Biochimica et Biophysica Acta 1822 (2012) 1216–1222

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Oxidative phosphorylation differences between mitochondrial DNA haplogroups modify the risk of Leber's hereditary optic neuropathy

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ARTICLE INFO

Article history:
Received 12 January 2012
Received in revised form 12 March 2012
Accepted 20 April 2012
Available online 26 April 2012

Keywords:
Leber's hereditary optic neuropathy
Oxidative phosphorylation
Mitochondrial DNA
Haplogroup
Cybrid

ABSTRACT

Leber's hereditary optic neuropathy is a maternally inherited optic atrophy caused by mitochondrial DNA point mutations. Previous epidemiological studies have shown that individuals from mitochondrial genetic backgrounds (haplogroups) J/Uk and H have a higher and a lower risk, respectively, of suffering this disorder. To analyze the bases of these associations at cellular and molecular levels, functional studies with cybrids provide high quality evidence. Cybrids from haplogroup J contain less mitochondrial deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and synthesize a smaller amount of mitochondrial DNA-encoded polypeptides than those from haplogroup H. Haplogroup J cybrids also display lower oxygen consumption, mitochondrial inner membrane potential and total adenosine-5′-triphosphate (ATP) levels. Moreover, mitochondrial DNA levels correlate with many parameters of the oxidative phosphorylation system. These results suggest that the mitochondrial DNA amount determines oxidative phosphorylation capacity and, along with other recently published observations, support the possibility that mitochondrial DNA levels may be responsible for the bias of the disorder toward males, for the incomplete penetrance of mutations causing Leber's hereditary optic neuropathy and for the association of the disease with particular mitochondrial DNA haplogroups.

1. Introduction

Leber's hereditary optic neuropathy (LHON, OMIM 535000) is the most frequent mitochondrial DNA (mtDNA) disease. The endpoint of the disease is an optic atrophy associated with loss of central vision. Most LHON patients harbor one of the three primary mtDNA point mutations in genes from respiratory complex I (CI): m.3460G>A/*MT-ND1*/p.MT-ND1:A52T, m.11778G>A/*MT-ND4*/p.MT-ND4:R340H and m.14484T>C/*MT-ND6*/p.MT-ND6:M64V. While the pathomechanisms for these mutations are beginning to unravel [1], why mainly males are affected with LHON and why many homoplasmic mutant maternal relatives do not develop the disease is still unknown.

Since the middle of the 1990s, different studies have found epidemiologic associations between LHON and particular phylogenetically related mtDNA genotypes, known as mtDNA haplogroups. In 1997, analyzing North American, German, Finish and Italian white patients, four different groups found that haplogroup J was overrepresented in LHON patients who harbored the pathologic mtDNA mutations m.11778G>A or m.14484T>C [2–5]. The risk of visual failure was also increased in haplogroup Uk patients who had m.3460G>A [6]. In contrast, haplogroup H appears to be a LHON resistance factor [7]. Combined, these results suggest that single nucleotide polymorphisms (SNPs) defining these haplogroups contribute to the pathologic phenotype.

Because it is impossible to control all environmental factors that affect humans, epidemiological studies inherently involve a heterogeneous subject pool. Moreover, nuclear DNA (nDNA) represents 99.9995% of the human genome and, therefore, many nuclear SNPs could affect the results of epidemiological studies on mtDNA. These disadvantages are only partially counteracted by increasing the sample size, and this is a difficult task for rare diseases such as LHON [8]. To overcome such problems, and analyze the bases of these associations at cellular and molecular levels, an appropriate model such as the transmitochondrial cell line, cytoplasmic hybrid or cybrid model [9] was required. Cybrid cell lines share the same nuclear genetic background and environmental conditions but differ in their mtDNA genotype. Using this approach, we have recently shown that mtDNA and mtRNA levels, mitochondrial protein synthesis, cytochrome oxidase activity and amount, normalized oxygen consumption, mitochondrial inner membrane potential and growth capacity are different between cybrids of haplogroup H and Uk [10].

Because haplogroups J/Uk and H are susceptibility and resistance factors, respectively, for LHON disease and that haplogroups H and Uk are different from a biochemical phenotype point of view, we used the cybrid model to investigate whether epidemiologic

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0925-4439/$ – see front matter
doi:10.1016/j.bbadis.2012.04.014

associations of haplogroup J were based on the same biochemical events as those of haplogroup Uk. Our results confirm that the mtDNA levels determine, in a large part, the oxidative phosphorylation (OXPHOS) capacity of the cell and that, if the lower amount of mtDNA in haplogroup J and Uk cybrids mirrors an individual's situation, then this lower amount might be responsible for higher susceptibilities of individuals from these haplogroups to develop LHON.

