Pathomechanisms in Coenzyme Q₁₀-Deficient Human Fibroblasts

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Key Words

Apoptosis · ATP · Coenzyme Q₁₀ deficiency · Mitochondria · Mitophagy · Molecular basis of disease · mtDNA · Oxidative stress · Ubiquinone

Abstract

Primary coenzyme Q₁₀ (CoQ₁₀) deficiency is a rare mitochondrial disorder associated with 5 major clinical phenotypes: (1) encephalomyopathy, (2) severe infantile multisystemic disease, (3) cerebellar ataxia, (4) isolated myopathy, and (5) steroid-resistant nephrotic syndrome. Growth retardation, deafness and hearing loss have also been described in CoQ₁₀-deficient patients. This heterogeneity in the clinical presentations suggests that multiple pathomechanisms may exist. To investigate the biochemical and molecular consequences of CoQ₁₀ deficiency, different laboratories have studied cultures of skin fibroblasts from patients with CoQ₁₀ deficiency. In this review, we summarize the results obtained in these studies over the last decade.

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The first clinical report of coenzyme Q₁₀ (CoQ₁₀) deficiency was published by Ogasahara et al. [1989], who described 2 sisters with recurrent rhabdomyolysis associ-

ated with seizures and mental retardation. Myopathy was the predominant clinical feature in these patients. In their muscle, the activities of mitochondrial respiratory complexes I+III and II+III were markedly reduced, and their CoQ₁₀ levels were 3.7 and 5.4% of controls [Ogasahara et al., 1989]. From subsequent reports of patients with CoQ₁₀ deficiency, striking clinical heterogeneity of the condition has emerged [Quinzii et al., 2008a]. To date, CoQ₁₀ deficiency syndrome (MIM 607426) has been associated with 5 major clinical phenotypes: (1) encephalomyopathy, (2) severe infantile multisystemic disease, (3) cerebellar ataxia, (4) isolated myopathy, and (5) steroid-resistant nephrotic syndrome [Quinzii and Hirano, 2010]. Moreover, growth retardation, deafness and hearing loss have been described in some CoQ₁₀-deficient patients.

Since 2006, multiple molecular causes of ubiquinone deficiencies have been identified. Primary CoQ₁₀ deficiencies are due to pathogenic mutations in genes involved in the biosynthesis of CoQ₁₀. To date, 33 patients have been identified with primary CoQ₁₀ deficiency due to mutations in *COQ2* [Quinzii et al., 2006; Diomedici-Camassei et al., 2007; Mollet et al., 2007], *PDSS1* [Mollet et al., 2007], *PDSS2* [López et al., 2006], *COQ4* [Salviati et al., 2012], *COQ6* [Heeringa et al., 2011], *ADCK3* [Lagier-Tourenne et al., 2008; Mollet et al., 2008], and *COQ9*

[Duncan et al., 2009]. CoQ₁₀ deficiencies may also result from pathogenic mutations in genes not directly related to CoQ₁₀ biosynthesis, and such cases are referred to as secondary CoQ₁₀ deficiencies [Turunen et al., 2004; Montero et al., 2005, 2009; Quinzii et al., 2005; Aeby et al., 2007; Gempel et al., 2007; Le Ber et al., 2007; Miles et al., 2008; Sacconi et al., 2010; Côtan et al., 2011; Quinzii and Hirano, 2011].

The definitive diagnosis of CoQ₁₀ deficiency, in most patients, is based on measurements of CoQ₁₀ levels in muscle. Additionally, patients with primary CoQ₁₀ deficiency frequently show decreased CoQ₁₀ levels in skin fibroblasts. Consequently, cultured skin fibroblasts from patients with primary CoQ₁₀ deficiency have been utilized for biochemical characterization of CoQ₁₀ biosynthetic defects, in vitro evaluation of pathogenic mechanisms and in vitro assessments of efficacy of CoQ analogs supplementation.

