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Mitochondrial iron and energetic dysfunction distinguish fibroblasts and induced neurons from pantothenate kinase-associated neurodegeneration patients

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ABSTRACT

Pantothenate kinase-associated neurodegeneration is an early onset autosomal recessive movement disorder caused by mutation of the pantothenate kinase-2 gene, which encodes a mitochondrial enzyme involved in coenzyme A synthesis. The disorder is characterised by high iron levels in the brain, although the pathological mechanism leading to this accumulation is unknown. To address this question, we tested primary skin fibroblasts from three patients and three healthy subjects, as well as neurons induced by direct fibroblast reprogramming, for oxidative status, mitochondrial functionality and iron parameters. The patients' fibroblasts showed altered oxidative status, reduced antioxidant defence, and impaired cytosolic and mitochondrial aconitase activities compared to control cells. Mitochondrial iron homeostasis and functionality analysis of patient fibroblasts indicated increased labile iron pool content and reactive oxygen species development, altered mitochondrial shape, decreased membrane potential and reduced ATP levels. Furthermore, analysis of induced neurons, performed at a single cell level, confirmed some of the results obtained in fibroblasts, indicating an altered oxidative status and signs of mitochondrial dysfunction, possibly due to iron mishandling. Thus, for the first time, altered biological processes have been identified in vitro in live diseased neurons. Moreover, the obtained induced neurons can be considered a suitable human neuronal model for the identification of candidate therapeutic compounds for this disease.

Introduction

Neurodegeneration with brain iron accumulation (NBIA) is a heterogeneous group of genetic disorders characterised by radiological evidence of focal accumulation of iron in the brain, usually in the basal ganglia and extrapyramidal dysfunction (Schneider et al., 2013; Levi and Finazzi, 2014). These disorders are characterised by early or late onset, with the main symptoms associated with problems in movement, spasticity and cognitive impairment. Approximately 50% of cases of NBIA can be explained by mutations in the *PANK2* gene that cause an autosomal–recessive form of the disease, termed pantothenate kinase-associated neurodegeneration (PKAN or NBIA type I; OMIM 234200) (Hayflick, 2014). Magnetic resonance imaging (MRI) is particularly useful for distinguishing the cases of PKAN from other NBIA forms. In the majority of PKAN patients, the T2-weighted images show a hyperintense lesion of the globus pallidus, surrounded by a hypointense area (Angelini et al., 1992). This combination of hyper- and hypointense areas in the globus pallidus gives rise to a pattern defined as the "eye of the tiger", which is almost pathognomonic of the disease. The brain regions in which iron accumulates at pathological levels are the globus pallidus and substantia nigra, where iron-positive spheroidal bodies are visible, usually in the vicinity of swollen axons. Other neuropathological signs include demyelination, neuronal loss and gliosis (Kruer et al., 2011).

PANK2 codes for the pantothenate kinase-2 (PANK2), a mitochondrial enzyme that catalyses the first limiting step of the de novo biosynthesis of coenzyme A (CoA). CoA is a key factor in several cellular processes, including mitochondrial energy metabolism, anabolism and catabolism of fatty acids, as well as protein biosynthesis (Leonardi et al., 2005). In humans, 4

Abbreviations: DCF, dichlorofluorescein; DFO, deferoxamine; DHR-123, dihydrorhodamine 123; FAC, ferric ammonium citrate; iNs, induced neurons; IRP1, iron regulatory protein 1; LIP, labile iron pool; NBIA, neurodegeneration with brain iron accumulation; PKAN, pantothenate kinase associated neurodegeneration; PIH, pyridoxal isonicotinoyl hydrazone; ROS, reactive oxygen species; RPA, rhodamine B-[(1,10-phenanthroline-5-yl)-aminocarbonyl]benzyl ester; RPAC, rhodamine B-[(phenanthren-9-yl)-aminocarbonyl]-benzylester; TMRM, tetramethylrhodamine methyl ester.

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genes code for pantothenate kinases, but only PANK2 has mitochondrial localisation. Mutations in *PANK2*, which is located on chromosome 20, are spread over all 7 exons and include missense, nonsense, frameshift and splicing site mutations (Hartig et al., 2006). Mutations in *PANK2* result in enzyme deficiency, leading to insufficiency of the final product and accumulation of upstream substrates, such as N-pantotenoil-cysteine and pantetheine, which are potentially toxic (Leoni et al., 2012). In particular, cysteine is a potent iron chelator, and it has been proposed that high local levels of cysteine are the basis of the subsequent accumulation of iron, resulting in increased oxidative stress (Perry et al., 1985). The other hypothesis to explain the observed iron accumulation suggests that alterations in phospholipid metabolism due to CoA-deficiency may injure the membranes, with consequent oxidative stress that leads to iron dyshomeostasis (Leonardi et al., 2007). Different animal disease-models have been developed using *Drosophila melanogaster* and *Mus musculus*. *Drosophila* PKAN models partially recapitulate the human phenotype, showing locomotor dysfunction and neurodegeneration (Rana et al., 2010). In addition, a *Pank2* KO mouse model does not fully recapitulate the human phenotype (Kuo et al., 2005), unless particular dietary conditions are used (Brunetti et al., 2014). The double knock-out of more than one *Pank* in mice produces a very drastic phenotype resembling a metabolic syndrome (Garcia et al., 2012). However, these models do not show iron accumulation in the brain and are not useful for studying the pathogenetic mechanism leading to iron imbalance, which is hallmark sign in the brains of patients. Two in vitro studies on cellular models have attempted to explain the relationship between *PANK2* deficiency and iron deregulation. In HeLa, HepG2 and SH-SY5Y cells, the specific siRNA silencing of *PANK2* affects cell proliferation, induces cellular iron deficiency and increases the expression of the iron exporter ferroportin (Poli et al., 2010). PKAN fibroblasts, maintained in chronic iron supplementation, showed disturbed iron sensor protein iron regulatory protein 1 (IRP1) activity, resulting in deregulation of ferritin and transferrin receptor1 (TfR1), as well as a larger intracellular bioactive labile iron pool (LIP). This effect results in higher reactive oxygen species (ROS) development and leads to increased cellular oxidative status (Campanella et al., 2012).