2. Materials and methods

2.1. Biological samples and growth conditions

After winning the approval of the Ethics Committee of the Government of Aragón (Acta n817/2008) and securing signed informed consent from the healthy volunteers, blood was obtained, and platelets of five and nine individuals from mtDNA haplogroups H and J, respectively, were used to build cybrids with the osteosarcoma 143B TK$^-$ rho^0 206 nuclear background from Attardi's laboratory.

Experiments were performed with cell lines grown in Dulbecco's modified eagle medium (DMEM) containing glucose (4.5 g/l), pyruvate (0.11 g/l) and fetal bovine serum (FBS) (5%) without antibiotics. When cells were grown with an antioxidant, 5 mM of N-acetyl cysteine (NAC) was used in the culture medium.

2.2. Molecular–genetic analysis

Samples from the volunteers were genetically characterized by performing PCR-RFLP for mitochondrial haplogroup-defining SNPs in the coding region and sequencing the hypervariable regions I and II. The complete mtDNA sequences were obtained by using the BigDye Terminator v 3.1 Cycle Sequencing Kit (Applera Rockville, MD, USA) and an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). The mtDNA content was measured by the qPCR method using an Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block (Applied Biosystems). The mtDNA levels were determined in triplicate in three to six independent experiments [10].

To assess the mRNA levels, total RNA was isolated from exponentially growing cells using a RNA isolation kit (NucleoSpin® RNA II) from Macherey–Nagel according to the manufacturer's protocol; 2.5 μg of total RNA was reversed-transcribed into cDNA with a high capacity cDNA reverse transcription kit (Applied Biosystems), using the manufacturer's conditions. The mRNA levels of manganese superoxide dismutase (MnSOD), 6-phosphofructo-1-kinase-L (PFKL) and mtRNAs were determined in triplicate in two independent experiments by RTqPCR using the One-Step Real-Time system (Applied Biosystems). The mRNA levels of mitochondrial DNA polymerase subunit B (POLG2), TWINKLE helicase, single stranded DNA-binding protein (mtSSB), mitochondrial RNA polymerase (POLRMT), mitochondrial transcription factor B2 (TFB2M) and mitochondrial termination factor (MTERF) were determined using probes described previously [11]. The expression levels were normalized using the 18S rRNA reference gene. The comparative *Cq* method was used for relative quantification of gene expression as described by the real-time PCR machine manual. Differences in the *Cq* values (d*Cq*) of the transcript of interest and the reference gene were used to determine the relative expression of the gene in each sample. The d*Cq* method was used to calculate fold expression. StepOne software version 2.0 (Applied Biosystems) was used for data analysis [10].

2.3. MtDNA-encoded protein synthesis

The mitochondrial protein synthesis was analyzed as previously described with minor modifications [10]. Electrophoresis was performed with a Protean II xi system (BIORAD). As a load control, we dyed the gel for 15 min with fixing solution (30% methanol, 10% acetic acid) plus 0.025% of Brilliant Blue R (Coomassie Blue) (Sigma). Then, the gel was washed several times with a 50% methanol, 10% acetic acid solution and left overnight in a fixing solution. Finally, it was treated for 20 min with the Amplify solution (AMERSHAM), dried and used for autoradiography. The band intensities from appropriate exposures of the fluorogram from at least two independent gels were quantified by densitometric analysis with the Gelpro analyzer v 4.0. Three bands, corresponding to p.MT-CO1, p.MT-ND2 and p.MT-ND3 (upper, middle and lower parts of the gel, respectively) polypeptides were selected to quantification.

2.4. Oxygen consumption and respiratory complex activities and levels

Oxygen consumption was analyzed using the high-resolution oxygraph OROBOROS®. To reduce experimental manipulation, such as cell permeabilization, and its effects on the final results, intact cells were used. Exponentially growing cells were collected by trypsinization, washed, counted and resuspended at 1.5×10^6 cells/ml. Endogenous, leaking (with oligomycin added at 49 nM) and uncoupled (with FCCP added at 1.2 μM) respiration analyses were performed. To correct for the oxygen consumption that is not due to an electron transport chain (ETC), respiration inhibition by KCN was performed. Each cell line was measured three to four times in DMEM glucose. Respiration was measured at 37 °C with chamber volumes set at 2 ml. The software DatLab (Oroboros Instrument, Innsbruck, Austria) was used for data acquisition (1 s time intervals) and analysis [10].

Mitoprofile® Human Complex IV Activity and Quantity from Mitosciences (Invitrogen) was used according to the manufacturer's instructions for the determination of CIV activity and levels. The enzymatic activities of respiratory complex II (CII) and IV (CIV) and citrate synthase (CS) were assayed in a NovoStar MBG Labtech microplate instrument [10].

