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Regulation of the biogenesis of OXPHOS complexes in cell transition from replicating to quiescent state
Involvement of PKA and effect of hydroxytyrosol

Anna Signorile [a,*,1], Loris Micelli [a,1], Domenico De Rasmo [b], Arcangela Santeramo [a], Francesco Papa [a], Romina Ficarella [a], Giuliano Gattoni [a], Salvatore Scacco [a], Sergio Papa [a,b]

[a] Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari "Aldo Moro", Bari 70124,Italy
[b] Institute of Biomembrane and Bioenergetics, CNR, Bari, Italy

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ABSTRACT

A study is presented on the expression of mitochondrial oxidative phosphorylation complexes in exponentially growing and serum-starved, quiescent human fibroblast cultures. The functional levels of respiratory complexes I and III and complex V (adenosine triphosphate (ATP) synthase) were found to be severely depressed in serum-starved fibroblasts. The depression of oxidative phosphorylation system (OXPHOS) complexes was associated with reduced levels of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and the down-stream nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factors (TFAM). In serum-starved fibroblasts decrease of the catalytic activity of AMP cyclic dependent protein kinase (PKA) and phosphorylation of cAMP response element-binding protein (CREB), the transcription coactivator of the PGC-1α gene, was found. Hydroxytyrosol prevented the decline in the expression of the PGC-1α transcription cascade of OXPHOS complexes in serum-starved fibroblast cultures. The positive effect of HT was associated with activation of PKA and CREB phosphorylation. These results show involvement of PKA, CREB and PGC-1α in the regulation of OXPHOS in cell transition from the replicating to the quiescent state.

1. Introduction

Different types of human somatic cells, like fibroblasts and lymphocytes, cycle through reversible quiescent and proliferative phases [1]. It is of central relevance to determine the functional state at which housekeeping metabolic processes are set up in the quiescent state, so to preserve cell integrity, viability and the capacity to re-enter the replicative phase when cells are induced to do so. In-vitro fibroblast cultures are widely used to study cell exit into a reversible quiescent state. For this purpose fibroblast cultures, in the exponentially growing phase, can be brought in the quiescent phase by serum withdrawal from the cultivation medium and subsequently reintroduced in the replicating phase by adding back the serum [2]. If fibroblast culture permanence in the quiescent state is prolonged for too many days, the capacity to re-enter proliferation is, however lost [3].

Previous studies from our laboratory have revealed a marked depression of complex I (NADH-ubiquinone oxidoreductase) of the mitochondrial respiratory chain, and increase in the cellular level of reactive oxygen species (ROS), when human and murine fibroblasts, as well as different cancer cells are brought from the exponential growth phase to the quiescent phase by serum starvation [4–6]. Results of recent investigations have shown that the cAMP/PKA system regulates the functional capacity of respiratory chain complexes in mammalian cells, both at post-translational [7–10] and transcriptional level [6,11,12]. cAMP produced in the cytosol by the plasma membrane adenylyl cyclase, and that produced in the mitochondrial matrix by the bicarbonate-activated, soluble adenylyl cyclase (sAC) [8,13], contribute to these effects. All of the factors responsible for reversible cAMP-dependent protein phosphorylation, such as AKAP, PKA [14,15], protein phosphatase(s) [16], sAC [8], and cAMP-phosphodiesterase [17] are also present in the inner mitochondrial compartment.

In the present work we have analyzed the activity and the expression level of mitochondrial oxidative phosphorylation (OXPHOS) complexes in exponentially growing and serum-starved, quiescent fibroblast cultures. The functional level of respiratory complexes I and III and complex V (ATP synthase) was severely depressed in serum-starved fibroblasts, with a drop in the rate of ATP production by OXPHOS, but no change in

