Biochimica et Biophysica Acta 1817 (2012) 1037–1043

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Compensatory upregulation of respiratory chain complexes III and IV in isolated deficiency of ATP synthase due to *TMEM70* mutation

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ARTICLE INFO

Article history:
Received 6 October 2011
Received in revised form 4 March 2012
Accepted 5 March 2012
Available online 10 March 2012

Keywords:
ATP synthase
TMEM70
Disease
Gene expression profiling
Oxidative phosphorylation
Mitochondrial biogenesis

ABSTRACT

Early onset mitochondrial encephalo-cardiomyopathy due to isolated deficiency of ATP synthase is frequently caused by mutations in *TMEM70* gene encoding enzyme-specific ancillary factor. Diminished ATP synthase results in low ATP production, elevated mitochondrial membrane potential and increased ROS production. To test whether the patient cells may react to metabolic disbalance by changes in oxidative phosphorylation system, we performed a quantitative analysis of respiratory chain complexes and intramitochondrial proteases involved in their turnover. SDS- and BN-PAGE Western blot analysis of fibroblasts from 10 patients with *TMEM70* 317-2A>G homozygous mutation showed a significant 82–89% decrease of ATP synthase and 50–162% increase of respiratory chain complex IV and 22–53% increase of complex III. The content of Lon protease, paraplegin and prohibitins 1 and 2 was not significantly changed. Whole genome expression profiling revealed a generalized upregulation of transcriptional activity, but did not show any consistent changes in mRNA levels of structural subunits, specific assembly factors of respiratory chain complexes, or in regulatory genes of mitochondrial biogenesis which would parallel the protein data. The mtDNA content in patient cells was also not changed. The results indicate involvement of posttranscriptional events in the adaptive regulation of mitochondrial biogenesis that allows for the compensatory increase of respiratory chain complexes III and IV in response to deficiency of ATP synthase.

1. Introduction

Isolated deficiency of ATP synthase belongs to autosomally transmitted mitochondrial diseases that typically affect paediatric population and present with early onset and often fatal outcome [1]. Nuclear genetic origin of ATP synthase deficiency was first demonstrated in 1999 [2] and up to now more than 30 cases have been diagnosed. Within the last few years, mutations in *ATP12* (*ATPAF2*) [3] and *TMEM70* [4] genes, encoding two ATP synthase ancillary factors have been identified as a cause of the disease. Most recently we have found that a mutation in *ATP5E* gene coding for ATP synthase F_1 epsilon subunit can also downregulate enzyme biogenesis resulting in a mitochondrial disease [5]. While *ATP12* and *ATP5E* mutations remain limited to one unique described case, mutations in *TMEM70* were present in numerous patients [4,6–9], thus representing the most frequent cause of ATP synthase deficiency. Up to now at least 8 different pathogenic mutations have been found in *TMEM70* gene [4,8–10]; however, most of the patients are homozygous for *TMEM70* 317-2A>G mutation thus forming a unique cohort of cases with an isolated defect of the key enzyme of mitochondrial ATP production, harboring an identical genetic defect.

TMEM70 is a 21 kDa mitochondrial protein of the inner mitochondrial membrane [11] synthesized as a 29 kDa precursor. It functions as an ancillary factor of mammalian ATP synthase biogenesis [12], and is uniquely specific for higher eukaryotes [4,13]. Its absence caused by the homozygous substitution in *TMEM70* gene (317-2A>G) results in an isolated decrease of the content of fully assembled ATP synthase and reduction of enzyme activity to less than 30% of control values. The clinical presentation of affected patients includes the early onset, lactic acidosis, frequent cardiomyopathy, variable CNS involvement and 3-methylglutaconic aciduria [1,2,4,14].

Diminished phosphorylating capacity of ATP synthase, with respect to respiratory chain capacity, results in low ATP production and insufficient discharge of mitochondrial proton gradient. Elevated levels of mitochondrial membrane potential ($\Delta\Psi_m$) thus stimulate mitochondrial ROS production and the overall metabolic disbalance is characterized by insufficient energy provision and increased oxidative stress in ATP synthase-deficient patient cells [1,15].

Abbreviations: OXPHOS, oxidative phosphorylation; ATP synthase, mitochondrial F_oF_1 ATPase; DDM, dodecyl maltoside; COX, cytochrome *c* oxidase; RT-PCR, real-time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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doi:10.1016/j.bbabio.2012.03.004

Assuming that these metabolic changes may influence the nucleo-mitochondrial signaling, in the present study we tested whether the patient cells may respond to ATP synthase deficiency and consequent metabolic disbalance by changes in biogenesis of mitochondrial OXPHOS system. To investigate possible compensatory/adaptive changes, we performed quantitative analysis of mitochondrial respiratory chain complexes I–V and intramitochondrial proteases (Lon protease, paraplegin, and prohibitins), quantified mtDNA content and compared the protein analysis data with the data from gene expression profiling analyses.

2. Materials and methods

2.1. Patients

Fibroblast cultures from 10 patients with isolated deficiency of ATP synthase (P4–P13 [4]) and 3 controls were used in this study. All the patients showed major clinical symptoms associated with ATP synthase deficiency and harbored a homozygous substitution 317-2A>G in gene *TMEM70* [4]. Relevant clinical, biochemical and molecular data on individual patients included in this study were described previously (see [2,4,6,14,16]).