2.5. Determination of mitochondrial inner membrane potential and surface

The determination of mitochondrial inner membrane potential (MIMP) was done in triplicate in at least three independent experiments using 3,3′-dihexyloxacarbocyanine [$DiOC_6(3)$]. Mitochondrial inner membrane surface (MIMS) was measured, based on the quantity of cardiolipin, four times in three independent experiments by using nonyl-acridine orange (NAO). A Beckman Coulter Cytomics FC500 cytometer was used for measurements of intracellular fluorescence [10].

2.6. Determination of ATP levels

ATP levels were measured four times in three independent experiments using the CellTiter-Glo® Luminiscent Cell Viability Assay (Promega) according to the manufacturer's instructions. Briefly, 20,000 cells/well were seeded 10–12 h before measurement. Then, cells were washed twice with PBS and incubated for 6 h in a record solution with either 5 mM glucose, 5 mM glucose plus 2.5 μg/ml oligomycin (glycolytic ATP generation), 5 mM 2-deoxy-D-glucose plus 1 mM pyruvate (oxidative ATP production) or 5 mM 2-deoxy-D-glucose plus 1 mM pyruvate plus 2.5 μg/ml oligomycin. Cells were lysed, and lysates were incubated with the luciferin/luciferase reagents. Samples were measured using a NovoStar MBG Labtech microplate luminometer, and the results referred to the protein quantity [10].

2.7. Statistics analysis

The statistical package StatView 6.0 was used to perform all the statistics. Data for mean, standard deviation and sample size are presented. The normal distribution was checked by the Kolmogorov–Smirnov test

and the unpaired two-tailed *t*-test was used to compare parameters. P-values of <0.05 were considered statistically significant.

3. Results

To analyze haplogroup J OXPHOS function attempting to avoid the introduction of clonal effects, we built nine different cybrids in the nuclear background of osteosarcoma 143B cells. The epidemiological differences described in the Introduction have been found in European or European-descended individuals, and the major European subhaplogroups J are J1c and J2. To minimize phenotypic effects due to subhaplogroup-defining SNPs, we built the same number of J1c- and J2-derived cybrids (Supplementary Fig. 1). We also included one sample from J1b, a less frequent subhaplogroup J in European populations [12]. The same five haplogroup H cybrids that we used to evaluate haplogroup Uk OXPHOS function [10] were also used here as the reference group; these transmitochondrial cell lines included haplotypes of three subhaplogroups H, two of which are widely represented in Europe.

3.1. MtDNA and mtRNA levels and mitochondrial protein synthesis are lower in cybrids from haplogroup J

It has been reported that one of the mtDNA variants that distinguishes haplogroups H and J (m.295C>T/*MT-DLOOP*) increases mitochondrial transcription factor A (TFAM) binding and mtDNA copy numbers. In fact, cybrids from haplogroup J were found to have two times more mtDNA than those of haplogroup H [13]. In contrast to the previous results, we observed that mtDNA levels were in fact 11% lower in cybrids from haplogroup J than in those of haplogroup H (Fig. 1A). Possible explanations for this discrepancy could be different nDNA–mtDNA interactions due to distinct nuclear donor rho^0 cell lines (lymphoblastoid Wal2A versus osteosarcoma 143B) or differences in mitochondrial genetic background (1 J1, 1 J1b2, 1 H1, 2 H6 and 2 H* versus 1 J1b1, 4 J1c, 4 J2, 3 H1, 1 H5 and 1 H13). Because cybrids from haplogroup J did not have other SNPs in sequences involved in mtDNA replication, we reasoned that some other mechanism was responsible for the differences in mtDNA levels.

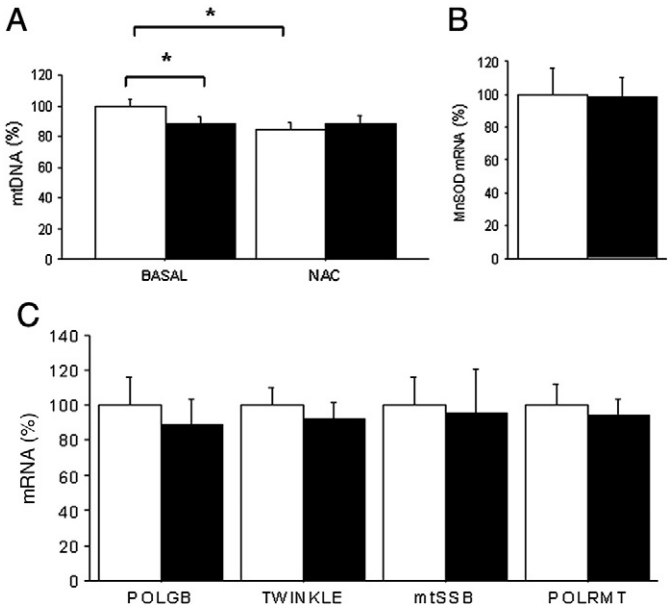

Fig. 1. mtDNA quantity and expression levels of genes associated with mtDNA replication. White and black bars represent mean values for cybrids H and J, respectively. The mean value for H cybrids has been set to 100%. A. mtDNA levels. The mean value for H cybrids not treated with NAC was set to 100%. *P = 0.0007. B. MnSOD mRNA levels. C. mRNA levels of different genes involved in mtDNA replication.