Skin Fibroblasts for Diagnosis of Primary CoQ₁₀ Deficiency

Once CoQ₁₀ deficiency is confirmed in patients' muscle and skin fibroblasts, assessment of CoQ₁₀ biosynthesis in cultured skin fibroblasts can be used to prove the defect in the CoQ₁₀ biosynthetic pathway. These assays utilize radiolabeled substrates for the biosynthesis of CoQ₁₀ [López et al., 2006; Quinzii et al., 2006; Mollet et al., 2007; Tekle et al., 2008]. Typically, [³H]-mevalonate, which is the initial substrate used in the formation of the decaprenyl tail, and [¹⁴C]-4-hydroxybenzoate (4HB), which is precursor of the benzoquinone ring of the ubiquinone, are employed in cell culture. In addition, [³H]-decaprenyl-pyrophosphate ([³H]-decaprenyl-PP), which is poorly cell penetrant due to high lipophilicity, is used in homogenized fibroblast extracts. The combined use of [¹⁴C]-4HB and [³H]-decaprenyl-PP may be useful to discriminate defects upstream or downstream of the reaction catalyzed by decaprenyl diphosphate synthase. For example, fibroblasts with defects of COQ2 (which condenses 4-HB with decaprenyl-PP) produce less radiolabeled CoQ₁₀ with both [¹⁴C]-4HB and [³H]-decaprenyl-PP substrates [Quinzii et al., 2006], while, in contrast, PDSS2 mutant fibroblasts synthesize less radiolabeled CoQ₁₀ with [¹⁴C]-4HB but a normal amount of CoQ₁₀ with [³H]-decaprenyl-PP [López et al., 2006]. Multiple steps in the CoQ₁₀ biosynthetic pathway downstream of COQ2 cannot be distinguished with the available assays, but this may become possible in the future by the genera-

tion of new radiolabeled intermediates or by using tandem mass spectrometry methods to identify the accumulation of abnormal metabolites [Xie et al., 2012]. In fact, the accumulation of an abnormal metabolite was found in the COQ9 mutant fibroblasts by the routine electrochemical-high-performance liquid chromatography methods [Duncan et al., 2009]. However, identification of the chemical structure of this metabolite is not possible with high-performance liquid chromatography techniques.

Bioenergetics Defects and Oxidative Stress

The disparate phenotypes of CoQ₁₀-deficient patients suggest divergent pathogenic mechanisms in this syndrome. To address this issue, research laboratories have studied various molecular and biochemical changes of cultured skin fibroblasts from patients with primary CoQ₁₀ deficiency. Initially, effects of CoQ₁₀ deficiency on mitochondrial bioenergetics were assessed because of its essential function for mitochondrial ATP synthesis as an electron carrier from mitochondrial complexes I and II to complex III [Geromel et al., 2001, 2002; Quinzii et al., 2008b, 2010]. In addition, inherited mitochondrial respiratory chain defects frequently result in increased oxidative stress [Kirkinezos and Moraes, 2001], and CoQ₁₀ is recognized as an important endogenous antioxidant, which protects the cell both directly by preventing lipid peroxidation and indirectly by regenerating other antioxidants such as vitamins C and E [Mellors and Tappel, 1966; Turunen et al., 2004]; therefore, a second focus has been on the consequences of CoQ₁₀ deficiency on the fibroblasts' oxidative status. Geromel et al. [2001, 2002], studying CoQ₁₀-deficient fibroblasts of unknown genetic etiology, showed mild defects of mitochondrial respiration and growth rate, but no signs of oxidative stress. López-Martín et al. [2007] also showed defects in the ubiquinone-dependent complexes activities, CI+III and CII+III, in CoQ₁₀ deficiency fibroblasts with a COQ2 mutation. Quinzii et al. [2008b] demonstrated that CoQ₁₀-deficient fibroblasts with PDSS2 mutations and ~20% of residual CoQ₁₀ showed a severe bioenergetics defect, but no signs of oxidative stress, whereas COQ2 mutant fibroblasts with ~40% of residual CoQ₁₀ showed less severe bioenergetics defects with striking signs of oxidative stress. Similar to the PDSS2 mutant fibroblasts, COQ9 mutant fibroblasts with ~15% residual CoQ₁₀ showed bioenergetics defects without oxidative stress, whereas fibroblasts from an additional patient with

Fig. 1. ROS production in CoQ₁₀-deficient fibroblasts depends on the residual CoQ₁₀ levels. 25–45% of residual CoQ₁₀ levels induce maximal ROS production with increased cell death.

COQ2 mutations and ~35% residual CoQ₁₀ manifested increased oxidative stress markers [Quinzii et al., 2010]. The study was also extended to fibroblasts from 3 different CoQ₁₀-deficient patients due to *ADCK3* mutations and >50% of residual CoQ₁₀. In these cases, very mild defects in ATP levels were observed, and no signs of oxidative stress were detected. Taken together, the results indicated that the levels of CoQ₁₀ correlate with reactive oxygen species (ROS) production and oxidative damage: 10–20% and >50% residual CoQ₁₀ are not associated with significant oxidative stress, whereas 25–45% residual CoQ₁₀ is accompanied by increased ROS production and oxidative damage (fig. 1). Nevertheless, because the increase of oxidative stress was only limited to COQ2 mutant fibroblasts, it was possible that defective 4-parahydroxybenzoate:polyprenyl transferase (COQ2) protein specifically produced ROS. For this reason, Quinzii et al. [2012] evaluated mitochondrial bioenergetics and oxidative stress in cultured fibroblasts with CoQ₁₀ deficiency induced by pharmacological inhibition of CoQ₁₀ biosynthesis. The drug used to inhibit CoQ₁₀ biosynthesis, 4-nitrobenzoate (4-NB), competes with 4-HB in the reaction catalyzed by COQ2 [Forsman et al., 2010]. Pharmacological inhibition of CoQ₁₀ biosynthesis led to 55–70% decreases of the endogenous CoQ₁₀ (30–45% residual CoQ₁₀ relative to normal) in both control and *ADCK3* mutant fibroblasts. As a consequence of this, there were

