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Mitochondrial Ca^{2+} uptake correlates with the severity of the symptoms in autosomal dominant optic atrophy

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ABSTRACT

The most frequent form of hereditary blindness, autosomal dominant optic atrophy (ADOA), is caused by the mutation of the mitochondrial protein Opa1 and the ensuing degeneration of retinal ganglion cells. Previously we found that knockdown of *OPA1* enhanced mitochondrial Ca^{2+} uptake (Fülöp et al., 2011). Therefore we studied mitochondrial Ca^{2+} metabolism in fibroblasts obtained from members of an ADOA family. Gene sequencing revealed heterozygosity for a splice site mutation (c. 984+1G>A) in intron 9 of the *OPA1* gene. ADOA cells showed a higher rate of apoptosis than control cells and their mitochondria displayed increased fragmentation when forced to oxidative metabolism. The ophthalmological parameters critical fusion frequency and ganglion cell–inner plexiform layer thickness were inversely correlated to the evoked mitochondrial Ca^{2+} signals. The present data indicate that enhanced mitochondrial Ca^{2+} uptake is a pathogenetic factor in the progress of ADOA.

1. Introduction

The most frequent form of hereditary blindness is autosomal dominant optic atrophy (ADOA, OMIM165500). The disorder is due to cell death confined to retinal ganglion cells which results in bilateral central visual loss, impaired color vision and central visual field defects [1,2]. The prevalence of ADOA is estimated to be between 1/50,000 and 1/10,000 [3,4]. The most frequent cause of type 1 ADOA is the mutation of the *OPA1* gene [5–7]. Opa1 is a dynamin-related GTPase protein and due to alternative splicing its gene is transcribed into 8 mRNA isoforms in human [7] encoding proteins of 924–1014 amino acids. The protein is tethered to the IMM [8–10] and localized in the IMS [8,9]. In Western blot analysis 5 separate bands (designated *a* to *e*) ranging from 94 to 86 kDa, can be detected. Two long isoforms are anchored to the IMM and three soluble short forms are located in the IMS. The soluble forms are the proteolytic products of the long isoforms [11,12]. (For further details see recently published excellent reviews [13,14].)

The fundamental pathology in ADOA is the degeneration of the small (parvo) retinal ganglion cells with subsequent atrophy of the optic nerve [13,15,16]. Nevertheless, the protein is expressed in all examined human tissues, explaining the accidental development of the so-called 'ADOA +' forms characterized by the association of the blindness with various neuromuscular disorders [17–19] or hearing loss [20,21]. The vulnerability of retinal ganglion cells and that of spiral ganglion cells in the inner ear [22] has been attributed to the impairment of ATP production as observed in fibroblasts [23,24] or skeletal muscle [25]. Since retinal ganglion cells are unique among neurons in that the are exposed to direct sunlight, the ensuing oxidative stress may potentiate the consequences of Opa1 dysfunction, leading to apoptosis of these cells [13]. In $OPA1^{+/-}$ mice the level of postsynaptic density protein 95 and the density of glutamatergic synaptic sites were reduced even without loss of mitochondrial membrane potential [26]. This observation indicates the significance of Opa1 in maintaining the synaptic architecture and connectivity of retinal ganglion cells.

Knockdown of *OPA1* in two human cell lines, in HeLa and H295R (adrenocortical) cells, induces enhanced mitochondrial Ca^{2+} uptake [27] and also mitochondrial Ca^{2+}-dependent hypersecretion

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of aldosterone (in H295R cells) [28]. On the other hand, excessive accumulation of Ca^{2+} by mitochondria leads to cell death [29–31]. In view of these observations it was reasonable to examine mitochondrial Ca^{2+} uptake in ADOA patients. Our present data showed a correlation between the Ca^{2+} sequestering ability of mitochondria and the grade of impairment of visual parameters.

2. Materials and methods

2.1. Materials

Bradykinin and digitonin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Silencing RNA products, Rhod-2 AM, Fluo-4 AM, Mito Tracker Deep Red (MTDR), tetramethylrhodamine ethyl ester (TMRE) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) were purchased from Life Technologies (Paisley, UK). Annexin V-FITC was from BD Biosciences, glucose-free medium (E15-079) was from GE Healthcare (Piscataway, NJ). D-galactose was purchased from Reanal (Budapest, Hungary). Fetal bovine serum (FBS) was obtained from Lonza (Basel, Switzerland). Antibodies were used as follows: anti-OPA1 mouse monoclonal antibody (612606, BD Biosciences), anti-protein disulphide isomerase mouse monoclonal antibody (ab2792, Abcam, Cambridge, UK), anti-mouse immunoglobulin-HRP (NA 931V, GE Healthcare).