It has been reported that high reactive oxygen species (ROS) levels enhanced mtDNA replication [14] and that the antioxidant NAC can decrease mtDNA levels [15]. The mRNA levels of the antioxidant enzyme MnSOD were the same in cybrids H and J (Fig. 1B), suggesting that the difference in mtDNA levels between the cybrids probably was not caused by differences in ROS levels. However, we did find that cybrid H's mtDNA levels were significantly reduced by treatment with NAC, though this compound left cybrid J's mtDNA levels largely unaltered (Fig. 1A).

It has been suggested that NAC may not function to scavenge ROS but rather to interfere with the formation of disulfide bonds involved in functional redox switches and signaling pathways [16]. If this was the case, it is possible that a NAC-sensitive regulatory pathway controlling mtDNA levels is differentially affected in the cybrids of the two different haplogroups. It has already been shown that expression levels of several nuclear genes are different between cybrids H and J [17,18] and that NAC affects the levels of some of them [19]. To check for potential differences in the expression of mtDNA replication genes, we measured mRNA levels for POLG2, TWINKLE, mtSSB and POLRMT [11]. However, we did not find differences between cybrids from the two haplogroups (Fig. 1C). Thus, NAC either acts directly at the protein level or the effects we observe involve some other cellular pathway.

After evaluating cybrid mtDNA levels, we then turned our attention to mtDNA gene expression. The levels of all mtRNAs were decreased in cybrids from haplogroup J; however, the differences for 16S rRNA, p.MT-ND4/ND4L, p.MT-ND6, p.MT-CO1, p.MT-CO3 and p.MT-ATP6/8 were the only ones that were statistically significant (Fig. 2A). The mean total mtRNA levels of cybrids from haplogroup J were 18% lower than those of haplogroup H (Fig. 2B). These levels were similarly lower for cybrids J RNAs from the three mtDNA transcription units (Fig. 2C); moreover, RNA levels of the three transcription units showed a significant positive correlation ($r \geq 0.77$, $P \leq 0.0007$). None of the genetic differences between cybrids H and J are in sequences involved in mtDNA transcription, and neither did the analysis of mRNA levels for proteins involved in mtDNA transcription, such as POLRMT, TFB2M and MTERF, show differences between cybrids H and J (Fig. 2D). Therefore, these results point to a general mechanism controlling these mtRNA levels. The relationship between mtDNA and mtRNA levels has been frequently reported [10,20–24], and it has been previously shown that p.MT-ATP6 and p.MT-CO1 mRNA levels correlate significantly with mtDNA copy number [24,25]. In our cybrids, RNA levels from transcription units L and H_1 correlate significantly with mtDNA levels ($r \geq 0.57$, $P \leq 0.031$), a result that further supports the interpretation that lower cybrid J mtRNA levels are due to reduced mtDNA amounts.

To determine the effect of lower mtRNA levels in J cybrids, we studied the synthesis of mtDNA-encoded polypeptides. The analysis of mitochondrial translation products showed a significant decrease in mitochondrial protein synthesis in J cybrids (Fig. 3). Only m.2706A>G, a mutation in the 16S rRNA gene (*MT-RNR2*), could explain the lower mitochondrial protein synthesis in J cybrids. However, we found that mtDNA amount and RNA levels for genes from transcription unit H1 were significantly correlated with mitochondrial protein synthesis ($r \geq 0.62$, $P \leq 0.016$). These correlations, along with previous observations [10,23], suggest that lower mitochondrial nucleic acid levels are probably responsible for the lower synthesis of mtDNA-encoded polypeptides.

3.2. The mitochondrial mass of haplogroups H and J cybrids is similar

The lower mtDNA and mtRNA levels and a decreased mitochondrial protein synthesis in J cybrids could result from having fewer mitochondria. To check for potential differences in the cell fraction occupied by mitochondria, we determined the specific activity of CS, a nDNA-encoded mitochondrial matrix enzyme that it is frequently used as a marker for the mitochondrial number or size. We also measured the specific activity of CII, a nDNA-encoded

