Dysfunctional Coq9 protein causes predominant encephalomyopathy associated with CoQ deficiency

Laura García-Corzo1,2, Marta Luna-Sánchez1,2, Carolina Doerrier1,2, José A. García1,2, Adela Guarás3, Rebeca Acín-Pérez3, Javier Bullejos-Peregrín1,2, Ana López1,2, Germaine Escames1,2, José A. Enríquez3, Darío Acuña-Castroviejo1,2 and Luis C. López1,2,∗

1 Instituto de Biotecnología, Centro de Investigación Biomédica, Parque Tecnológico de Ciencias de la Salud, Armilla, Granada, Spain, 2 Departamento de Fisiología, Facultad de Medicina, Universidad de Granada, Granada, Spain and 3 Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain

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Coenzyme Q10 (CoQ10) or ubiquinone is a well-known component of the mitochondrial respiratory chain. In humans, CoQ10 deficiency causes a mitochondrial syndrome with an unexplained variability in the clinical presentations. To try to understand this heterogeneity in the clinical phenotypes, we have generated a Coq9 Knockin (R239X) mouse model. The lack of a functional Coq9 protein in homozygous Coq9 mutant (Coq9X/X) mice causes a severe reduction in the Coq7 protein and, as consequence, a widespread CoQ deficiency and accumulation of demethoxyubiquinone. The deficit in CoQ induces a brain-specific impairment of mitochondrial bioenergetics performance, a reduction in respiratory control ratio, ATP levels and ATP/ADP ratio and specific loss of respiratory complex I. These effects lead to neuronal death and demyelinization with severe vacuolization and astrogliosis in the brain of Coq9X/X mice that consequently die between 3 and 6 months of age. These results suggest that the instability of mitochondrial complex I in the brain, as a primary event, triggers the development of mitochondrial encephalomyopathy associated with CoQ deficiency.

INTRODUCTION

Coenzyme Q (CoQ) biosynthesis is a complex process that is present in all mammalian tissues (1). In humans at least 11 genes codify proteins required for the biosynthesis of CoQ and have homologs in bacteria, yeast, nematodes and mice (1). One of these genes, coq9, was identified and characterized as a new gene that, when mutated in yeasts, results in a CoQ-deficient phenotype (2). However, the function of Coq9 protein in CoQ biosynthesis is not yet known.

Once CoQ molecules are synthesized, they localize in all tissue’s membranes, thereby indicating their involvement in multiple cellular functions. Basically, all these functions are based on their redox properties. In mitochondrial inner membrane, CoQ plays a central role in the function of the mitochondrial electron transport chain (mtETC) because it is required for the transfer of electrons between complexes I and III. CoQ and its oxidation by the mtETC is also required for the function of several enzymes that link the mtETC to the function of the TCA cycle by succinate dehydrogenase (EC 1.3.5.1), to β-oxidation by the electron-transfer flavoprotein-ubiquinone oxidoreductase (EC 1.5.5.1), to the shuttling of reduction equivalents from the cytoplasm by the glycerol-3-phosphate dehydrogenase (EC 1.1.99.5), to the synthesis of pyrimidines by the dihydroorotate dehydrogenase (EC 1.3.3.1), to the metabolism of glycine by the choline dehydrogenase (EC 1.1.99.1), to the arginine and proline metabolism by the proline dehydrogenase (EC 1.5.99.8) and to the selenoamino acid metabolism and sulfur assimilation by the sulfide CoQ reductase (EC 1.8.99.1). Thus, CoQ is a critical molecule in the production of ATP by the mitochondrial oxidative phosphorylation (OXPHOS)
system, which is apparently structured in mitochondrial supercomplexes (SC) that can be detected by blue native PAGE (3–5). In addition to the bioenergetics involvement, it is well known that CoQ is an important antioxidant and it participates in the redox regulation of the mitochondrial permeability transition pore and the uncoupling proteins (6).

Because of the fundamental functions of CoQ, the deficit in this vital molecule causes mitochondrial disorders with heterogeneous clinical presentations grouped in five major phenotypes: (i) an encephalomyopathy with brain involvement and recurrent myoglobinuria, (ii) an infantile multisystem disorder with encephalopathy usually associated with nephropathy and variable involvement of other organs, (iii) ataxic syndrome with cerebellar atrophy, (iv) an isolated myopathy and (v) a steroid-resistant nephrotic syndrome. Primary CoQ10 deficiency is transmitted as an autosomal recessive trait and may be caused by mutations in any of the known or in additional unknown genes required for its biosynthesis. To date, mutations in PDSS1, PDSS2, COQ2, COQ4, COQ6, COQ9 and ADCK3 have been identified (7). In vitro studies from these patients’ skin fibroblasts have indicated that the decrease in mitochondrial ATP production, the increase in oxidative stress and the differences in the induction of mitophagy and apoptosis may be critical factors in the development of the disease (8–12). Nevertheless, little is known about the cellular and tissue differences in these parameters following the deficiency in CoQ levels.

To explore the molecular and pathophysiologic consequences of CoQ deficiency and the tissue-specific differences, we have generated Coq9 knockin mouse model that develops predominant encephalomyopathy at 3 months of age. The function of the Coq9 protein in the CoQ biosynthetic pathway has also been examined.

RESULTS

Dysfunctional Coq9 protein, which regulates Coq7, produces widespread CoQ deficiency

The Coq9 gene was identified and characterized as a gene that is required for CoQ biosynthesis pathway in Saccharomyces cerevisiae (2,13). Later on, Duncan et al. (14) reported a patient with CoQ10 deficiency because of two point mutations in the COQ9 gene. In spite of these findings, the specific role of the Coq9 protein in the CoQ biosynthesis pathway is unclear. To study the function of Coq9 in mammals, we have generated a Coq9 mutant mouse using homologous recombination to introduce into mouse embryonic stem (ES) cells an R239X (c.715 C>T and c.717 T>A) mutation (see ‘Materials and Methods’ and Supplementary Material, Figs S1A–S1C) that is homologous to the human R244X Coq9 mutation (14). Mating of heterozygous mice (Coq9+/−) produced the expected mendelian distribution of wild-type (Coq9+/+), heterozygous mice (Coq9+/−) and homozygous knockin (Coq9XX) mice.