2.2. Clinical studies

Seven members of a family with genetically verified ADOA were subjected to detailed ophthalmological and neurological examinations including the tests for far and near visual acuity (spec. no. 2305 and 2307) (logarithmic visual acuity chart, from Precision Vision, Artesia, LA), measurement of thickness of retinal nerve fiber layer, ganglion cell and inner plexiform layer (optical coherence tomography, Cirrhus HD-OCT, Carl Zeiss Meditec, Dublin, LA) and critical flicker fusion frequency (CFF). Written informed consent was obtained from all patients and controls. As control, two healthy volunteers were also examined. After obtaining appropriate consent skin samples used for fibroblast culturing were excised from all five adult subjects. The research was approved by Hungarian Research Ethical Committee and carried out according to the Helsinki declaration.

2.3. Gene sequencing

Sequencing of the coding region and the exon–intron junctions of the *OPA1* gene (NM 015560.2) in patient I/3 was performed by Dr. Josseline Kaplan at the Service Medicale, GH Necker Enf. Malades, F-75743 Paris Cedex 15, France. Subsequently the segregation analysis of the mutation in the affected family members was performed in our laboratory using ABI Prism 3500 DNA Sequencer (Applied Biosystems, Foster City, CA). Genetic sequence was compared with the human reference genome (NM_001005360, ENSG00000079805) using NCBI's Blast® application.

2.4. Cell culture and transfection

Fibroblasts acquired from human skin biopsies were grown in DMEM containing 20% heat inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Passage numbers 3–7 were used.

2.5. Apoptosis

Control cells or cells exposed to 0.1 mM H_2O_2 for 4 h at 37 °C were dyed with FITC labeled annexin V (1:100) in a binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 min. The cells were examined with confocal microscopy.

2.6. Experimental conditions

The cells were superfused at room temperature with a modified Krebs-Ringer solution containing 140 mM Na^+, 4.5 mM K^+, 1.2 mM Ca^{2+}, 0.5 mM Mg^{2+}, 5 mM Hepes and 2 mM HCO_3^- (pH 7.4). Flow rate was ~1 ml/min. Permeabilization with 25 μM digitonin (for 8 min) was carried out in a cytosol-like medium (117 mM KCl, 6 mM NaCl, 1 mM KH_2PO_4, 2 mM Na^+ pyruvate, 2 mM Na^+ succinate, 2 mM K^+ ADP, 2 mM EGTA, 0.5 mM Mg^{2+} and 10 mM K^+ HEPES). The medium containing 5 μM free Ca^{2+} was composed with the Chelator software [32].

2.7. Confocal microscopy

Cytoplasmic and mitochondrial Ca^{2+} uptake (monitored with Fluo-4 and Rhod-2, respectively), mitochondrial membrane potential (TMRE or JC-1) and mitochondrial morphology (MTDR) were examined with confocal microscopy (LSM 510) as described [27]. Apoptosis was studied applying annexin V-FITC with a Zeiss LSM 710 confocal laser scanning microscope, equipped with a 63×/1.4 oil immersion objective (Plan-Apochromat, Zeiss) and operated with ZEN 9.0 software.

2.8. Electrophoresis and immunoblotting

SDS electrophoresis and immunoblotting were performed as described [27]. Anti-Opa1 and anti-PDI primary antibodies and anti-mouse secondary antibody were applied at a dilution of 1:500, 1:3000 and 1:5000, respectively. Protein content of the samples was measured with BCA assay.

2.9. Statistics

Means + S.E.M. are shown. For estimating significance of differences Kruskal–Wallis test (followed by Dunnett's test) was used. Correlation was calculated with Pearson's or Spearman's test. Data were analyzed with Statistica 11.

3. Results

3.1. Clinical examinations and genetic analysis

Seven members of a family with hereditary visual impairment were subjected to detailed ophthalmological and neurological examination (Fig. 1a, Table 1 and Suppl. Tables 1–5). The most severe progress of ADOA has been diagnosed in patient I/1. Patient I/1 and I/2 beside the visual impairment, had mild neurological symptoms. The four children did not have any neurological symptoms. We had no contact with the mother of the three adult siblings but based on the hetero-anamnestic data, she had serious visual impairment. The diagnosis has been confirmed in all the seven patients by genetic investigations which revealed a new heterozygous splice site mutation (c. 984+1G>A) in the *OPA1* gene. Western blot analysis of fibroblasts from patient I/1 detected reduced expression of Opa1 (as related to protein disulphide isomerase), however, there was no change in the pattern of OPA1 isoforms (Fig. 1b). The results of the Western blot are compatible with the ability of the antibody to recognize amino acids 708–830, a site downstream of the splice site mutation.

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For studying mitochondrial state and function, fibroblasts were cultured from the three adult patients (I/1, I/2 and I/3) who also