Abbreviations: AKAP, A-kinase anchor proteins; Akt, protein kinase B; CREB, cAMP response element-binding protein; HT, hydroxytyrosol; NHDF-neo, neonatal normal human dermal fibroblasts; NRF1, nuclear respiratory factor 1; OXPHOS, oxidative phosphorylation system; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PKA, AMP cyclic dependent protein kinase; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; sAC, soluble adenylyl cyclase; SOD, superoxide dismutase; SP, exponentially growing; SS, serum-starved; TFAM, mitochondrial transcription factor

* Corresponding author at: Department of Basic Medical Sciences, Neurosciences and Sense Organs, University of Bari "Aldo Moro", Policlinico, P.zza G. Cesare, 11, 70124 Bari, Italy. Tel.: +39 080 5448516; fax: +39 080 5448538.
E-mail address: anna.signorile@uniba.it (A. Signorile).

[1] A. Signorile and L. Micelli have equally contributed to the present work.

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complex IV was observed. The depression of OXPHOS complexes was associated with reduced level of PGC-1α, the master transcription factor, as well as of the down-stream NRF1 and TFAM transcription factors of the OXPHOS biogenetic cascade [6,18]. In quiescent as compared to replicating fibroblasts depression of the catalytic activity of PKA and phosphorylation of CREB, the transcription coactivator of the *PGC-1α* gene was found.

It has recently been reported that hydroxytyrosol (HT), a phenol of olive oil, prolongs the duration of quiescence of human fibroblasts, in which the capacity to re-enter the proliferative condition is retained [3]. It has also been observed that HT promotes the PGC-1α mediated expression of OXPHOS complexes in adipocyte cultures [19]. We show here that HT prevents the depression in serum-starved fibroblast cultures of PKA, P-CREB and the PGC-1α transcription cascade of OXPHOS complexes. These results show involvement of PKA, CREB and PGC-1α in the regulation of OXPHOS in mammalian cell transition from the replicating to the quiescent state.

2. Material and methods

2.1. Cell cultures

Neonatal normal human dermal fibroblasts (NHDF-neo, Cambrex #CC-2509, East Rutherford, NJ, USA) were grown at 37 °C in the exponential phase in high glucose Dulbecco's modified Eagle's medium (DMEM, EuroClone) supplemented with 10% fetal bovine serum (FBS) this condition indicated as SP, plus 2 mM glutamine (Euroclone), 100 IU/ml penicillin (Euroclone) and 100 IU/ml streptomycin (Euroclone), 5% CO_2. In the present work a limited serum starvation of the fibroblast cultures was adopted: where indicated, once fibroblasts were at 70% confluence, the medium was replaced with DMEM with 0.5% FBS and cells cultivated for additional hours, this condition indicated as SS (for other details see [4]). Further specifications are given in the legends to figures.

NHDF-neo cells were harvested from Petri dishes with 0.05% trypsin, 0.02% EDTA and pelleted by centrifugation at 500 ×*g*. The pellet was resuspended in PBS plus 0.25 mM phenylmethylsulfonyl fluoride (PMSF).

Cell numbers were counted using an Automated Cell Counter (Scepter, Millipore). Cell population doubling times were calculated using the following equation: Td = 0.693 t / ln(Nt / N0) where t is time in hours, and Nt and N0 represent cell numbers at time t and the initial time, respectively.

Protein concentration was determined using a protein assay kit (Bio-Rad DC; Hercules, CA, USA).

2.2. H_2O_2 assay

H_2O_2 level was determined by the cell permeant probe 2′-7′-dichlorodihydrofluorescin diacetate (H_2DCFDA) [20]. Cells were incubated with 10 μM H_2DCFDA in DMEM in the dark at 37 °C for 20 min, collected by trypsinization, washed and resuspended in the assay buffer (100 mM potassium phosphate, pH 7.4, 2 mM $MgCl_2$). An aliquot was used for protein determination. The H_2O_2 dependent oxidation of the fluorescent probe (507 nm excitation and 530 nm emission wavelengths) was measured by a Jasco FP6200 spectrofluorimeter.