Murine Coq9 gene (GenBank Accession NP_080728) comprises 9 exons on chromosome 8 and is predicted to encode a 313 amino acid protein (UniProtKB/Swiss-Prot Q8K1Z0). The Coq9XX mice are predicted to produce a truncated protein lacking the C-terminal 75 amino acid residues. To confirm the presence of the truncated Coq9 protein in Coq9XX mice, we first performed an immunoblotting analysis using an anti-Coq9 antibody against the C-terminal region of the protein. As predicted, we detected a ~21 kDa protein in Coq9+/+ mice, but not in Coq9XX mice (Fig. 1A). Unfortunately, an anti-Coq9 antibody against N-terminal region of the protein was not available for this study, so we could not demonstrate the presence of a stable truncated protein in Coq9XX mice. Anyhow, if Coq9 is involved in CoQ biosynthesis, a dysfunctional Coq9 protein must produce a severe CoQ deficiency. As expected, Coq9XX mice showed a significant decrease in both CoQ9 (the major form of ubiquinone in rodents) and CoQ10 levels in tissue homogenates from Coq9+/+ (N = 10) and Coq9XX (N = 10) mice at 1, 3 and 5 months. Data are expressed as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.005 versus Coq9+/+.

Figure 1. Mutations in the murine Coq9 gene generate a dysfunctional Coq9 protein and a widespread CoQ deficiency. (A) Representative western blot analysis of Coq9 protein in heart homogenate from Coq9+/+ and Coq9XX mice. (B) CoQ9 levels in tissue homogenates from Coq9+/+ (N = 10) and Coq9XX (N = 10) mice at 1, 3 and 5 months. Data are expressed as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.005 versus Coq9+/+.
accumulation of two peaks in Coq9X/X mice. The retention times of these two additional peaks were 12.5 and 17.6 min, immediately before the CoQ8 (13.5 min) and CoQ10 (18.8 min) peaks, respectively (Fig. 2A and Supplementary Material, Fig. S3). Interestingly, these extra peaks were also observed in the skin fibroblasts belonging to the patient with COQ9 mutations (14). The mass spectral identification of the former lipid with a retention time of 12.5 min exhibited a molecular ion peak of 765.7 [M + H]+ (Fig. 2B) and could thus be identified as demethoxyubiquinone 9 (DMQ9)
DMQ9 was accumulated in all tissues of Coq9X/X mice, and the Coq9/DMQ9 ratio was lower in kidney, heart and skeletal muscle than in cerebrum and cerebellum (Supplementary Material, Fig. S4). DMQ9 is the substrate for the murine Coq7 protein that catalyzes its hydroxylation to produce 5-hydroxy-ubiquinone (Supplementary Material, Fig. S5). Consequently, homozygous Coq7 knockout mice accumulated DMQ9 with no production of CoQ9 (15,16). However, the tissues belonging to Coq9X/X mice contained both DMQ9 and CoQ9. Therefore, our results suggest that Coq9 may regulate Coq7. By virtue of that, we measured the protein levels of the Coq7, revealing that Coq9X/X mice showed a severe reduction in the Coq7 levels when compared with Coq9+/+ mice (Fig. 2C). The reduction in the Coq7 levels in the Coq9X/X mice is very specific because the protein levels of Coq6 and Adck3 did not differ between Coq9+/+ and Coq9X/X mice (Fig. 2D and E).

Coq9X/X mice show clinical signs of predominant encephalomyopathy

The reduction in CoQ levels in Coq9X/X mice tissues implies that this mouse model may recapitulate some of the clinical phenotypes associated with human CoQ10 deficiency (7). As a result, we performed an exhaustive characterization of this mouse model. Coq9X/X mice were indistinguishable from Coq9+/+ mice, which corresponds with the recessive transmission of the disease (7). By crossbreeding Coq9+/X mice, a normal mendelian distribution was obtained in the offspring. Coq9X/X mice pups had normal birth weights, sex distribution and early growth. Following the first month of life, Coq9X/X mice went through a developmental delay evidenced by a reduction in the body and tissues sizes as well as in the body weight (Fig. 3A–C). Curiously, by postnatal day 20–22, the Coq9X/X mice lost their body hair (Fig. 3D), but it grew back during the next hair-growth cycle. A similar observation was provided by a complex I-deficient mouse model (17,18). Locomotor activity was lower in 3 months old Coq9X/X mice, with a significant decline at 5 months (Fig. 3E). In a similar way, the numbers of vertical rears were mildly lowered at 3 months and significantly lowered at 5 months (Fig. 3F). No differences in urine albumin, creatinine, glucose and magnesium levels were found between Coq9X/X and Coq9+/+ mice (Table S2). Between 3 and 6 months, Coq9X/X mice developed a rapid and progressive leg paralysis, suggesting an involvement of the central nervous system in the pathology of the disease. In this range of age (3–6 months), Coq9X/X mice started manifesting moderate

Figure 3. Phenotypic characterization of Coq9X/X mice. (A) Image of 3 months old Coq9+/+ and Coq9X/X mice showing the differences in their sizes. (B) Body weight of males and females Coq9+/+ (N = 20) and Coq9X/X (N = 20) mice at 3 and 5 months old animals. Data are expressed as mean ± SD. ***, P < 0.005 versus male Coq9+/+, ###P < 0.005 versus female Coq9+/+. (C) Image of the sizes of brain, heart, kidney, liver and muscle of Coq9+/+ (left panel) and Coq9X/X mice (right panel) at 3 months of age. (D) Photo of a Coq9X/X mouse at 20–22nd postnatal day showing the loss of the body hair. (E and F) Open field test of Coq9+/+ (N = 16) and Coq9X/X (N = 16) mice at 3 and 5 months. Traveled distance was measured by intervals of 30 min. (D) Numbers of vertical rears were evaluated by intervals of 5 min during 30 min. (E) Data are expressed as mean ± SD. *P < 0.05; ***, P < 0.005 versus Coq9+/+. (G) Lateral view of Coq9X/X mouse with severe symptom phenotype represented by a severe paralysis of the legs.
symptoms (MS) represented by weight loss and mild walking alterations (Supplementary Material, Movie S1) and it was followed by a progressive development of severe symptoms (SS) manifested by rapid and severe leg paralysis (Fig. 3G and Supplementary Material, Movie S2). These phenotypic changes observed in *Coq9*<sup>−/−</sup> mice were correlated with their early death: *Coq9*<sup>−/−</sup> mice started to die at 3 months, and by 4 months, 50% of them were dead. By month 6, all *Coq9*<sup>−/−</sup> mice were dead. These results suggest that *Coq9*<sup>−/−</sup> mice developed the encephalomyopathic variant associated with CoQ deficiency (7).

