Skin fibroblasts from pantothenate kinase-associated neurodegeneration patients show altered cellular oxidative status and have defective iron-handling properties

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Pantothenate kinase-associated neurodegeneration (PKAN) is a neurodegenerative disease belonging to the group of neurodegeneration with brain iron accumulation disorders. It is characterized by progressive impairments in movement, speech and cognition. The disease is inherited in a recessive manner due to mutations in the Pantothenate Kinase-2 (PANK2) gene that encodes a mitochondrial protein involved in Coenzyme A synthesis. To investigate the link between a PANK2 gene defect and iron accumulation, we analyzed primary skin fibroblasts from three PKAN patients and three unaffected subjects. The oxidative status of the cells and their ability to respond to iron were analyzed in both basal and iron supplementation conditions. In basal conditions, PKAN fibroblasts show an increase in carbonylated proteins and altered expression of antioxidant enzymes with respect to the controls. After iron supplementation, the PKAN fibroblasts had a defective response to the additional iron. Under these conditions, ferritins were up-regulated and Transferrin Receptor 1 (TfR1) was down-regulated to a minor extent in patients compared with the controls. Analysis of iron regulatory proteins (IRPs) reveals that, with respect to the controls, PKAN fibroblasts have a reduced amount of membrane-associated mRNA-bound IRP1, which responds imperfectly to iron. This accounts for the defective expression of ferritin and TfR1 in patients' cells. The inaccurate quantity of these proteins produced a higher bioactive labile iron pool and consequently increased iron-dependent reactive oxygen species formation. Our results suggest that Pank2 deficiency promotes an increased oxidative status that is further enhanced by the addition of iron, potentially causing damage in cells.

INTRODUCTION

Pantothenate kinase-associated neurodegeneration (PKAN, previously known as Hallervorden-Spatz disease, OMIM #234200) is an early-onset, autosomal recessive disorder with prevalence of ~1–3 per million (1–3). It is caused by mutations in the Pantothenate Kinase 2 gene (PANK2), and it is characterized by progressive impairments in movement, vision and cognition (1–3). PKAN is classified into the group of heterogeneous disorders called neurodegeneration with brain iron accumulation (NBIA). Classical forms of NBIA include very rare adult-onset disorders, such as neuroferritinopathy and aceruloplasminemia (1–3), the infantile neuroaxonal dystrophy (2,3), and the more recently described NBIA child-onset subtypes caused by mutations in the FA2H (4), ATP13A2 (5) and C19orf12 (6) genes. The common feature of these diseases is the iron overload in the brain as visualized by radiological and histopathological examinations (7), although some cases

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without signs of brain iron overload have been reported (8). In T2-weighted magnetic resonance imaging, the hallmark of PKAN disease is the eye-of-the-tiger signal, which is a low signal region associated with the accumulation of iron in the globus pallidus and a central hyperintense area resulting from tissue edema (2,7). Morphological and histochemical analyses of brain tissues from PKAN patients show degeneration of the globus pallidus and substantia nigra pars reticularis with a net loss of neurons and brown pigmentation associated with an excess of local iron (9). Pantothenate kinase 2 (Pank2) catalyzes the phosphorylation of vitamin B5, pantothenate in the first reaction of the Coenzyme A (CoA) biosynthetic pathway (10). In mammals, four active Pank enzymes have been described: Pank1, 2, 3 and 4. Pank1, 3 and 4 are localized exclusively in the cytosol, whereas Pank2 localization varies among species (11,12). Furthermore, alternative splicing of PANK2 mRNA generates multiple transcripts encoding different isoforms (13).

Attempts to generate animal models of the disease have produced incomplete phenotypes, possibly because the mitochondrial localization of Pank2 has only been reported in humans and primes and because many organisms, like Drosophila, have a single Pank (14). In mice, the Pank2 homolog was reported to localize in the cytosol (15); a PANK2 knockout exhibited reduced growth, retinal degeneration and male infertility as a result of azoosperma, but no movement disorders or signs of iron accumulation in the brain (16). However, a pantothenic acid-deficient diet caused movement disorders and azoosperma in mice without iron accumulation in the basal ganglia (17). In accordance with these results, suppression of the single Pank in Drosophila caused neurodegeneration (18) that could be rescued by pantethine (19), but no neuronal iron accumulation was observed.