decreases in ATP levels and increases in ROS production and oxidative damage [Quinzii et al., 2012]. These results confirmed that a range of 30–45% of residual CoQ₁₀ is the key factor in generating ROS (fig. 1). However, the fundamental mechanisms of ROS generation associated with CoQ₁₀ deficiency in human skin fibroblasts remain to be elucidated. In this regard, 2 possible mechanisms operating in mitochondria may be responsible: (1) reduction of CoQ-dependent CI+III activity may increase the NADH/NAD⁺ ratio in the matrix, leading to an increase of O₂ formation through the FMN group of the CI [Murphy, 2009]; or (2) increased proton motive force (e.g. increased mitochondrial membrane potential) without concomitant enhanced ATP synthesis, leading to increased ROS production [Korshunov et al., 1997; Murphy, 2009]. This second possibility is particularly relevant because hyperpolarization of mitochondria and increased ROS production have been observed in fibroblasts with 30–45% of residual CoQ₁₀ [Quinzii et al., 2010, 2012]. The reason for the increased mitochondrial membrane potential may apply to other respiratory chain defects because the F₁F₀-ATPase (complex V) activity is regulated by 2 factors: free energy of the adenosine phosphates and proton electrochemical potential. Thus, in the setting of impaired respiration, membrane potential is initially decreased, but if the cells have sufficient ATP supply, e.g. by the upregulation of glycolysis, complex V may operate as a proton-translocating ATPase, increasing the membrane potential by pumping protons in reverse [Scott and Nicholls, 1980; McKenzie et al., 2007]. Nevertheless, this mechanism does not explain how differences in mitochondrial membrane potential increase ROS generation in fibroblasts with 20–45% residual CoQ₁₀.

From a bioenergetics point of view, another interesting observation is the reduced levels of subunits of complex III and IV with normal levels of complex I subunits in immunoblots of extracts of CoQ₁₀-deficient fibroblasts with COQ2 mutations or other unknown etiological defects [Rodríguez-Hernández et al., 2009]. The authors suggested that CoQ₁₀ deficiency may affect the activity, organization and assembly of complex III as it has been reported in yeast [Santos-Ocaña et al., 2002], with a secondary effect on complex IV. Further experiments are needed to confirm these results in CoQ₁₀-deficient fibroblasts with different molecular defects and whether the destabilization of complex III is the cause or the effect of increased ROS production associated with CoQ₁₀ deficiency.

Fig. 2. Schematic diagram summarizing the pathomechanisms identified in CoQ₁₀-deficient fibroblasts. OXPHOS = Oxidative phosphorylation.

Apoptosis, Necrosis and Mitophagy

Increased oxidative stress and ATP depletion have been described as initial events promoting mitochondrial-mediated apoptotic cell death. In studies of CoQ₁₀-deficient skin fibroblasts, Quinzii et al. [2010] found that only the *COQ2* mutant fibroblasts showed increased apoptotic cell death. Because these cells also showed increased ROS production, the authors hypothesized that the induction of apoptosis was related to increased oxidative stress. Whether the oxidative stress-induced cell death was specific to *COQ2* defects or due to the severity of CoQ₁₀ deficiency was assessed by the pharmacological inhibition of CoQ₁₀ biosynthesis [Quinzii et al., 2012]. Once again, skin fibroblasts with 30–45% of residual CoQ₁₀ were associated with cell death as a consequence of the increased oxidative stress and independent of the molecular defect [Quinzii et al., 2012]. Another interesting observation of these studies is that, contrary to the initial expectations, *PDSS2* and *COQ9* mutant fibroblasts did not show increased cell death, despite the cells' severe depletion of ATP (fig. 2) [Quinzii et al., 2010]. One possible explanation is that cells with <20% of residual CoQ₁₀ acquire resistance to mitochondrial stress-induced apoptosis, compared to those with partial blockage of respiratory chain electron flux associated with a heightened sensitivity to cell death, as has been previously reported [Dey and Moraes, 2000; Park et al., 2004; Quinzii et al., 2010]. In support of this notion, studies have indicated that mitophagy can selectively degrade dysfunctional mitochondria

preventing the mitochondrial-dependent apoptotic cell death. In CoQ₁₀-deficient fibroblasts, Rodríguez-Hernández et al. [2009] observed increased levels of lysosomal markers and enhanced expression of transcriptional and translational levels of autophagic genes in cell lines carrying *COQ2* mutations and in 2 other cell lines from patients with CoQ₁₀ deficiency and unknown molecular defects. Because inhibition of autophagy resulted in apoptotic cell death, the authors suggested that autophagy is a protective mechanism involved in the degradation of dysfunctional mitochondria (fig. 2) [Rodríguez-Hernández et al., 2009]. Thus, *PDSS2* and *COQ9* mutant fibroblasts may have increased mitophagy to remove dysfunctional mitochondria. Additionally, compensatory production of ATP via upregulation of glycolysis may also protect cells by decreasing oxidative stress and associated cell death [Quinzii et al., 2008b].