To determine whether the phenotype observed in *Coq9*<sup>−/−</sup> mice was the result of anatomopathologic changes, we performed a histopathologic analysis of different tissues sections. Hematoxylin and eosin (H&E) stain of the brain showed an intense vacuolization in the white matter and medulla oblongata from *Coq9*<sup>−/−</sup> mice with SS (upper panel, scale bars, 100 μm; lower panel, scale bars, 500 μm). (A) PAS stain did not reveal histologic alterations in the kidney of both wild-type and homozygous mice (top panel and scale bar, 50 μm). The Masson’s TCM stain in the muscle showed nerve fibers with severe demyelination in *Coq9*<sup>−/−</sup> mice with SS (middle panel and scale bar, 50 μm). The heart of *Coq9*<sup>−/−</sup> mice showed an increase in connective tissue between cardiac cells (bottom panel and scale bar, 50 μm).
a profound demyelination and an increase in glial cells in the pons and the medulla oblongata of Coq9\textsuperscript{XX} mice. Likewise, it is possible to distinguish a decrease in the Nissl bodies of the neurons. These changes were more evident in mice with SS (Fig. 4A). SS Masson’s trichrome (TCM) stain in the hind legs skeletal muscle (vastus lateralis) showed nerve fibers with severe demyelination in Coq9\textsuperscript{XX} mice (Fig. 4B). The heart showed signs of fibrosis, represented by an increase in connective tissue between cardiac cells (Fig. 4B). Finally, the Periodic acid-Schiff (PAS) stain did not reveal histologic alterations in the kidney of both wild-type and homozygous mice (Fig. 4B). In the brain, the immunohistochemistry with primary anti-glial fibrillary acid protein (GFAP) antibody revealed numerous astrocytes with enlarged cell bodies in Coq9\textsuperscript{XX} mice, particularly in the pons and cerebellum (Fig. 5A and B) and also in the encephalon. The astrocytes proliferation was more pronounced in mice with SS (Fig. 5A and B), where zones with scars formation were observed (Fig. 5B). Immunohistochemistry with primary tubulin beta III (TUJ1) antibody revealed loss of neuron’s dendrites in
Coq9<sup>XX</sup> mice, more evident in mice with SS (Fig. 5A and B). On the contrary, immunohistochemistry with primary anti-oligodendrocytes antibody did not show any difference between Coq9+/+ and Coq9<sup>XX</sup> mice (Fig. 5A), except for the damaged area in the pons that was not stained with any of the techniques used. Therefore, the histopathologic results in Coq9<sup>XX</sup> mice showed signs of predominant encephalomyopathy with astrogliosis and neuronal death.

**Defect in the mitochondrial bioenergetics correlates with the encephalomyopathic phenotype of Coq9<sup>XX</sup> mice**

Despite a general reduction in CoQ levels in all tissues, the more impaired organ by large in Coq9<sup>XX</sup> mice is the brain. Interestingly, an analysis of the steady state level of ATP in different tissues revealed a significant decrease only in the brain (Fig. 6A). This decrease is not due to an overall decrease...
in the adenine nucleotide pool but to a decrease in the ATP/ADP ratio (Fig. 6B), suggesting that the rate of ATP synthesis may be significantly impaired in the brain. Because CoQ is a key component of mitochondrial respiratory chain, the ATP depletion in the brain may reflect impairment in mitochondrial bioenergetics. The ADP-stimulated respiration (state 3) was reduced in cerebrum of Coq9VX mice using substrates for both mitochondrial complex I (glutamate plus malate) (Supplementary Material, Table S3A) and complex II (succinate) (Supplementary Material, Table S3B). However, state 4 was increased using the substrates for mitochondrial complex I (Supplementary Material, Table S3A) while it was decreased using the substrate for mitochondrial complex II (Supplementary Material, Table S3B). These results suggest that respiration through complex I is partially uncoupled in cerebrum of Coq9VX mice. As a consequence, the respiratory control ratios (RCRs) were significantly decreased in cerebrum of Coq9VX mice (Fig. 6C and D), but the decrease in the RCR by glutamate plus malate was higher (64%) than the decrease in RCR by succinate (36%). This strongly suggests that in the brain of Coq9VX mice, the complex I-dependent respiration is substantially more impaired than the complex II-dependent respiration. Mitochondria from skeletal muscle and heart of Coq9VX mice showed a global reduction in oxygen consumption (Supplementary Material, Tables S3A and S3B), but no changes in the RCR (Fig. 6C and D). Kidneys of Coq9VX mice did not show differences in mitochondrial respiration through complex I (Supplementary Material, Table S3A), whereas states 3 and 4 through complex II were slightly decreased (Supplementary Material, Table S3B). The RCR were not different in the kidneys from Coq9+/+ and Coq9VX mice (Fig. 6C and D). The bioenergetics defects in the cerebrum of Coq9VX mice was confirmed by the measurement of the CI + III activity (Supplementary Material, Fig. S6). Moreover, the addition of decylubiquinone partially restored the CI + III activity in the cerebrum of Coq9VX mice (Supplementary Material, Fig. S6).