Nevertheless, patients affected by PKAN have the most severe brain iron overload among the NBIA diseases, and the mechanisms leading to iron overload and its pathophysiological role remain unclear. In cells, iron is mainly utilized in the mitochondria to sustain the Fe/S cluster and heme biosynthesis (20). Iron homeostasis is mainly regulated at the post-transcriptional level by iron sensor proteins, namely iron regulatory proteins (IRPs). IRPs bind iron responsive elements (IREs) in the messenger RNAs of cellular iron import proteins (TfR1 and DMT1), iron storage proteins (cytosolic ferritins) and iron export proteins (ferroportin). Iron excess reduced the binding of IRPs to IREs, allowing the translation of iron storage and exporter protein mRNAs and the degradation of iron importer protein mRNAs. The regulatory response of IRPs is reversed in iron deficiency (21).

The link between Pank2 mutations and iron metabolism is largely enigmatic. The Pank2 enzyme is not expected to use iron as a cofactor, nor does iron seem to be involved in the other steps of the CoA biosynthesis pathway. A Pank2 deficiency may cause cysteine accumulation, and cysteines may act as an iron ligand and cause iron accumulation (22). Alternatively, a negative effect on membrane synthesis, caused by a CoA deficiency, may indirectly lead to alterations in iron homeostasis and/or to oxidative stress (23). The latter hypothesis is supported by the finding that genes involved in NBIA, like PLA2G6, FA2H and ATP13A2, encode proteins that are essential for the maintenance and repair of cellular membranes (3). However, how defects in lipid metabolism might cause iron deposition in the brain is unclear. Recent evidence has suggested that the deregulation of ferroportin, the membrane-bound cellular iron exporter, is related to a Pank2 deficiency, at least in some cell types (24).

To further identify iron metabolism alterations in PKAN, we studied iron homeostasis under basal growth conditions and after iron supplementation in fibroblasts from three PKAN-affected patients and three controls. Under basal conditions, PKAN fibroblasts showed a detectable alteration in their oxidative status, even if they did not display an alteration of iron parameters. In response to long-term iron supplementation, PKAN fibroblasts were defective in controlling the coordinated expression of Tfr1 and ferritins. This defect caused an increase in intracellular-free iron, leading to a further enhancement of iron-dependent reactive oxygen species (ROS) formation. Our results suggest that a Pank2 deficiency induces an alteration of the cellular oxidative status and that iron plays a role in aggravating the oxidative stress and damage.

RESULTS

To investigate the link between PANK2 gene defects and iron metabolism, we analyzed the oxidative status and iron homeostasis in skin fibroblasts isolated from three PKAN patients and three healthy subjects. One patient, coded 1265, was homozygous for a single amino acid substitution (R286C) located on the protein surface (25). Two patients, coded 1527 and 1535, were homozygous for a frame shift mutation that affects the catalytic region and leads to premature termination (F419fsX472) (Fig. 1A). Control fibroblasts are coded C1, C2 and C3 in the text. Quantitative RT–PCR analysis indicated that both the controls’ and patients’ fibroblasts showed detectable and similar expression levels of PANK2 mRNA (Fig. 1B). The probe that we used matched the first 183 nucleotides of the full PANK2 coding sequence (12); thus, these data proved that all fibroblasts express the long transcript that generates the precursor peptide, although this does not exclude the existence of the recently described shorter transcripts (13). The controls and patient 1265 showed the expression of the mature 47 kDa peptide, as detected by immunoblotting. In contrast, the truncated peptide of ~36 kDa, expected in fibroblasts 1527 and 1535, was undetectable (Fig. 1C).