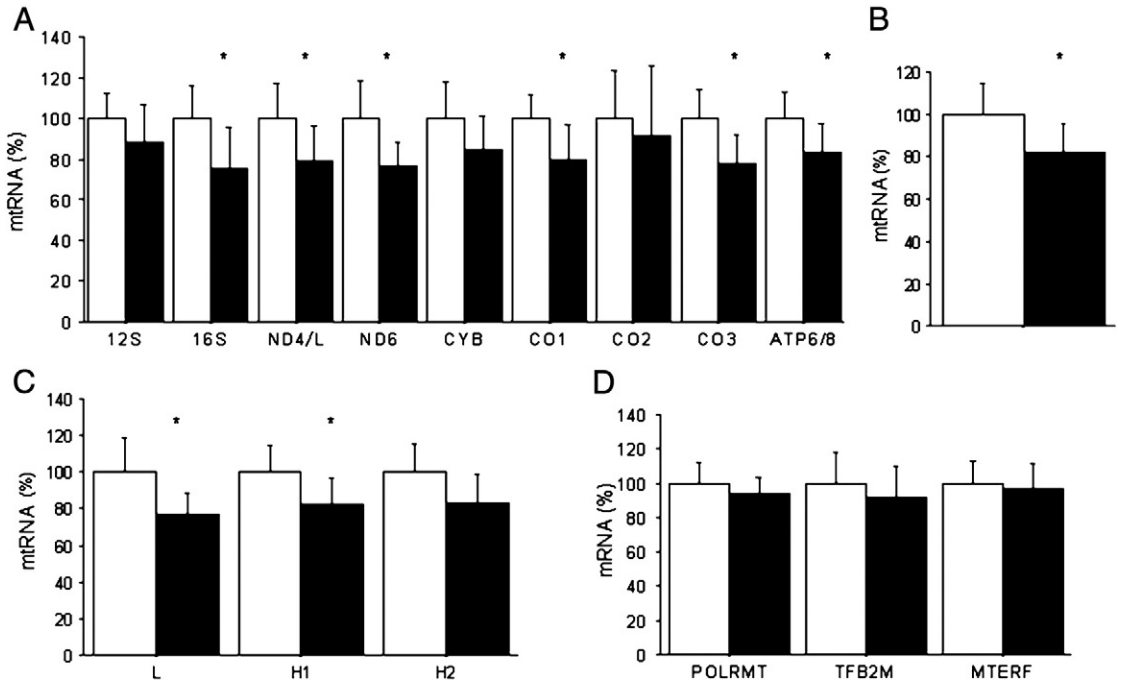

Fig. 2. mtRNA quantity and expression levels of genes associated with mtDNA transcription. White and black bars represent mean values for cybrids H and J, respectively. The mean value for H cybrids was set to 100%. *P ≤ 0.05. A. mtRNA levels. B. Total mtRNA levels. C. Levels of mtRNA products according to transcription unit. D. mRNA levels of genes involved in mtDNA transcription.

mitochondrial inner membrane enzyme, as well as MIMS. These experiments, however, did not reveal any differences between H and J cybrid values for the parameters tested (Fig. 4A and B). Therefore, the lower mitochondrial nucleic acid and protein levels in J cybrids are not due to having fewer mitochondria.

It has recently been found that mtDNA levels correlate with CIV activity [26,27]. Although we demonstrate that CIV amount and activity are indeed correlated ($r = 0.81$, $P = 0.0002$), there were no significant differences in these values between both haplogroups (Fig. 4C). Interestingly, a general tendency for a faster recovery and higher final CIV levels after treatment with doxycycline were previously observed in haplogroup J LHON mutant cybrids [28]. These results suggest the possibility that the mtDNA genetic background of J cybrids somehow influences CIV assembly or stability. Such an observation could be the reason that the CIV of H and J cybrids is of similar quantity and activity even though mitochondrial gene expression in J cybrids is lower.

3.3. Oxygen consumption and mitochondrial inner membrane potential are lower in cybrids from haplogroup J

The absence of differences in CIV activity and quantity between cybrids H and J does not rule out the possibility for differences in other respiratory complexes and OXPHOS function. In fact, it has been shown that mtDNA levels also correlate with respiratory complex III (CIII) activity [26]. ETC is the main oxygen consumer in the cell. Oxidation of substrates generates reducing power and these electrons will travel through the ETC complexes (CI–IV), which ultimately lead to the reduction of oxygen to water. We therefore measured oxygen consumption of our cybrids and found significant differences in

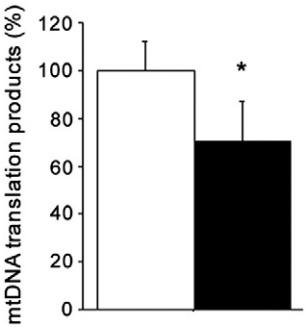

Fig. 3. mtDNA-encoded protein levels. White and black bars represent mean values for cybrids H and J, respectively. The mean value for H cybrids was set to 100%. *P = 0.005.