Mitochondrial respiratory complexes could assemble into efficient supramolecular associations or SC that behave as functional and physiologic entities (4,5). Interestingly, recent studies have revealed the presence of CoQ in some SC variants (4). The analysis by blue native gel electrophoresis (BNGE) and the subsequent immunoblotting with an anti-NDUFA9 (complex I subunit) antibody showed that the overall amount of complex I substantially forming SC did not change in the mitochondria from different tissues of 3 months old Coq9VX mice when compared with Coq9+/+ mice (Fig. 6E). However, in cerebrum and cerebellum, complex I was decreased (Fig. 6E). In addition, in the immunodetection of complex III (anti-core I antibody), we observed significant differences in cerebrum and cerebellum of Coq9VX mice when compared with Coq9+/+ mice, whereas no changes in any other tissues were appreciated (Fig. 6E). Explicitly, cerebrum and cerebellum from Coq9VX mice exhibited higher levels of free complex III than its form bound to the SC (Fig. 6E). This is in agreement with the reduction in complex I that would release complex III by the decrease in the amount of SC containing I/III. Therefore, the brain of Coq9VX mice showed a direct correlation between the deficit in CoQ, complex I reduction, mitochondrial respiration and ATP synthesis.

**Oxidative damage in the brain of Coq9VX mice**

Deficiencies in mitochondrial respiratory chain components are, in some cases, associated with an increase in the production of reactive oxygen species (ROS) with the subsequent oxidative damage to biomolecules. Specifically, CoQ-deficient cells have shown increased oxidative stress when the residual CoQ levels were between 30 and 45% in human skin fibroblasts (10–12) or 46 and 76% in a human neuronal cell line (19). To investigate whether the oxidative stress participates in the brain pathology of Coq9VX mice, we performed an immunohistochemistry analysis of brain sections using antibodies against 8-hydroxyguanosine (8-OHdG), a marker of nucleic acid oxidation, and 4-hydroxynonenal (4-HNE), a marker of lipid peroxidation. Coq9VX mice showed an increased number of positive cells for 8-OHdG (Fig. 7A and B) and increased intensity in the staining, which is especially evident in the pons (Fig. 7C–F). The immunostaining against 4-HNE did not show profound differences between...
Coq9+/+ and Coq9<sup>VX</sup> mice. However, some neurons in the encephalon of Coq9<sup>VX</sup> mice showed an increase in 4-HNE stains (Fig. 7G and H).

The energy deficit in neurons triggers caspase-independent apoptosis

Defects in the mitochondrial function are the more plausible explanation for the impossibility of brain tissue to maintain normal levels of ATP. This energetic failure may cause neuronal death and be the primary cause of the histopathologic features on Coq<sup>VX</sup> mice. In fact, the histologic study of the brain showed the presence of dead neurons in the Coq<sup>VX</sup> mice with SS. To verify how neuronal death occurred, we performed the TUNEL assay in different brain sections. Brain images of Coq<sup>VX</sup> mice with MS did not reveal the presence of apoptotic neurons (Fig. 8A). However, brain images from Coq<sup>VX</sup> mice with SS revealed the presence of apoptotic cells in the pons and in the encephalon (Fig. 8A). Following that, we investigated the mechanisms by which apoptosis was developed. First, we detect low levels of caspase 3 and p53 in mutant mice, as well as overexpression of the antiapoptotic protein bcl2 (Fig. 8B), implying that a caspase-dependent cell death pathway was not activated. Intrigued by our finding that pointed to an apoptotic process in the cerebrum of the Coq<sup>VX</sup> mice, we measured the expression of the apoptosis induction factor (AIF) in this tissue to check whether a caspase-independent cell death pathway was activated. Interestingly, we found a significant translocation of AIF from the mitochondria to the nucleus suggesting that the apoptotic process triggered was in fact a caspase-independent pathway (Fig. 8C).

DISCUSSION

Following the first reports of human CoQ<sub>10</sub> deficiency, there has been an increased interest in discovering the different steps and regulation aspects of CoQ biosynthetic pathway, as well as the pathways that are associated with CoQ<sub>10</sub> deficiency. In the current study, the generation and characterization of Coq<sup>VX</sup> mice favor some important conclusions regarding these inquiries: (i) Coq<sup>9</sup> protein specifically regulates Coq7 protein in the CoQ biosynthetic pathway, (ii) the presence of a dysfunctional Coq9 protein and/or the deficit in Coq9 in the brain causes loss of complex I and an increase in free complex III, leading to a decrease in mitochondrial respiration and ATP synthesis, (iii) mitochondrial dysfunction in the brain induces a caspase-independent apoptotic cell death and (iv) the encephalomyopathic form of CoQ deficiency is progressive and takes place with neuronal death, severe reactive astrogliosis and spongiform degeneration.

CoQ biosynthesis is a complex process that is known in part, thanks to research in bacteria and yeast. One of the proteins involved in this biosynthetic pathway is Coq9, but its specific function is unknown. The involvement of Coq9 in CoQ biosynthesis was initially discovered in yeasts because coq9 mutant yeasts did not produce CoQ<sub>6</sub> (2). Interestingly, coq9 point mutant yeasts harboring adck3 (coq8/abc1) on a multicopy plasmid have partial CoQ<sub>6</sub> biosynthesis and accumulation of DMQ<sub>6</sub> (2, 20). Coq9<sup>VX</sup> mice have constitutive levels of Adck3, and they show, similar to coq9 point mutant yeasts harboring adck3 on a multicopy plasmid (2, 20), reduced CoQ<sub>6</sub> levels and accumulation of DMQ<sub>6</sub>. Because DMQ<sub>6</sub> is the substrate for the reaction catalyzed by Coq7, we hypothesized that Coq7 may require Coq9 for its normal function. The reduced levels of Coq7 together with the normal levels of Coq6 and Adck3 other enzymes in the CoQ biosynthesis, confirmed this premise. Coq9 protein has no homology with proteins of known function, but the prediction indicates that it works as a cation/ion/metal-binding protein (Kihara Bioinformatics Laboratory, IN, USA). Because Coq7 possess a di-iron center (21), we may speculate that Coq9 is necessary for the proper conformation of this di-iron center in the Coq7 protein. The co-localization of Coq7 and Coq9 in rat molecular podocytes would reinforce this idea (22).