Pank2 deficiency induces an alteration in cellular oxidative status

PKAN fibroblasts showed a higher amount of carbonylated proteins with respect to the control fibroblasts (Fig. 2A). To evaluate whether this altered oxidative status was linked to the modified expression of scavenger proteins, we analyzed SOD1, SOD2 and catalase levels by both qRT–PCR and immunoblotting with specific antibodies. Quantification of mRNAs showed high variability in the expression of these peptides and did not reveal any statistically significant differences between PKAN and control subjects (data not shown). Nevertheless, immunoblotting analysis suggested that, in the cytosolic compartment, the levels of catalase, but not SOD1, were increased in patients compared with controls, likely in response...
to oxidative input (Fig. 2B). In the mitochondria, at least in patients 1535 and 1265, SOD2 protein levels were significantly reduced (Fig. 2C), suggesting the possible impairment of the organelle. We proceeded to investigate whether the biosynthesis of the Fe-S cluster, one of the main markers of mitochondrial functionality linked to cellular iron metabolism, was affected by Pank-2 deficiency. We checked the activities of two mitochondrial Fe-S cluster enzymes, aconitase and succinate dehydrogenase (SDH). Patients 1527 and 1535 showed SDH activity reduced by 40% compared with controls, whereas the SDH activity was unaffected in patient 1265 (Fig. 2D). The protein level was similar in controls and patients, as detected by immunoblotting (Fig. 2D, lower panel). A reduction in enzymatic activity was not detected for mitochondrial aconitase or for the non Fe-S cluster enzyme malate dehydrogenase (data not shown). Interestingly, SDH activity was not recovered by growing cells in the medium supplemented with iron [20 \( \mu \text{M} \) ferric ammonium citrate (FAC) and 10 \( \mu \text{M} \) ascorbic acid for 18 h] or in glucose-free medium supplemented with 5 \( \mu \text{M} \) galactose to stimulate mitochondrial functionality (data not shown). This result is suggestive of a phenotype not related to mitochondrial iron availability.

Pank2 deficiency induces defective modulation of iron parameters after iron supplementation

Iron homeostasis is a determinant of oxidative status. To verify whether PKAN fibroblasts were able to manage excess iron as well as controls, we studied cellular \( ^{55}\text{Fe} \) incorporation. Fibroblasts were grown in the medium supplemented with 2 \( \mu \text{M} \) \( ^{55}\text{FeAC} \) plus 18 \( \mu \text{M} \) FAC and 10 \( \mu \text{M} \) ascorbic acid for 72 h. Total iron incorporation was measured and compared with the amount of iron safely stored in ferritins. All the cells showed similar amounts of total \( ^{55}\text{Fe} \) incorporation (Fig. 3A), although PKAN fibroblasts stored a lower amount of \( ^{55}\text{Fe} \) in ferritins with respect to controls (Fig. 3B). Under basal conditions, all the cells showed similar FtH levels (Fig. 3C). Moreover, the addition of iron caused an up-regulation of FtH, as expected, but the mean increase was 2.9-fold for controls and only 1.8-fold for the PKAN patients (Fig. 3C). Thus, the minor amount of \( ^{55}\text{Fe} \) found in ferritin in patients, shown in Figure 3B, is not due to reduced ferritin enzymatic activity, but rather to a lower protein amount; in fact, the ratio between ferritin-iron versus ferritin-protein is similar in patients and controls (data not shown). This suggests that patients’ fibroblasts could have a high amount of potentially toxic ferritin-free iron, commonly known as a labile iron pool (LIP), as also indicated by the ratio between total cellular \( ^{55}\text{Fe} \) versus \( ^{55}\text{Fe-ferritin} \), which was higher in patients than in controls (Fig. 3D). To test this hypothesis, we measured the LIP by incubating cells with the iron-sensitive fluorescent probe Calcein-AM. Untreated fibroblasts showed a similar amount of LIP in controls and patients (data not shown). After 72 h of iron supplementation, the amount of LIP in the controls was lower than that in the untreated cells, likely a consequence of the up-regulation of ferritins (Fig. 3E). In patients, this reduction did not occur and the LIP remained at the same level as the untreated cells (Fig. 3E). Iron supplementation usually causes the coordinated up-regulation of FtH and decreased expression of TIR1 to reduce cytosolic iron levels. Under basal conditions, all cells showed similar TIR1 protein levels (Fig. 3F, basal lanes). After 4 h of iron supplementation, TIR1 levels remained unchanged in all cells, while after 72 h TIR1 was reduced by 50% in controls, but only by 20% in PKAN patients (Fig. 3F).
Thus, after long-term iron supplementation, patients’ cells responded by up-regulating ferritins and down-regulating TfR1 to a lesser extent than controls.