2.2. Cell culture and isolation of mitochondria

Fibroblast cultures were established from skin biopsies and were grown at 37 °C in 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (DMEM, PAA) supplemented with 10% (v/v) fetal calf serum. When indicated, cultivation was also performed in DMEM without glucose (Sigma) that was supplemented with 5.5 mM galactose and 10% dialyzed fetal calf serum. Cells were harvested with 0.05% trypsin and 0.02% EDTA and washed twice with phosphate-buffered saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4). The protein content was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories), using BSA as standard.

Mitochondria were isolated as in [5] by hypotonic shock cell disruption [17].

2.3. mtDNA quantification

Genomic DNA was isolated by QIAamp DNA Mini kit (Qiagen). To quantify the mtDNA content, we selected two mitochondrial target sequences—16S rRNA and D-loop, and GAPDH as a nuclear target. RT-PCR (LightCycler 480 instrument, Roche Diagnostics) was performed with SYBR Green Master kit (Roche) using the following primers—16S (5′ -3′): F-CCAAACCCACTCCACCTTAC, R-TCATCTTTCCCTTGCGGTA; D-loop: F-CACCATCCTCCGTGAAATCAA, R-GCGAGGAGAGTAGCACTCTTGTG; GAPDH: F-TTCAACAGCGACACCCACT, R-CCAGCCACTACCAGGAAAT [18]. The mtDNA content was calculated from threshold cycle (C_T) ratio of C_{TmtDNA}/C_{TnDNA}.

2.4. Electrophoresis and immunoblot analysis

Tricine SDS polyacrylamide gel electrophoresis (SDS-PAGE) [19] was performed on 10% (w/v) polyacrylamide slab minigels (Mini Protean, Bio-Rad). The samples were incubated for 20 min at 40 °C in 2% (v/v) mercaptoethanol, 4% (w/v) SDS, 10 mM Tris-HCl (pH 7.0) and 10% (v/v) glycerol. Bis-Tris blue-native electrophoresis (BN-PAGE) was performed on 4–13% polyacrylamide minigels [20]. Fibroblasts were solubilized by dodecyl maltoside (DDM, 2 g/1 g of protein) for 15 min at 4 °C in 1.75 mM 6-aminohexanoic acid, 2 mM EDTA, 75 mM Bis-Tris (pH 7). Samples were centrifuged for 20 min at 30000 *g* and 4 °C, Coomassie Brilliant Blue G-250 (8:1, DDM:dye) and 5% glycerol were added to the supernatants and electrophoresis was run for 30 min at 45 V and then at 90 V at 4 °C.

The separated proteins were blotted onto PVDF membranes (Immobilon-P, Millipore) by semi-dry electro transfer for 1 h at 0.8 mA/cm^2. The membranes were blocked with 5% (w/v) non-fat milk in TBS, 0.1% (v/v) Tween-20 and then incubated for 2 h or overnight with subunit specific antibodies. We used monoclonal antibodies from Mitosciences against complex I (NDUFA9-MS111, NDUFS3-MS112), complex II (SDH70-MS204), complex III (Core1-MS303, Core2-MS304), complex IV (Cox1-MS404, Cox2-MS405, Cox4-MS408, Cox5a-MS409), complex V-ATP synthase (F1-β-MS503), and against porin (MSA03). For detection of proteases, the polyclonal antibodies to Lon (kindly provided by Dr. E. Kutejova), paraplegin (kindly provided by Dr. T. Langer), prohibitin 1 (Lab Vision/NeoMarkers) and prohibitin 2 (Bethyl, A300-657A) were used. Quantitative detection was performed using infrared IRDye®-labeled secondary antibodies (goat anti-mouse IgG, Alexa Fluor 680 (A21058) and goat anti-rabbit IgG, Alexa Fluor 680 (A21109), Invitrogen) and Odyssey Infrared Imager (Li-Cor); the signal was quantified by AIDA 3.21 Image Analyzer software (Raytest).

2.5. Gene expression analysis

RNA isolations and RNA quality control were performed as previously described [4]. Total RNA (500 ng) was reverse transcribed, labeled and hybridized onto Agilent 44 k human genome microarray using Two-color Microarray Based Gene Expression Analysis Kit (Agilent). Patient samples and controls (Cy5-labeled) were hybridized against common Cy3-labeled reference RNA isolated from HeLa cell lines. The hybridized slides were scanned with Agilent scanner with PMT gains adjusted to obtain highest intensity unsaturated images. Gene PixPro software (Axon Instruments) was used for image analysis of the TIFF files generated by the scanner. Comparative microarray analysis was performed according to MIAME guidelines [21]. Normalization was performed in R statistic environment (http://www.r-project.org) using Limma package [22], a part of Bioconductor project (http://www.bioconductor.org). Raw data from individual arrays were analyzed as one color data and processed using loess normalization and normexp background correction. Quantile was used for normalization between arrays. Linear model was fitted for each gene given a series of arrays using lmFit function. The empirical Bayes method was used to rank differential expression of genes using eBayes function. Multiple testing correction was performed using the method of Benjamini and Hochberg [23].

2.6. Data accession

Expression data reported in this study are stored and available in Gene Expression Omnibus repository under accessions GPL4133 and GSE10956.

2.7. Protein/transcript correlation

Gene expression signals were background corrected, log2 transformed and normalized using the quantile normalization method. Relative protein levels (ratio to porin) were mean centered, averaged and log2 transformed. For all possible pairs of genes and proteins, we calculated the Pearson correlation coefficient and its significance levels using correlation test function in R statistical language.

2.8. Ethics

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Medical Ethics at both collaborating institutions. The informed consent was obtained from parents.