2.3. Measurement of MnSOD and Cu/ZnSOD activities

MnSOD and Cu/ZnSOD activities were determined by native gel activity-stain [21]. Fibroblasts 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 proteins were separated by native gel-electrophoresis, and SOD activity was assayed by incubating the gel with nitrobluetetrazolium. MnSOD was distinguished from cyanide-sensitive Cu/ZnSOD, by the addition of 2 mM cyanide. Band intensity relative to SOD activity was calculated densitometrically using Quantity One-4.4.1 imaging software (Bio-Rad Laboratories). Immunoblot analysis of the same samples with antibody against β-actin was used to verify equal loading.

2.4. Mitoplast preparation

NHDF-neo cells were collected by trypsinization, pelleted by centrifugation at 500 ×*g* and resuspended in phosphate-buffered saline, pH 7.4 (PBS). The cell suspension was exposed for 10 min on ice to 2 mg of digitonin/mg cellular proteins. The mitoplast fraction, obtained by digitonin cell disruption, was pelleted at 14,000 ×*g* and resuspended in PBS.

2.5. Enzymatic spectrophotometric assay

Mitoplasts were exposed to ultrasound energy for 15 s at 0 °C. The NADH-UQ oxidoreductase activity (complex I) was measured in 40 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, in the presence of 3 mM KCN, 1 μg/ml antimycin, 200 μM decylubiquinone, using 50 μg of mitoplast proteins, by following the oxidation of 100 μM NADH at 340–425 nm ($\Delta\varepsilon = 6.81\ mM^{-1}\ cm^{-1}$). The activity was corrected for the residual activity measured in the presence of 1 μg/ml rotenone (see also [4]).

Cytochrome c oxidase (complex IV) activity was measured by following the oxidation of 10 μM ferrocytochrome c at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$). Enzymatic activity was measured in 10 mM PBS, using 30 μg mitoplast proteins [22].

Succinate-cytochrome c oxidoreductase (complex II + III) activity was measured at 550–540 nm ($\Delta\varepsilon = 19.1\ mM^{-1}\ cm^{-1}$) as initial rate of cytochrome c reduction. Proteins (100 μg/ml) were incubated for 10 min in the assay buffer (25 mM potassium phosphate, pH 7.2, 5 mM $MgCl_2$) in the presence of 20 mM succinate, 3 μg/ml rotenone, 2 mM KCN and 65 mM decylubiquinone. The reaction, started by the addition of 20 μM cytochrome c, was corrected for the residual activity measured in the presence of 2 μg/ml antimycin A.

For citrate synthase activity, 20 μg mitoplast proteins, supplemented with 0.5 mM Acetyl CoA and 5′5′ dithiobis-2 nitrobenzoate (DTNB) were suspended in 100 mM Tris–HCl buffer, pH 8.0. The reaction was started by the addition of 0.5 mM oxaloacetate and initial rate was measured following the reduction of DTNB at 419 nm ($\Delta\varepsilon = 163\ mM^{-1}\ cm-^{1}$).

2.6. Measurement of mitochondrial ATP production rate

The rate of ATP production by oxidative phosphorylation was determined in digitonin-permeabilized cells, essentially as described in [23]. Briefly, aliquots of trypsinized fibroblasts, washed with PBS, were suspended in 1 ml of medium consisting of 210 mM mannitol, 70 mM sucrose, 20 mM Tris/HCl, 5 mM KH_2PO_4/K_2HPO_4, (pH 7.4), and 3 mM $MgCl_2$ in the presence of the ATP detecting system (ATP-ds) consisting of 2.5 mM glucose, 2 e.u hexokinase (HK), 1 e.u. glucose 6-phosphate dehydrogenase (G6P-DH) and 0.25 mM $NADP_+$ in the presence of 3 μM rotenone and 5 mM succinate as energy substrate, plus 10 μM diadenosinepentaphosphate (Ap5A), to inhibit adenylate kinase [24]. After 5 min of incubation with digitonin (30 μg/10^6 cells) at 37 °C, 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. The rate of ATP production by Complex V was corrected for the residual ATP production measured in the presence of 2 μg/mg protein oligomycin.