Ferritin-free iron is a potent inducer of oxidative stress (26). In all fibroblasts, the amount of carbonylated proteins, measured by oxyblot after iron treatment, was highly increased compared with untreated cells, although no differences were observed between controls and patients (data not shown). We therefore used a different approach to directly measure ROS generation induced by iron excess. Fibroblasts grown in the presence of iron for 72 h were loaded with the ROS-sensitive mitochondrial probe DHR 123 and then the kinetics of ROS formation were followed for 1 h with, or without, fresh iron supplementation (20 μM FAC and 10 μM ascorbic acid). In both controls and patients, the addition of fresh iron caused an increase in ROS production; however, ROS levels were 20% higher in patients than in controls (Fig. 3G).

**Pank2 deficiency induces a defect in IRPs activity**

IRPs exist in an mRNA-free form in the cytosol, measurable by electro mobility shift assay (EMSA), or in an mRNA-
Figure 3. Regulation of iron homeostasis in fibroblasts. Comparative analysis of (A) total iron incorporation, (B) ferritin-iron incorporation and (C) Ferritin H (FtH) protein content in controls’ and patients’ fibroblasts after incubation with 2 μM ferric ammonium citrate (55FeAC) plus 18 μM FAC and 10 μM ascorbic acid for 72 h. Error bars indicate the standard deviation of three measurements. The analysis was repeated three times, and one representative autoradiography experiment is shown. (D) Representative ratio between total iron incorporation and iron stored in ferritins calculated by data collected in the experiments presented in (A) and (B). (E) LIP determination in fibroblasts that were untreated or subjected to 20 μM FAC and 10 μM ascorbic acid for 72 h. The plot summarizes the mean and standard deviations of three independent experiments for each fibroblast population. (F) Representative pictures of Transferrin Receptor 1 (TfR1) protein content, analyzed by immunoblotting in cellular extracts of fibroblasts that were untreated (Basal) or subjected to 20 μM FAC and 10 μM ascorbic acid for 4 and 72 h. Plots summarize TfR1 levels measured by densitometry and normalized to β-actin in three independent experiments for each fibroblast population. Error bars indicate standard deviations. (G) Representative kinetics of ROS generation in fibroblasts pretreated with iron and incubated with freshly added 20 μM FAC and 10 μM ascorbic acid for 60 min. Error bars indicate standard deviations of 24 independent measurements (8 for each controls’ and patients’ fibroblasts population). The difference between controls and patients was statistically significant after 3 min. *P < 0.05. Horizontal bars show statistically significant differences determined by the Student’s t-test. **P < 0.01.
bound form, mainly associated with membranes (27). We first used EMSA to evaluate whether IRPs/IRE binding was defective in patients’ cells. As expected, iron supplementation (20 μM FAC and 10 μM ascorbic acid for 4 h) reduced the activity of IRPs to 40 and 20% of the original in controls and patients, respectively (Fig. 4A). After 72 h of iron treatment, the activity of IRPs returned to basal levels in both cell types (Fig. 4A). This result indicated that the IRPs were functional and had similar activity in cells from controls and patients.

Thus, we proceeded to determine the IRP2-specific signal via an EMSA and the total amount of IRPs protein by immunoblotting. The IRP1 and IRP2 signals were distinguished in the EMSA by the addition of the specific antibody against IRP2. The results indicated that IRP2 did not contribute to the EMSA signals in all fibroblasts analyzed (data not shown). The total protein level of IRP1 was highly variable and the difference between controls and patients was not statistically significant (Fig. 4B).