Once CoQ molecules are synthesized, they play a central role in the OXPHOS system transferring electrons from complexes I and II to complex III (3, 23). The OXPHOS system is organized into efficient supramolecular associations or SC. Interestingly, Acin-Perez et al. (24) demonstrated that complex I, almost in its totality, is forming physical association with complex III in vivo to the point that the amination of complex III destabilizes complex I and promotes its degradation. It is also described that the depletion of complex I does not destabilize complex III, but modifies its distribution between free and supercomplex-associated complex III (24). Coq9<sup>VX</sup> mice showed reduction in complex I with a parallel increase in free complex III only in the brain. This reduction in the complex I amount correlates with the partial restoration of the CI + III activity after the addition of decyclubiquinone. In other CoQ-deficient tissues with stable mitochondrial I/III ratios, no differences in mitochondria coupled respiration and ATP levels were found between Coq9<sup>VX</sup> and Coq9<sup>VX</sup> mice. Therefore, our study suggests that the destabilization of the mitochondrial complex I is a critical event triggered by the CoQ deficiency that reduces mitochondrial ATP synthesis. It is unclear why this tissue has a specific defect, but the differences (~40%) in the protein composition of mitochondria from different mouse tissues suggest that there are differences in the mitochondria organization and function depending on the tissue (25). One possibility is a tissue-specific role of Coq7, which is reduced in the Coq9<sup>VX</sup> mice, in the distribution of CoQ through the outer and inner mitochondrial membranes, as it has been recently suggested (26). Therefore, further investigations will be needed to clarify whether the loss of complex I in brain is a molecular-specific or CoQ levels-specific phenomenon.

The accumulation of DMQ<sub>6</sub> in Coq9<sup>VX</sup> mice tissues does not seem to be a critical factor for the cerebellum-specific bioenergetics impairment because cerebrum and cerebellum showed higher CoQ<sub>6</sub>/DMQ<sub>6</sub> ratios than other tissues. DMQ<sub>6</sub> is not functional in the mitochondrial respiratory chain, (27) and mice with accumulation of DMQ<sub>6</sub> and no production of CoQ<sub>6</sub> are embryonically lethal (15, 16). Moreover, the accumulation of DMQ<sub>6</sub> does not seem to produce detrimental effects in the mitochondrial respiratory chain because Coq9 and Coq7 mutant yeasts are not functionally distinguishable from other ubiquinone-deficient mutant yeasts (1, 28).
The consequence of energy depletion in the brain is an increase in apoptotic neuronal death triggered by translocation of AIF from mitochondria to the nucleus, a route of cell death pathway mitochondrial dependent, but caspase independent. The AIF-dependent pathway seems to be particularly important in the induction of apoptosis in neurons (29–31). Similar to our results, the cell death induced as a consequence of the lack of mitochondrial glutathione peroxidase 4 (GPx4) was mediated through the AIF translocation to the nucleus (31). Moreover, caspase 3 was not activated in the GPx4−/− cells, and the overexpression of Bcl2 did not prevent the apoptosis cell death (31). Similar outcomes were found in the brain of Coq9−/− mice. Another question, however, is whether the energy depletion is the only cause of the caspase-independent cell death. In this regard, the cell death in CoQ10-deficient fibroblasts has been associated with a combination of
increased oxidative stress and a decreased ATP synthesis (11,32). Takahashi and et al. (33) suggested that the increase in apoptosis in the embryos of Coq7 knockout mice was caused by the bioenergetics failure. Other important issue is that the mechanisms to induce apoptosis may be different depending on the cell type or tissue. In cortical neurons, for example, it has been suggested that both energy failure and oxidative stress would contribute to the caspase-independent neuronal death (34). This circumstance may be also plausible in the brain of Coq9X/X mice, where an overall increase in 8-OHdG and accumulation of 4-HNE in some neurons were observed. The increased oxidative damage detected in the brain of Coq9X/X mice and in the kidney of Pdss2 mutant mice suggests that oxidative stress is involved in the pathology of CoQ deficiency in vivo (35,36). Future studies with an exhaustive evaluation of the ROS generation, oxidative damage and antioxidant defenses in Coq9X/X mice tissues, as well as the effects of antioxidant therapies, will provide the detailed mechanisms of the induction of the caspase-independent neuronal death.

Finally, the histopathologic evaluation of Coq9X/X mice showed loss of neuronal dendrites and neuronal death, demyelination in the white matter and the ponds of the brain, as well as in the nerve fibers of the hind leg skeletal muscle, and signs of fibrosis in the heart. The neuronal death also induced reactive astrogliosis, a process that can exert beneficial effects in the initial phase, supporting neuronal survival; however, when the response progresses over time, the reactive astrogliosis may be detrimental (37). This last phase of reactive astrogliosis can be identified by a typical scar formation that is clearly apparent in the brain of Coq9X/X mice with SS.

The causes of the clinical heterogeneity associated with human CoQ10 deficiency are poorly understood. The main progress in this area has been developed in cellular models because of the lack of suitable animal models with the different clinical phenotypes. The Coq9X/X mice described here is the first animal model showing biochemical, molecular, histologic and behavioral signs resembling human mitochondrial encephalomyopathy associated with CoQ deficiency. The tissue-specific pathomechanisms described in Coq9X/X mice unlock new possibilities for the mitochondrial organization and function. Given that some of these mechanisms are common to other mitochondrial encephalomyopathies as well as neurodegenerative and neuromuscular disorders, this Coq9X/X mouse model will be very useful not only for developing and testing therapies for CoQ deficiency but also for other illnesses related to mitochondria.