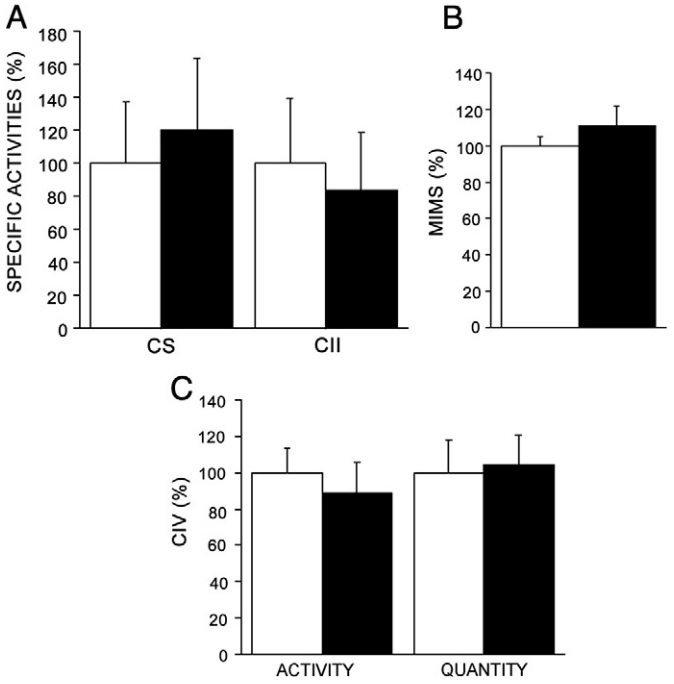

Fig. 4. Cell fraction occupied by mitochondria. White and black bars represent mean values for cybrids H and J, respectively. The mean value for H cybrids was set to 100%. A. Specific activities of nDNA-encoded mitochondrial enzymes. B. MIMS. C. CIV specific activities and levels.

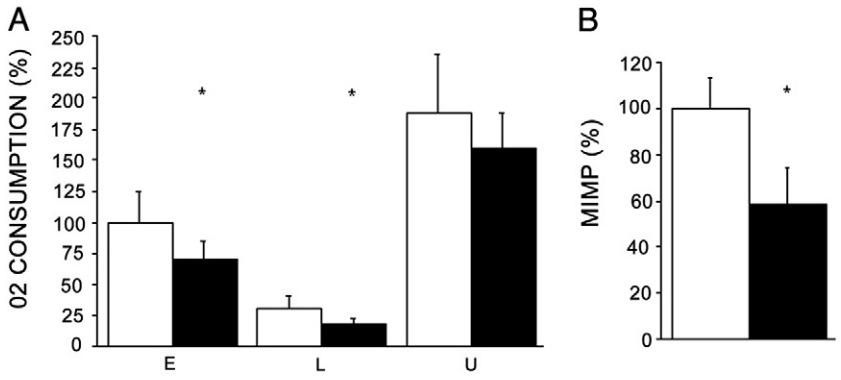

Fig. 5. Oxygen consumption and MIMP. White and black bars represent mean values for cybrids H and J, respectively. A. Oxygen consumption. E, L and U stand for endogenous, leaking and uncoupled respiration, respectively. The mean value for E respiration in H cybrids was set to 100%. *P = 0.01. B. MIMP. The mean value for H cybrids was set to 100%. *P = 0.006.

endogenous respiration between cybrids H and J (expressed as fmol/min/cell) (Fig. 5A). Interestingly, we have previously observed that healthy Spanish males of haplogroup J have significantly lower VO_{2max} than non-J individuals [29] and H individuals, in particular [30]. Moreover, it has been reported that mtDNA levels correlate with VO_{2max} [21] and with cell oxygen consumption [31,32]. Despite having similar CIV values, the different cybrids showed a significant positive correlation between CIV specific activity and endogenous respiration ($r = 0.65$, $P = 0.009$).

Respiratory ETC is coupled to the pumping of protons into the intermembrane space, a process that generates an electrochemical gradient across the mitochondrial inner membrane. This gradient can, by feedback, inhibit the proton pumping and, as a consequence, oxygen consumption. To rule out indirect effects of the electrochemical gradient on oxygen consumption, we treated the cybrids with the ATP synthase inhibitor oligomycin (leaking respiration) or the uncoupler FCCP (uncoupled respiration), compounds that, respectively increase or decrease the electrochemical gradient. This analysis, however, showed that uncoupled respiration was not significantly lower in J cybrids. Interestingly, others have described a correlation between mtDNA levels and uncoupled oxygen consumption in mouse cybrids [33]. Leaking respiration was significantly lower in cybrids from haplogroup J (Fig. 5A). There was a positive correlation between CIV specific activity and leaking respiration ($r = 0.60$, $P = 0.020$) as well as between endogenous and leaking or uncoupled respiration ($r \geq 0.65$, $P \leq 0.010$). Thus, the lower oxygen consumption of J cybrids was apparently not due to a higher electrochemical gradient. In fact, MIMP of cybrids from haplogroup J was significantly lower (Fig. 5B), and MIMP was significantly correlated with mtDNA levels, endogenous respiration and leaking respiration ($r \geq 0.56$, $P \leq 0.042$). It is possible that the lower MIMP of J cybrids results secondarily from reduced proton pumping, because of a lower electron flow rate through the ETC complexes, which in turn causes lowered oxygen consumption. Supporting this interpretation, rotenone inhibition of CI from osteosarcoma 143B cells inhibited cell respiration and decreased MIMP [34]. Moreover, a mutation in the *MT-ND5* gene of osteosarcoma 143B cybrids resulted in decreases in both respiration and MIMP [35].