Figure 4. Analysis of IRPs in fibroblasts. (A) IRP-binding activity on 32P-labeled IRE was measured by EMSA in controls’ and patients’ fibroblasts grown without the addition of iron (NT) or after 20 μM FAC and 10 μM ascorbic acid supplementation for 4 and 72 h. Upper panels: representative pictures of IRE-binding activities (IRE/IRPs) and total activities obtained by addition of 2% β-mercaptoethanol (β-ME) to cellular extracts. Bottom panels: IRE-binding activity expressed as percentage with respect to total activity. The plots summarize data collected in three independent experiments for each fibroblasts population. (B) Relative quantification of total IRP1 protein content by immunoblotting. A representative of three experiments is shown. Plots summarize the mean and standard deviations of IRP1 levels measured by densitometry and normalized on β-actin in the three replicates. (C) Evaluation of the mRNA-bound IRP1 fraction. Upper panel: immunoblotting after non-denaturing PAGE of soluble precipitate extracts prepared as described in the Materials and Methods and incubated for 30 min with 2 μg RNase-A. Lower panel: densitometry of band intensity; one representative of three experiments is shown. (D) Variation of the mRNA-bound IRP1 fraction, induced by 72 h of iron treatment, measured in all the three replicates of the experiment presented in (B). Error bars indicate the standard deviations. Statistically significant differences were determined by the Student’s t-test. *P = 0.039.
IRP1 is able to regulate protein expression when it is bound to mRNA (mRNA-bound IRP1), although this form represents a minor fraction of the total IRP1. This form is mainly associated with the membrane and the amount changes in conjunction with the variation in iron levels (27,28). Therefore, we analyzed the amount of mRNA-bound IRP1 by immunoblotting after differential centrifugation (27). Under basal growth conditions, a lower amount of mRNA-bound IRP1 was detected in patients than in controls (Fig. 4C). Iron supplementation reduced the mRNA-bound IRP1 by 60% in controls, as expected, but it did not affect the levels in patients’ cells (Fig. 4C and D). Overall, the data indicated that the IRPs/IRE machinery was able to respond to increases in the extracellular iron concentration. However, after 72 h of iron treatment, the decrease in the mRNA-bound IRP1 associated with the membrane fraction that was observed in controls was not observed in patients, which explains the defective regulation of ferritins and TIR1.

**DISCUSSION**

An overload of iron in the brain is pathognomonic for PKAN disease. MRI scans in patients reveal iron accumulation in the globus pallidus and also to a minor extent in the substantia nigra (29). These data have been validated by several histological studies (30,31); the most recent one accurately described the iron deposition in the cytoplasm of neurons, glia and macrophages in the globus pallidus of six well-characterized PKAN cases (32). However, the mechanisms leading to iron overload and its pathological role are still largely undefined. Animal models of the disease have failed to reproduce any signs of iron accumulation, and detailed studies of this topic using primary cells have yet to be performed. Our analysis of fibroblasts from PKAN patients indicated that an alteration of the cellular oxidative status and iron homeostasis can be detected in these cells. Many features were common to all patients’ cells, such as an increase in carboxylated proteins, the LIP level and ROS generation. Others appeared to be associated with specific mutations, such as an SDH deficiency, which occurs in the two patients carrying the F419fsX472 mutation. Thus, it seems that Pank2 mutations influence cellular functions via different mechanisms possibly depending on the severity of the protein alteration. An *in silico* model of the Pank3 protein, based on the crystal structure of Pank3 (25), suggests that the F419fsX472 mutation produces a truncated protein without enzymatic activity and with the potential to stress the mitochondrial protein quality control machinery. This is confirmed by the absence of the predicted peptide in the immunoblotting analysis (Fig. 1C). The structural protein model indicates that the missense mutation (R286C) alters a residue on the peptide surface (25), and the protein is believed to maintain enzymatic activity (33).

Alterations of iron homeostasis were common in all patients. PKAN fibroblasts failed to adjust FtH and TIR1 protein expression to manage continuous high iron concentration (20 μM). Interestingly, this condition is similar to the serum iron concentrations found in children, which range between 9 and 20 μM. This finding suggests that physiological iron concentrations could be sufficient to become potentially toxic for patients’ cells *in vivo*. In patients, the unusually low amount of ferritin was unable to store all intracellular iron, and this excess remained in the bioactive LIP for a longer duration, which might induce ROS (Fig. 5). Thus, the altered response to variations in iron concentration may cause oxidative damage and severe phenotypes. We were able to directly measure increased ROS generation immediately after iron addition. The difference was not measurable under basal growth conditions or after prolonged exposure to iron, most likely because of the highly transient nature of ROS. Nevertheless, we detected a typical effect induced by an increase in ROS, specifically the accumulation of oxidative protein modifications. Oxidative stress is associated with the dysfunction of the mitochondria and endoplasmic reticulum, inducing apoptosis and protein misfolding in neurons (34). In fact, ROS have been implicated in a wide range of neurodegenerative processes, including amyotrophic lateral sclerosis, Alzheimer’s disease, Parkinson’s disease and aging (34).