**MATERIALS AND METHODS**

**Generation of knockout Coq9 mouse**

A positively identified C57BL/6 BAC clone, RP23-128L6, was used to isolate the Coq9 genomic sequence for vector construction. A 7.72 kb fragment spanning from the upstream of exon 4 to downstream of exon 9 was first subcloned into a backbone vector derived from vector plasmid pSP72 (Promega) using the recombinering technology. The mutation CGT > TGA (R239X) within exon 7 was generated by three-step PCR mutagenesis. Four mutagenesis primers [PT1, PT2, PT3 and PT4 (BsiWI)] were designed to amplify a fragment with the size of 1165 bp, which incorporates the mutation at the desired location (Supplementary Material, Fig. S1A). The PCR fragment containing the mutation was then used to replace the wild-type sequence using an endogenous EcoRV site and an engineered BsiWI site. The neomycin drug selection cassette flanked by both LoxP and flippase recognition target (FRT) sites is inserted 205 bp downstream of exon 7. The final targeting vector is composed of a long homology arm extending ~5.2 kb 5’ to the point mutation in exon 7, a short homology arm extending 2.17 kb 3’ to the neomycin cassette, the engineered mutation Arg > stop and the neomycin selection cassette flanked by LoxP and FRT sites (Supplementary Material, Fig. S1B). The total size of the targeting construct (including the backbone vector) is 11.82 kb. It contains both ampicillin- and kanamycin-resistant genes for bacteria transformation. The targeting vector was confirmed by restriction analysis and sequencing after each modification. The vector was linearized by NotI restriction enzyme digestion and then transfected into IC1 C57BL/6 ES cells by electroporation to generate targeted ES cell lines (inGenious Targeting Laboratory, NY, USA).

After selection with G418 antibiotic, approximately 200 resistant clones were isolated and screened by PCR using Coq9 primers outside of the targeting sequence with neomycin-resistant gene primers. Homologous recombinants were confirmed by Southern blot analyses using probes recognizing Coq9 genomic sequences outside of the targeting vector (Supplementary Material, Fig. S1C) (primers A1, LAN1, COOK3 and UNI). Presence of the c.715 C>T and c.717 T>A mutations were confirmed by sequencing (primer COOK4). Cells from two to four ES clones harboring the homologous recombinants were submitted for injection into C57BL/6j blastocysts and generation of chimeric mutant mice. The germline transmission of the mutation was confirmed by PCR, Southern blot (Supplementary Material, Fig. S1D) (primers PT1, F7, A1 and F3) and sequencing (primer COOK4). Heterozygous mice were subsequently bred with FLPe mice (38) on a C57BL/6 background to remove the Neo cassette. The deletion of the Neo cassette was confirmed by PCR using the Neo Del1 and Neo Del2a primers. The FLPe transgene was eventually eliminated from the pedigree by further breeding. For all experiments, heterozygous mice for the knockout allele were crossbred to obtain wild-type controls (Coq9+/+ = Coq9+/−), heterozygous knockin (Coq9R239X/R239X = Coq9+/R239X) or homozygous knockin (Coq9R239X/R239X = Coq9R239X) mice. Mice were housed in the Animal Facility of the University of Granada under an SPF zone with lights on at 7:00 am and off at 7:00 pm. Mice had unlimited access to water and rodent chow. Institutional Animal Care and Use Committees approved all experiments.

**Quantification of CoQ9 and CoQ10 levels in mice tissues**

CoQ9 and CoQ10 from mice tissues were extracted by mixing tissue extracts with 1-propanol. After 2 min vortex, the solution was centrifuged at 11 300 g for 5 min. The resultant supernatant was injected in a HPLC system (Gilson, WI, USA), and the lipid components were separated by a reverse phase symmetry C18 3.5 μm, 4.6 × 150 mm column (Waters, Spain), using a mobile phase consisting of methanol,
ethanol, 2-propanol, acetic acid (500:500:15:15) and 50 mM sodium acetate at a flow rate of 0.9 ml/min. The electrochemical detector consisted of an ESA Coulochem III with the following setting: guard cell (upstream of the injector) at +900 mV, conditioning cell at ~600 mV (downstream of the column) and followed by the analytical cell at +350 mV (32). CoQ6 and CoQ10 concentrations were estimated by comparison of the peak areas with those of standard solutions of known concentrations. The results were expressed in pmol CoQ/mg protein.

**Ultra carrying out liquid chromatography- mass spectrometer (MS/MS) analysis of intermediate metabolites**

Lipid extracts were obtained as described above for the CoQ quantification. Samples were analyzed using an Acquity ultra carrying out liquid chromatography system coupled to a Xevo TQS detector of mass spectrometer (MS/MS) with an electrospray ionization (Waters Corporation). The analytical separation column was a BEH C18, 1.7 × 50 mm column (Waters, Spain) (39). The mobile phase consisted of methanol and 5 mM ammonium acetate at the constant flow rate of 0.3 ml/min. Source and desolvation temperatures were set at 150 and 300°C, respectively. Nitrogen was used as both cone gas (150 l/h) and desolvation gas (500 l/h), and argon was used as collision gas (0.14 ml/min). Mass spectrometry analyses were carried out in full scan mode between 600 and 950 uma to improve the sensitivity of the analysis.

**Evaluation of SC formation by BNGE**

BNGE was performed on mitochondrial fraction from mouse tissues. Mitochondrial isolation was performed as previously described (40). Tissues were homogenized in a glass–teflon homogenizer. Liver and kidney were homogenized (1: 4, w/v) in the homogenization medium A [0.32M sucrose, 1 mM EDTA and 10 mM Tris–HCl (pH 7.4)]; cerebrum and cerebellum were homogenized (1:5, w/v) in the homogenization medium A plus 0.2% free fatty acids BSA; heart was homogenized (1:10, w/v) in the homogenization medium B [0.075 M sucrose, 0.225 mM sorbitol, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1% free fatty acids BSA, 10 mM Tris–HCl (pH 7.4)]; and skeletal muscle was homogenized (1:20, w/v) with ultratrex in homogenization medium C [0.12 mM KCl, 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM MgCl2, 1 mM EGTA and 5 mg/ml free fatty acids BSA]. Liver, kidney, cerebrum, cerebellum and heart homogenates were centrifuged at 1000g for 5 min at 4°C to remove nuclei and debris. Mitochondria were collected from supernatants after centrifuging at 14 400g for 2 min at 4°C. Skeletal muscle homogenate was centrifuged at 600g for 10 min at 4°C. The supernatant (s1) was kept on ice, and the pellet was re-suspended in eight volumes of buffer A and centrifuged at 600g for 10 min at 4°C. The supernatant (s2) was combined with s1 and centrifuged at 17 000g for 10 min at 4°C. The pellet obtained was re-suspended in 10 volumes of medium A and centrifuged at 7000g for 10 min at 4°C. The pellet was re-suspended in one volume of medium B (0.3 M sucrose, 2 mM HEPES and 0.1 mM EGTA) and centrifuged at 3000g for 10 min at 4°C.