Between cybrids H and J, there are nine different SNPs in mtDNA protein-coding genes that could potentially explain the differences we observe in oxygen consumption. While four of the SNPs are synonymous changes (m.7028C>T, m.11251A>G, m.11719G>A and m.12612A>G), three of the non-synonymous SNPs affect CI subunits (m.4216T>C/p.MT-ND1:Y304H, m.10398A>G/p.MT-ND3:T114A and m.13708G>A/p.MT-ND5:A458T). It was shown that a cybrid harboring threonine at p.MT-ND3 residue 114 (as in H cybrids) had increased CI activity [36] and *Escherichia coli* harboring histidine at NuoH residue 316 (homologous to human p.MT-ND1 residue 304) had a markedly decreased hexamine ruthenium reductase activity, indicating that structural integrity of CI was affected [37]. The two other non-synonymous SNPs are in p.MT-CYB (m.14766C>T/p.MT-CYB:T7I and m.15452C>A/p.MT-CYB:L236I), and it has been suggested that Thr7 in p.MT-CYB, like in H cybrids, can increase the efficiency of the Q-cycle and the ETC overall [38].

3.4. ATP levels are also lower in cybrids from haplogroup J

Because mitochondrial ATP synthesis is dependent on MIMP, we decided to measure ATP levels in our cybrids and found that ATP levels were significantly lower in cybrids from haplogroup J when grown in the presence of glucose (Fig. 6A). Under these conditions, there was also a significant correlation between ATP levels and MIMP ($r = 0.80$, $P = 0.007$). In contrast, we did not find any difference in ATP levels when the cybrids were grown in deoxyglucose with pyruvate, a glycolytic inhibitor and a substrate that is consumed by the OXPHOS system. ATP levels were also significantly different when the

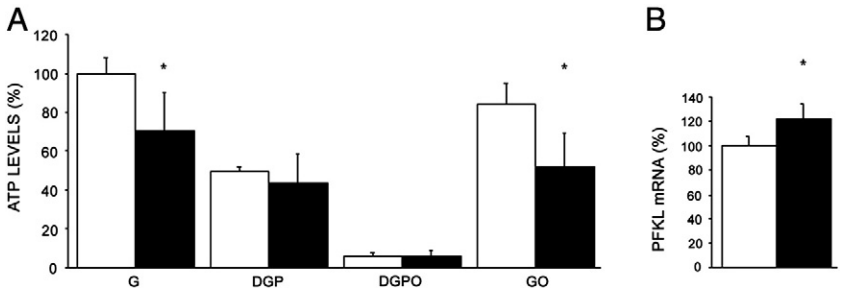

Fig. 6. ATP and PFKL mRNA levels. A. ATP levels. G, DGP, DGPO, GO are abbreviations for glucose, 2-deoxy-glucose plus 1 mM pyruvate, 2-deoxy-glucose plus 1 mM pyruvate and 2.5 μg/ml oligomycin and glucose plus 2.5 μg/ml oligomycin, respectively. The mean value for H cybrids in glucose was set to 100%. *P = 0.003. B. PFKL mRNA levels. The mean value for H cybrids was set to 100%. *P = 0.006.

cybrids were grown in glucose with oligomycin (Fig. 6A). These results therefore suggest that J cybrids are defective in some aspect of glycolysis. To test this hypothesis, we first analyzed the mRNA levels of the main glycolytic regulator enzyme PFKL. Contrary to our expectations, PFKL levels were significantly higher in J cybrids (Fig. 6B), an effect that probably results from a compensatory response. However, it has been suggested that glycolytic ATP can supply energy for maintaining MIMP [35,39]. Therefore, it is possible that the lower ATP levels in J cybrids is the combined result of an incomplete compensatory glycolytic response and the increased demand for ATP needed to maintain MIMP. Because glycolytic enzymes are nDNA-encoded, this result also suggests that OXPHOS differences due to mtDNA SNPs can influence the expression of nuclear genes, as has been shown previously by others [40].