Alterations in antioxidant enzymes might contribute to increased oxidative stress due to the reduction in the cellular antioxidant potential. In patients’ cells, even given subject variability, the cytosolic scavenger catalase appears to respond to changes in the oxidative status, while in the mitochondrial compartment the expression of SOD2 does not. ROS formation might induce SOD2 degradation, as demonstrated in isolated rat brain mitochondria (35), and its decrease further promotes ROS formation. Considering that the SOD2 expression level is extremely variable among the human subjects (36), an individual who has low SOD2 levels might exhibit a more severe phenotype because of an additive effect. These data, together with the reduction in SDH enzymatic activity detected in two
patients, denote a selective impairment of mitochondrial functionality that occurs in individual PKAN fibroblasts.

Another effect that can be ascribed to oxidative stress is the impairment of IRPs/IRE functionality in PKAN fibroblasts. This machinery is regulated not only by intracellular iron but also by ROS. Several studies (37,38) note that an increased ROS level causes the loss of both the aconitase and IRE-binding activities of IRP1, by inducing the formation of a degradation-prone form, and promotes the degradation of ubiquitinated IRP2 by the proteasome (39). The reduction in IRPs is aimed at decreasing TfR1 and increasing the ferritin level, to diminish the LIP and to prevent the enhanced formation of ROS (40). This oxidative-dependent IRPs degradation might be responsible for the reduced amount of mRNA-bound IRP1 detected in PKAN fibroblasts in basal condition, and it may partially contribute to the insufficient cellular response to the addition of iron (Fig. 5). Thus, the deficient response to iron and oxidative stress appears to produce a vicious cycle that synergizes each other (Fig. 5).

Recent findings that defects in genes involved in lipid metabolism are the cause of other forms of neurodegeneration with iron accumulation in the brain (3) suggest that iron misregulation may not be the primary actor in PKAN pathogenesis. However, the damage induced by iron misregulation can lead to, or at least contribute to, neurodegeneration, particularly in the dopaminergic neurons of the basal ganglia. These cells are known to manage the highest amount of iron inside the central nervous system and naturally accumulate iron during aging (41). Interestingly, our data highlight defects in iron metabolism that might be associated with lipids. Pank2 deficiency is thought to affect CoA, which is involved in many biological reactions that are essential for energy production and lipid synthesis. To date, the link between these pathways and iron metabolism is far from being defined because no reaction involved in the regulation of iron homeostasis directly uses CoA as a cofactor. However, there is some indirect evidence of such a link. First, the SDH activity is strongly reduced in the absence of a decrease in the protein. SDH is a multimeric protein that is anchored to mitochondrial crests, and it is possible that the observed reduction in activity could account for protein assembly defects. The finding that mitochondrial aconitase activity is not modified in these cells suggests a minor involvement of the iron sulfur cluster synthesis machinery. Moreover, our EMSA results showed that the iron sulfur cluster was correctly inserted in IRP1 after iron supplementation. Nevertheless, PKAN fibroblasts showed lower mRNA-bound IRP1 levels than controls under basal conditions, and they were unable to reduce this fraction after the addition of iron. The latter analysis appeared to be more accurate than the EMSA, which measured the potential activation of mRNA-free IRP1 for IRE binding and not the amount of protein bound to mRNAs (27). Among the target mRNAs that bind IRPs, TTR1 mRNA is membrane associated (42), and ferritins mRNAs are found in both the cytosol and endoplasmic reticulum (43). Therefore, the defect results are more related to an altered subcellular distribution of IRP1 between membrane-free and membrane-linked conformations than to dysfunction in the IRPs/IRE system. These data suggest that a minimal alteration of IRP distribution is sufficient to increase free iron and ROS. Furthermore, we show, for the first time, that membrane defects could be associated with a faulty response to iron addition in PKAN primary cells.