The mitochondrial pellets from all tissues were suspended in 75 μl (heart and cerebellum), 120 μl (skeletal muscle), 140 μl (cerebrum and kidney) and 800 μl (liver) in the corresponding homogenization buffers. An aliquot of each sample was used for protein determination. The remaining samples were then centrifuged at 17 000g for 3 min at 4°C. Mitochondrial pellets were suspended in an appropriate volume of buffer D [1 M 6-aminohexanoic acid, 50 mM Bis–Tris–HCl (pH 7.0)] to be at 10 mg/ml, and the membrane proteins were solubilized by the addition of digitonin (4 g/g) and incubated for 5 min in ice. After 30 min of centrifugation at 13 000g, the supernatant was collected, and 3 μl of 5% Brilliant Blue G dye prepared in 1 M 6-aminohexanoic acid was added (4). Mitochondrial proteins (100 μg) were then applied and run on a 3–13% gradient native gel using electrophoresis system mini-PROTEAN Tetra Cell (Bio-rad). Western blot was performed using a mini Trans-Blot Cell onto polyvinylidene difluoride membranes and probes with specific antibodies against complex I, anti-NUDFA9 (Abcam, ab14713) and complex III, anti-ubiquinol-Cytochrome C Reductase Core Protein I (Abcam, ab110252) (4).

**Sample preparation and western blot analysis**

Western blot analyses were performed in cerebrum and heart homogenates, as well as mitochondrial and nuclei fractions. For the experiments in total homogenate samples, cerebrum and heart were homogenized in homogenization buffer A (50 mM Tris–HCl, 1% Triton X-100 and 1 mM Dithiothreitol, pH 7.6, protease inhibitor cocktail) at 1100 r.p.m in a glass–teflon homogenizer. Tissue homogenate was sonicated and centrifuged at 1000g for 5 min at 4°C, and the resultant supernatant was used for western blot analysis. For mitochondria isolation, the cerebrum was homogenized in homogenization buffer B (0.32 M sucrose, 10 mM Tris–HCl, 1 mM EDTA and 0.2% fatty acid-free BSA, pH 7.4, protease inhibitor cocktail) at 600 r.p.m in a glass–teflon homogenizer. Cerebrum homogenate was centrifuged at 1000g for 5 min at 4°C. The supernatant was then centrifuged at 17 000g for 2 min at 4°C. The resultant pellet containing the mitochondrial fraction was washed in 1.5 ml of homogenization buffer B under similar conditions. The mitochondrial pellet was re-suspended in 100 μl of homogenization buffer A and stored in aliquots at ~80°C. For nuclei isolation, cerebrum was excised, weighed, washed with saline and homogenized in 0.5 ml phosphate inhibitor buffer (10 mM phosphate buffer, 150 mM NaCl, 2.7 mM KCl, 125 mM NaF, 250 mM β-glycerophosphate, 250 mM p-nitrophenyl phosphate and 25 mM NaVO3, pH 7.5) at 600 r.p.m with a Stuart Scientific SS2 stirrer with a teflon pestle. The homogenate was centrifuged at 300g for 5 min at 4°C, and the pellet obtained was re-suspended in 0.25 ml of ice-cold hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM NaF, 5 μl of 0.1 M Na2MoO4 and 0.1 mM EDTA). After incubation for 15 min at 4°C, 50 μl of 10% IGEPA was added, and the sample was then centrifuged at 14 000g for 1 min at 4°C. The nuclear pellet was re-suspended in 100 μl of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM NaF, 0.5 mM dithiothreitol, 1 μg/ml leupeptin and 0.2 mM phenylmethylsulfonyl
Mitochondrial respiration

To isolate fresh mitochondria, mice were sacrificed, and the organs were extracted rapidly on ice. Muscle and heart were submerged in 1 mg/ml proteinase K solution (for 30 s in heart and 60 s in hind leg skeletal muscle). Then, tissues were homogenized (1:10, w/v) in isolation buffer (250 mM sucrose, 2 mM EDTA, 10 mM Tris and 0.5% free fatty acids albumin, pH 7.4) at 800 r.p.m at 4°C with a glass–teflon homogenizer. The homogenate of each tissue was centrifuged for 5 min at 4°C, and the supernatant was centrifuged for 10 min at 4°C. The pellet was then centrifuged at 4°C for 10 min at 4°C. The pellet was re-suspended in buffer A and centrifuged again at 6000 g for 10 min at 4°C. The final crude mitochondrial pellet was re-suspended in MiR05 medium. Mitochondrial pellets were suspended in MiR05 medium.

Mitochondrial respiration was measured by high-resolution respirometry using an OROBOROS Oxygraph-2k instrument at 37°C (42). Respiration in isolated mitochondria (0.09–0.1 mg protein/ml in cerebrum; 0.2–0.6 mg protein/ml in kidney; 0.01–0.03 mg of protein/ml in heart; 0.01–0.03 mg protein/ml in muscle) was measured in 2 ml MiR05 medium that was previously equilibrated in the chamber with air at 37°C and stirred at 750 r.p.m until a stable signal at air saturation was obtained. The sequence of measurements of respiratory states was as follows: initial state 2 was measured in the presence of 10 mM glutamate plus 2 mM malate (respiration through complex I) or 10 mM succinate + 2.5 μM rotenone (respiration through complex II); then, maximal mitochondrial respiration (state 3) was stimulated by adding 0.25 mM ADP, and state 4 was determined after state 3 in brain mitochondria, when all added ADP was consumed. In kidney, heart and skeletal muscle mitochondria, state 4 was measured after the addition of 3 μg/ml of oligomycin (state 4s). State 3 and state 4 (or state 4s) were expressed in pmol O2/(s × mg protein). The RCR was calculated as the ratio of state 3 to state 4 (or state 4s).

