG-treated lymphoblastoid cell extracts. The levels of histone acetylation are calculated as ratio of AC-H3/H3. (G) Representative immunoblotting analysis and (H) densitometric analysis of acetylated-lysine of immunoprecipitated PGC-1 α from CT and DS untreated and EGCG-treated lymphoblastoid cells. The levels of PGC-1 α acetylation are calculated as ratio of acetylated-lysine vs immunoprecipitated PGC-1 α . Values are the mean (\pm S.E.) of three independent experiments on five normal and six DS samples from lymphoblastoid cell lines. Significant among groups are indicated with asterisks (* = $p < 0.01$).

Table 1
Changes in pyridine nucleotide metabolism in DS cells and after treatment with EGCG.

	CT	DS	DS + EGCG
NAD ⁺ (nmol/mg prot.)	1.3 \pm 0.2	2.7* \pm 0.2	4.4* \pm 0.2
NADH (nmol/mg prot.)	0.22 \pm 0.06	0.32 \pm 0.05	0.37 \pm 0.07
NAD ⁺ /NADH	5.9 \pm 0.5	8.4* \pm 0.4	11.9* \pm 0.6

NAD⁺ and NADH content were determined in both DS and control (CT) lymphoblastoid cells, incubated in the absence or in the presence of 20 μ M EGCG for 24 h, using NAD/NADH Assay Kit from Abcam. All values are means \pm S.E. of three different measurements on six DS and five CT cell lines. Significant differences between groups are indicated with asterisks (* = $p < 0.01$).

overproduction of ROS in DS cells [9]. The present data show that EGCG counteracts oxidative stress, not acting on the ROS scavenging enzyme activities, but preventing mitochondrial ROS overproduction by rescuing the efficiency of OXPHOS, in particular of complex I.

Alterations in the post-translational cAMP/PKA-mediated regulation of the catalytic activity of mitochondrial proteins involved in oxidative phosphorylation are involved in mitochondrial energy deficit in DS cells [8,9]. Given that EGCG has been found to be a powerful inhibitor of the activity of the cyclic nucleotide phosphodiesterase [20] and an activator of adenylyl cyclase [21], we tested the effect of EGCG on cAMP/PKA signaling pathway in DS cells. Our results show that EGCG

promotes in DS cells an increase of cAMP levels and PKA activity with respect to untreated cells, which results in increase in NDUFS4 phosphorylation, a subunit of complex I, and hence in complex I activation.

An effective strategy helping to minimize a damage attributable to mitochondrial energy impairment, is to increase the OXPHOS capacity and the number of mitochondria [49]. As known, mitochondrial biogenesis involves the integration of multiple transcriptional pathways controlling both nuclear and mitochondrial gene expression. The transcription factor NRF-1 plays a key role in integrating the transcription of nuclear- and mitochondrial-encoded genes [34]. NRF-1 target genes include subunits of OXPHOS complexes, assembly factors for the respiratory apparatus, components of mitochondrial protein import, and key mtDNA replication and transcription factors such as T-FAM [50]. The expression of NRF-1 is promoted by the coactivator PGC-1 α [39]. Increase of PGC-1 α activity, effected at both the expression and post-translational levels, results in increased mitochondrial mass and overall mitochondrial function [38]. In DS cells, as expected, we found an increase (about 50% with respect to normal cells) in the protein level of the chromosome 21 encoded DYRK1A, a protein kinase involved in several signal transduction pathways (for Refs. see [51]). It is known that DYRK1A phosphorylates and activates both CREB and Sirt1 in different cell types [42,52]. Consistently, the present results show an increase of P-CREB protein level and, interestingly, of sirtuin-dependent histone deacetylation activity and PGC-1 α deacetylation in DS cells under basal conditions. This could account for the increased mitochondrial biogenesis of DS cells, to partially compensate the mitochondrial energy deficit.

Recent studies have shown that exogenously supplied factors may be able to drive mitochondrial biogenesis by augmenting endogenous signaling responses [40,53]. The present study shows that in DS cells EGCG upregulates NRF-1 and T-FAM and strongly increases the level of OXPHOS proteins and mtDNA copy number. This promoting effect exerted by EGCG did not involve up-stream stimulation of CREB phosphorylation neither expression of PGC-1 α which were instead both depressed by EGCG. In addition, EGCG treatment in DS cells, although does not affect DYRK1A expression, as shown in a mouse model of DS [24], affects the activity of DYRK1A decreasing the phosphorylation of CREB, a downstream DYRK1A target. On the other hand, EGCG did induce a further increase of the histone deacetylation activity and PGC-1 α deacetylation in both DS and control cells. These results indicate that the EGCG inducing increase of NRF-1, T-FAM and OXPHOS proteins in DS cells can be due to sirtuin-catalyzed post-translational deacetylation of PGC-1 α and consequent promotion of its down-stream co-transcriptional activity.

The observed increase of cellular NAD⁺ levels induced by EGCG in DS cells, probably due to a stimulation of NAD⁺ biosynthesis [54], can be involved in the activation of sirtuin(s).

These findings are consistent with reports showing that polyphenols, like resveratrol and EGCG modulate Sirt1 activity [19,43]. Sirt1 activation can also occur through PKA-mediated phosphorylation [55]. Further work is in progress to verify whether the EGCG-dependent PKA activation correlates with promotion of Sirt1 activity.

It is interesting to note that, at ultrastructural level, EGCG treatment induces considerable changes in RER structure and organization and that several RER-mitochondria interconnection can be observed. As RER-mitochondria connections are directly involved in the regulation of mitochondrial membrane biosynthesis, genome replication, Ca²⁺ signaling and protein import [56,57] our data further indicate that EGCG protects DS cells ameliorating their mitochondrial biology. It is interesting to note that the same structural reorganization occurs also in normal cells substantiating the positive effects of this green tea extract on cell metabolism.

In conclusion, the present report gives new indication on molecular mechanisms occurring in DS cells leading to compensative events towards the energy deficit and identifies a novel activity of EGCG to counteract mitochondrial energy deficit in DS. In particular, EGCG,

stimulating both cAMP/PKA and Sirt1/PGC1 α signaling pathways, is able to restore oxidative phosphorylation capacity and promote mitochondrial biogenesis in fibroblast and lymphoblastoid cells obtained from DS subject. These findings provide a useful background for in vivo studies and suggest that EGCG, through the promoting effect on mitochondrial bioenergetic functions in DS cells, could contribute to the therapeutic treatment of this disease.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbdis.2012.12.011>.

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