From the overall data, we propose that brain iron accumulation, even if it is an epiphenomenon, is involved in the pathogenesis of the disease, and it should be treated because of its potential toxic effects. In conclusion, our data highlight a possible molecular relationship between Pank2 deficiency and iron misregulation. The effect of a Pank2 deficiency on LIP and ROS generation is mild in fibroblasts but could be vital in the dopaminergic neurons of the basal ganglia, which are affected in PKAN disease.

MATERIALS AND METHODS

Cell culture

We analyzed primary skin fibroblasts from three unaffected subjects (control: 143, 304 and 1459) and 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 1525 and 1535) were brothers who are homozygous for the same frame shift mutation that results in a truncated Pank2 protein (F419fsX472). The third, 1265, was homozygous for a missense mutation that produced the amino acid substitution R286C. Fibroblasts were grown in the glucose-free medium supplemented with 5 mM galactose, 10% FBS, 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin.

RNA extraction and real-time PCR

Total RNA was isolated from fibroblasts cultured in T75 flasks (80% confluence) using the Qiagen RNeasy Kit protocol for adherent cells. RNA quantity was measured using a Nanodrop (Nanodrop Technologies) and 1 μg of each sample was incubated with 1 U DNaseI (Invitrogen) for 15 min at RT. After DNase inactivation at 65°C for 10 min, RNA was used as a template to generate complementary DNA (cDNA) by a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse-transcription samples, without reverse transcriptase, were also included as negative controls. Reverse transcriptase products were used in real-time PCR to evaluate the expression level of Pank2 (primers: 5′ATCACTCTCTTCTGGGCTAC3′ and 3′CTAACTCGAGGAGGATTGC3′), Catalase (primers: Hs_CAT_1_SG), SOD1 (primers: Hs_SOD1_1_SG) and SOD2 (primers: Hs_SOD2_va.1_SG) relative to GAPDH with the SYBR Green PCR Master Mix (Applied Biosystems) system.

Preparation of cell extracts and immunosassays

Soluble cellular extracts for immunoblotting, ELISA and EMSA assays were obtained by lysing cells in 20 mM Tris–HCl pH 7.4 and 0.5% Triton X-100 followed by centrifugation at 16000g for 10 min. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting was performed using specific antibodies for SDH (Santa Cruz), Catalase (Calbiochem), SOD1 (Santa Cruz), SOD2 (Santa Cruz), IRP1 (homemade rabbit antiserum), TTR1 (Zymed) and β-actin (Sigma). The
FtH and FtL contents were determined in soluble cellular extracts by ELISA using rH02 and LF03 monoclonal antibodies, and the assay was calibrated for recombinant human homopolymers (44). To evaluate mRNA-bound IRP1 levels, soluble cellular extracts were centrifuged at 100 000g for 1 h and pellets were resuspended in 20 mM Tris–HCl pH 7.4 and 1% Triton X-100. Samples were incubated for 30 min with 2 μg RNase-A and loaded onto a 6% non-denaturing polyacrylamide gel. Immunoblotting analysis was performed using mouse serum anti-IRP1 (27).

The mitochondrial fraction (MF) and post-mitochondrial fraction (PMF) used for enzyme activity analyses were obtained by lysing cells in 20 mM Tris–HCl pH 7.4, 250 mM sucrose and 0.007% digitonin. After lysis, samples were centrifuged at 1000g for 5 min, and the soluble fractions were centrifuged at 4000g for 10 min to obtain mitochondria-enriched pellets (MF). The resulting soluble fractions were further centrifuged at 16 000g for 10 min to obtain the cytosolic soluble cellular extracts (PMF) that were used for ACO1 activity analysis. SDH activity quantifications were performed on MF suspended extracts (PMF) that were used for ACO1 activity analysis. SDH activity quantifications were performed on MF suspended in 20 mM Tris–HCl pH 7.4 with 250 mM sucrose. Measurements of ACO2 and MDH activities were performed on MF suspended in 20 mM Tris–HCl pH 7.4 with 0.5% Triton X-100 and centrifuged at 16 000g for 10 min to obtain mitochondrial soluble cellular extracts. During sample preparation, the SDH activity of mitochondria-containing fractions was determined. The activities of aconitases (ACO1/IRP1 and ACO2), SDH and MDH were measured spectrophotometrically following standard protocols (45–47). ACO activity was assayed by measuring the disappearance of cis-aconitate at 240 nm. SDH was assayed by following the reduction in para-iodonitrotetrazolium violet (INT) to INT formazan at 500 nm. MDH was assayed by monitoring the disappearance of NAD at 340 nm.