4. Discussion

The correlations found between mtDNA levels and many other OXPHOS parameters, such as mtRNA levels, mtDNA-encoded protein amount, respiratory complex activities, oxygen consumption and MIMP, confirm that mtDNA amount largely determines the OXPHOS function. Cybrids from haplogroup Uk [10] and haplogroup J contain less mtDNA than those from haplogroup H. Considering that the nuclear genetic background and culture conditions are the same in all these cybrids, the differences between them must be due to particular mtDNA SNPs. Because none of the mtDNA SNPs examined were in sequences involved in mtDNA replication and because mtDNA encodes only OXPHOS proteins, it is likely that the lower mtDNA amounts we observe are the result of an indirect OXPHOS effect due to other mtDNA SNPs. OXPHOS is a metabolic pathway involved in many cell functions, including the regulation of cell red-ox state and the levels of ROS, calcium and ATP. These compounds act as second messengers that trigger retrograde signaling responses [41,42]. Thus, the mtDNA genotype must be able to provoke differences in these parameters. In support of this hypothesis, it has been shown that intracellular calcium dynamics are different in cybrids from mtDNA haplogroups N and non-N [43]. Similar to our study, it was recently reported that ATP levels were higher in $CD4^+$ cells from haplogroup H versus non-H patients suffering from Huntington disease [44]. Therefore, these differences in second messengers might alter some of the many cellular pathways regulated by these compounds.

One of the five SNPs that differentiate haplogroups J and Uk from haplogroup H is located in the mtDNA control region but does not affect sequences that are functionally important. While two of the other SNPs are synonymous, the remaining two, m.2706A>G/*MT-RNR2* and m.14766C>T/*MT-CYB*/p.MT-CYB:T7I, are not. In cybrids Uk [10] and J, these latter two SNPs are suggested to indirectly reduce mtDNA, mtRNA and mitochondrial protein levels as well as MIMP. Alternatively, it is possible that the OXPHOS differences we observe are instead determined by SNPs defining haplogroups J1c, J2 and Uk. Haplogroups Uk and J1c share a non-synonymous SNP in the cytochrome *b* gene (m.14798T>C/p.MT-CYB:F18L), while haplogroup J2 is defined by a different amino acid change in this protein (m.15257G>A/p.MT-CYB:D171N). Interestingly, such a scenario in which J1c and J2 were the key OXPHOS determinants would explain why haplogroup J and LHON are not correlated in Iran [45], as the major subhaplogroup J in this region is J1b [46]. The haplogroup J1b is not defined by amino acid changes in cytochrome *b*, and cybrids from this haplogroup have higher mtDNA levels than those from haplogroup H [13].

Our results suggest that individuals from haplogroups Uk [10], J1c and J2 are more susceptible to LHON because they have reduced OXPHOS capacity, which results in part from lower mtDNA levels. The mtDNA amounts may also explain why LHON is more highly prevalent in males. It has recently been reported that estrogens can increase mtDNA, mtRNA and mtDNA-encoded polypeptide levels and enhance oxygen consumption and ATP levels in a glucose medium [47]. Moreover, mtDNA levels are lower in LHON-affected individuals as compared to their unaffected homoplasmic mutant maternal relatives [48]. Thus, a complete compensatory response could be achieved by increasing mtDNA amounts. According to this model, individuals incapable of increasing mtDNA amounts sufficiently will develop LHON, a relationship that could very well explain why LHON has incomplete penetrance. Environmental factors precipitate the loss in vision and contribute to explain the marked incomplete penetrance of LHON. A strong and consistent association between loss in vision and smoking has recently been reported in LHON pedigrees [49], and it has been observed that smokers have fewer copies of mtDNA per nuclear genome [50].

Our results confirm that ancient, highly frequent and inherited mtDNA genetic variants, such as those defining mtDNA haplogroups, differently affect OXPHOS function and human health. However, recent, rare and somatic mtDNA mutations can also alter this function and manifest phenotypical effects in the organism [33]. Therefore, an important part of our susceptibility to different pathologies appears to be mtDNA-mediated.

Conflict of interest statement

No conflict of interest.

Acknowledgements

This work was supported by grants from Instituto de Salud Carlos III (FIS-PI10/00662 to JM; PI08/0264 and PI11/01301 to ER-P); Departamento de Ciencia, Tecnología y Universidad del Gobierno de Aragón y Fondo Social Europeo (Grupos Consolidados B33 to JM); Instituto Aragonés de Ciencias de la Salud (PIPAMER10-010 to ER-P); Fundación ARAID (Programa de Apoyo a la I + D + I para jóvenes investigadores 2010 to EL-G); and Asociación de Enfermos de Patología Mitocondrial (AEPMI) to AG-D. The CIBERER is an initiative of the ISCIII.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbadis.2012.04.014.

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