Histology and immunohistochemistry

Mice tissues were formalin fixed and paraffin embedded. Multiple sections (4 μm thickness) were deparaffinized with xylene and stained with H&E, Masson’s TCM, PAS and LFB. Immunohistochemistry was carried out in the same sections, using the following primary antibodies: Glial fibrillary acidic protein or anti-GFAP (Millipore, MAB360), anti-oligodendrocytes (Millipore, MAB1580), Neuronal Class III β-tubulin anti-TUJ1 (Covance, MMS-435P), 8-OhDg (QED Bioscience, 12501) and 4-HNE (Alpha Diagnostic, HNE11-S) (42). The TUNEL method was performed in these samples using a commercial kit (ApopTag® Plus Peroxidase in situ Apoptosis Detection Kit, Code: S7101, Millipore) (43). Dako Animal Research Kit for mouse primary antibodies fluoride, followed by incubation at 4°C for 30 min on a shaking platform. After vortex mixing, the resulting suspension was centrifuged at 14 000 g for 10 min at 4°C. The supernatant (nuclear fraction) was stored in aliquots at -80°C (41).

Fourteen micrograms of proteins from the sample extracts were electrophoresed in a PhastGel™ Homogeneous 12.5% using a PhastSystem instrument (GE Healthcare Europe GmbH, Spain). The proteins were transferred to an Amersham Hybond™ ECL™ nitrocellulose membrane (GE Healthcare Europe GmbH, Spain) and probed with target antibodies. Protein–antibody interactions were detected with peroxidase-conjugated horse anti-mouse, anti-rabbit or anti-goat IgG antibodies (Santa Cruz Biotechnology, Inc.), using Amersham conjugated horse anti-mouse, anti-rabbit or anti-goat IgG antibodies. Bands quantification were carried out using an Image Station 2000R (Kodak, Spain) and the Kodak 1D 3.6 software. Quantitative values of the bands were normalized by GAPDH, actin, β-actin, VDAC1 or Lamin A/C (Santa Cruz Biotechnology, sc6215).

Mitochondrial respiration was measured by high-resolution respirometry using an OROBOROS Oxygraph-2k instrument at 37°C (42). The mitochondrial respiration buffer B (250 mM Sucrose, 0.5 mM Na2EDTA and 10 mM Tris), and an aliquot was used for protein determination. The remaining sample was then centrifuged at 6000 g for 10 min at 4°C. The pellet was re-suspended in buffer A and centrifuged again at 6000 g for 10 min at 4°C. The final crude mitochondrial pellet was re-suspended in MiR05 medium. Cerebrum was homogenized (1:10, w/v) in a respiration buffer C (0.32 mM Sucrose, 1 mM EDTA-K+ and 10 mM Tris–HCl, pH 7.4) at 500 r.p.m at 4°C in a glass–teflon homogenizer. The homogenate was centrifuged at 13 000 g for 3 min at 4°C. S1 was kept on ice, and the pellet was re-suspended in 5 ml of buffer A and centrifuged at 13 000 g for 3 min at 4°C. The subsequent supernatant (s2) was combined with s1 and centrifuged at 21 200 g for 10 min at 4°C. Mitochondrial pellet of this step was re-suspended in 0.85 ml of extraction buffer A containing 15% Percoll, poured into ultracentrifuge tubes containing a Percoll gradient formed by 1 ml 40% Percoll and 1 ml 23% Percoll in buffer A and centrifuged at 63 000 g for 30 min at 4°C. Pure mitochondria, corresponding to the fraction between 23 and 40% Percoll, were collected and washed twice with 1 ml of buffer A at 10 300 g for 10 min at 4°C. Mitochondrial pellets were suspended in MiR05 medium.
Measurement of adenine nucleotides levels

Adenine nucleotides were separated and quantified by a Gilson HPLC-UV (44). Briefly, mice were sacrificed by cervical dislocation, and the whole animal was immediately frozen in liquid nitrogen. Tissues were quickly dissected on a cold plate, and 50 µg of each piece of tissue was homogenized in 500 µl of ice-cold 0.5 M perchloric acid, kept on ice for 10 min and then centrifuged at 25,000g for 10 min at 4°C. The pellets were stored at −80°C for protein measurement. The supernatants were neutralized with 50 µl of 4 M K2CO3, kept on ice for 5 min and centrifuged at 12,000g for 10 min at 4°C. The resulting supernatants were kept at −80°C until injection into the HPLC. The mobile phase consisted of 100 mM potassium phosphate (pH 7), 1 mM tetrabutylammonium hydrogen sulfate and 1.5% acetonitrile. Sample was run isocratically at a flow rate of 1.5 ml/min through a Kromasil® C18 (5 µm; 4.6 × 250 mm) reverse-phase column. The retention times for AMP, ADP and ATP were 3.4, 6.9, and 11.8 min, respectively. Standard curves for AMP, ADP and ATP were constructed with 15, 30 and 60 mM of each nucleotide. Absorbance of the samples was measured with an UV detector at 254 nm wavelength, and the concentration of each nucleotide in the samples was calculated based on the peak area. Adenine nucleotide levels were expressed in nmol/mg protein.

Determination of the metabolite profile in urine

Urine samples were collected for 24 h and analyzed in a BS-200 Clinical Chemistry Analyzer (Mindray Medical España S.L., Spain) at 37°C. The following colorimetric tests were performed: urea, magnesium, creatinine, glucose, calcium Arsenazo III and albumin, microalbumin (Linear Chemicals S.L., Spain).

Open-field test

To check spontaneous locomotor activity, we used the open-field test. The apparatus consisted of a square arena in a ground space of 25 × 25 × 25 cm. Walls were opaque, so the animals cannot see the room. Each mouse was placed in the center of the square arena, and its movement monitored through the video-tracking system SMART® (Panlab S.L., Spain) for 30 min after an adaption period of 30 min. (45). For each mouse, distance traveled (cm) and vertical rears (numbers) were quantified. Those tests were performed between 8 and 9 p.m. under red light exposure. At the end of each trial, any defecation was removed, and the apparatus was wiped with 70% ethanol.

Statistical analysis

Data are expressed as the mean ± SD of 520 experiments per group. Two-tailed Student’s t-test was used to compare the mean between groups. A P-value of 0.05 was considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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