The total protein contents were measured using the BCA™ protein assay (Pierce) calibrated with bovine serum albumin.

**Oxidized protein detection**

Oxidized proteins were detected using the Oxyblot Protein Oxidation Detection Kit (Chemicon International) following the manufacturer’s instructions. Briefly, the soluble cellular extracts were obtained as described above with the exception of the addition of 50 mM DTT. The extracts were then derivatized to 2,4-dinitrophenylhydrazine, and 1 μg was loaded on 12% SDS–PAGE, blotted and incubated with an anti-DPN antibody. The bound activity was revealed by ECL Advance (Amerham).

**Determination of ROS**

ROS levels were determined by incubating cells with the redox-sensitive probe DHR 123, which is converted by oxidation to fluorescent Rhodamine 123 (48). Briefly, fibroblasts were plated in 96-well plates and incubated with Hank’s balanced saline solution (HBSS) supplemented with 10 mM glucose and 30 μM DHR 123 for 15 min at 37°C. After two washes, cells were maintained in HBSS supplemented with 10 mM glucose. Fluorescence was determined using the Victor3 Multilabel Counter (Wallac, Perkin Elmer) at 485 nm (excitation) and 535 nm (emission). Results were normalized to the protein content. To measure the ROS generation induced by the addition of fresh FAC after the determination of basal fluorescence, cells were incubated with 20 μM FAC and 10 μM ascorbic acid at RT for 60 min, and the fluorescence kinetics were determined.

**Electro mobility shift assay (EMSA)**

To perform the EMSA, cell extracts (2 μg of total protein) were incubated with a molar excess of [32P]-labeled IRE probe (100 000 cpm) in the presence or absence of 2% β-mercaptoethanol. The IRE probe was generated by in vitro transcription of the plasmid pSPT-fer. The plasmid was linearized with BamHI and transcribed by T7 RNA polymerase in the presence of 80 μCi [32P]UTP (ICN) (27).

**Radiolabeled iron incorporation**

Fibroblasts were incubated for 72 h with 2 μM 55Fe-Ammonium Citrate (Perkin Elmer), 18 μM FAC and 10 μM ascorbic acid (Sigma) (Fe:citrate 1:2). Cells were lysed in 20 mM Tris–HCl pH 7.4 with 0.5% Triton X-100 and centrifuged at 16 000g for 10 min. Samples (10 μl) from the soluble fraction were measured by liquid scintillation and 10 μg of total protein was loaded onto a 6% non-denaturing PAGE. The gel was dried and exposed to autoradiography.

**LIP determination**

The LIP was measured using the iron-sensitive fluorescent probe Calcein-AM (Molecular Probes) (49). Briefly, fibroblasts were plated in 96-well plates and incubated with or without 20 μM FAC and 10 μM ascorbic acid (Sigma) for 72 h. Cells were incubated in αMEM supplemented with 1 mg/ml BSA and 0.25 μM Calcein-AM at 37°C for 15 min. After two washes with HBSS, cells were maintained in HBSS supplemented with 10 mM glucose for 10 min. Basal fluorescence was measured using a Victor3 Multilabel Counter (Wallac, Perkin Elmer) at 485 nm (excitation) and 535 nm (emission). Cells were supplemented with the specific iron chelator Salicyladehyde Isonicotinoyl Hydrazine (final concentration 0.1 mM) for 15 min. Fluorescence was followed during incubation with the chelator, and when a plateau was reached, that value was considered to be the LIP value. Finally, the results were normalized to the protein content.

**Statistical analyses**

Except where otherwise indicated, the data are reported as the mean ± SD values of at least three independent experiments for each fibroblast population. Statistically significant differences between controls and patients were determined by the Student’s t-test, which was considered significant when \( P < 0.05 \).

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REFERENCES