Mitochondria are the main sites of iron utilisation in the cell (Levi and Rovida, 2009). This organelle employs the metal to sustain the biosynthesis of the iron sulphur cluster (ISC) and heme cofactors, which are prosthetic groups of widespread proteins involved in key biological processes, such as electron transfer, DNA synthesis and repair, metabolic and regulatory processes (Stehling et al., 2014). Thus, mitochondria play a central role in cell life, not only for energy supply but also for cellular iron handling. This important role is highlighted by the fact that defects in mitochondrial iron homeostasis lead to pathological phenotypes and cell death (Levi and Rovida, 2009). Here, we evaluated mitochondrial functionality in terms of iron handling and energetic profile to investigate whether the iron-dependent oxidative status alteration, previously revealed in PKAN patients' fibroblasts (Campanella et al., 2012), also affects the mitochondrial compartment. Furthermore, by taking advantage of the recently developed technology (Amamoto and Arlotta, 2014; Caiazzo et al., 2011) that allows neurons to be directly transdifferentiated from fibroblasts, we generated induced neurons (iNs) from PKAN patients to establish a suitable disease model in which to study the consequences of *PANK2* dysfunction.

Material and methods

Cell culture

We used primary skin fibroblasts from three unaffected subjects (controls 1, 2 and 3, two neonatals and one adult) purchased from ATCC and from three PKAN patients selected from the Movement Disorders Bio-Bank available at the Neurogenetics Unit of the Neurological Institute 'Carlo Besta' (INCB), Milan, Italy. Two PKAN patients (marked F419fsX472(a) and F419fsX472(b), biopsy was made at the age of two and four years old) (Campanella et al., 2012) were brothers who are homozygous for the same frame shift mutation that results in a truncated PANK2 protein (F419fsX472). The third, Y190X (Hartig et al., 2006), was homozygous for a mutation that produced a truncated amino acid chain. The fibroblasts were grown in DMEM (Lonza) supplemented with 10% FBS (Lonza), 100 mg/ml streptomycin, 100 U/ml penicillin and 4 mM L-glutamine (Sigma).

Generation of iNs from human fibroblasts by direct reprogramming

Human fibroblasts from patients and controls were grown in medium for fibroblasts (DMEM, FBS, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin) plated onto Matrigel-coated 24-well plates (5×10^4 cells/well). For the immune-histochemical analysis, some of these cells were plated onto Matrigel-coated glass coverslips. On the second day, the fibroblasts were infected by lentivirus in which cDNAs for transcription factors (Mash1, Nurr1, and Lmx1a) had been cloned (Caiazzo et al., 2011) under the control of a tetracycline-responsive promoter. Sixteen to twenty hours after infection, the cells were switched into fresh fibroblast medium containing doxycycline (2 mg/ml), and after a further 48 h, the medium was replaced with neuronal inducing medium (DMEM F12, 25 μg/ml insulin, 50 μg/ml transferrin, 30 nM sodium selenite, 20 nM progesterone, and 100 nM putrescine and penicillin/streptomycin) containing doxycycline (all from Sigma). The medium was changed every 2–3 days for a further 20 days.

Immunoblotting

Soluble cellular extracts for immunoblotting were obtained by lysing cells in 20 mM Tris–HCl, pH 7.4, 1% Triton X-100, and protease inhibitor cocktail (Roche) followed by centrifugation at 16,000 *g* for 10 min. Fifteen micrograms of total proteins was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed using specific antibodies: anti-citosolic aconitase (cAco) (Campanella et al., 2012) was used at a dilution of 1:500; anti-mitochondrial aconitase (mAco) (Antibody Verify) was used at a final concentration of 1 μg/ml; the anti-β-actin antibody (Sigma) was used at a dilution of 1:6000; the mouse monoclonal anti-PANK2 (Origene) antibody was used at a dilution of 1:2000; and the mouse monoclonal anti-SDH (70 kDa subunit) (MitoScience) was used at a final concentration of 0.1 μg/ml, followed by peroxidase-labelled secondary antibodies (Sigma-Aldrich). Band intensity was revealed by the ECL-chemiluminescence kit (GE Healthcare). The total protein contents were measured using the BCA protein assay (Thermo Fisher Scientific) calibrated with bovine serum albumin.

Determination of aconitase activity

Aconitase activity was in-gel assayed as described in Tong and Rouault (2006). The patient and control fibroblasts were grown in DMEM, harvested, washed in PBS and lysed in 20 mM Tris–HCl buffer, pH 7.4, 1% Triton X-100, protease inhibitor cocktail, 2 mM citrate, 0.6 mM MnCl2, and 40 mM KCl. Soluble extracts (40 μg) in 25 mM Tris–HCl, pH 8.0, 10% glycerol, bromophenol blue, were loaded on PAGE gels containing 8% acrylamide, 132 mM Tris base, 132 mM borate, and 3.6 mM citrate in the separating gel; and 4% acrylamide, 67 mM Tris base, 67 mM borate, 3.6 mM citrate in the stacking gel. The run was performed at 180 V for 2.5 h at 4 °C. Aconitase activity was determined in the dark at 37 °C by incubating the gel in 100 mM Tris–HCl, pH 8.0, 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM MgCl2, 1.2 mM MTT, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase. The quantification of the signal was performed using the NIH image software ImageJ.

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