Pyrimidine Metabolism

In addition to its bioenergetics and antioxidant role, CoQ₁₀ is also a cofactor in the reaction catalyzed by the mitochondrial protein dihydroorotate dehydrogenase [Rawls et al., 2000]. This enzyme catalyzes the oxidation of dihydroorotate to orotate at the FMN group, which is reduced to FMNH₂. FMNH₂ is then re-oxidized by reaction with ubiquinone, which is reduced to ubiquinol [Rawls et al., 2000]. The product of the reaction, orotate, is an intermediate of the de novo synthesis of the pyrimidines by the conversion to the ribonucleotide UMP, which can be converted through subsequent reactions in pyrimidine ribonucleotides and deoxyribonucleotides [Löffler et al., 1997; Rawls et al., 2000]. The conversion of ribonucleotides into deoxyribonucleotides is catalyzed by the ribonucleotide reductases [Nordlund and Reichard, 2006]. Until recently, it was thought that mtDNA replication and repair in quiescent cells was dependent on salvage synthesis of deoxynucleotide triphosphates [Ferraro et al., 2005], but the scenario changed suddenly with the identification of mutations in a p53 inducible small ribonucleotide reductase subunit, called p53R2 [Tanaka et al., 2000] in patients with mtDNA depletion [Bourdon et al., 2007]. Subsequent experiments confirmed that the de novo supply of deoxynucleotide triphosphates is required for replication and repair of mtDNA in cells and mice [Bourdon et al., 2007; Pontarin et al., 2008]. As a consequence of these findings, it has been postulated that the mtDNA depletion identified in the muscle of a patient with CoQ₁₀ deficiency may be the result of the reduction

in the deoxynucleotide triphosphates supply [Montero et al., 2009]. mtDNA depletion has also been described in skin fibroblasts with *COQ2* mutations [Quinzii et al., 2010], and the slow rates of growth observed in these fibroblasts were restored by addition of uridine in the culture medium [López-Martín et al., 2007] (fig. 2). Alternatively, it is possible that the uridine effect in the growth rate of the *COQ2* mutant fibroblasts may be due to the role of uridine metabolism in the glycolysis through the catabolism of galactose [Petry and Reichardt, 1998].

Treatment Evaluation in CoQ₁₀-Deficient Skin Fibroblasts

The only therapeutic option currently available for CoQ₁₀ deficiency syndrome is exogenous CoQ₁₀ supplementation. However, of patients with identified mutations, only 20% improved after exogenous CoQ₁₀ supplementation [Rahman et al., 2001; López et al., 2006; Diomedi-Camassei et al., 2007; Mollet et al., 2007, 2008; Lagier-Tourenne et al., 2008; Montini et al., 2008; Heeringa et al., 2011]. In order to understand the low percentage of successful treatments, studies have evaluated the effects of CoQ₁₀ supplementation in CoQ₁₀-deficient fibroblasts. We attempted to counteract the bioenergetics defect and oxidative stress of the CoQ₁₀-deficient fibroblasts by adding 5 µM of CoQ₁₀ in culture cells [López et al., 2010]. The results showed that CoQ₁₀ supplementation for 1 week, but not for 24 h, improved ATP levels and ATP/ADP ratio. Similar to these results, yeast *coq* mutants showed an inefficient uptake of exogenous CoQ₆ to the mitochondrial inner membrane, which was reflected in a low succinate cytochrome *c* reductase activity after 2–15 µM CoQ₆ supplementation for 48 h [Do et al., 2001; Santos-Ocaña et al., 2002]. The complete rescue of growth of the yeast *coq* mutants supplemented with 15 µM CoQ₆ was only possible after 6–8 days [Do et al., 2001; Jonassen et al., 2002; Santos-Ocaña et al., 2002]. In contrast to these results, López-Martín et al. [2007] noted normalization of mitochondrial complexes I+III and II+III activities in *COQ2* mutant fibroblasts after 24 h of 10 µM CoQ₁₀ supplementation. Paradoxically, the same authors, using the same *COQ2* mutant cells and other genetically undefined CoQ₁₀-deficient fibroblasts, found that the activities of complex II+III increased only slightly and remained below control values after 72 h of 100 µM CoQ₁₀ supplementation [Rodríguez-Hernández et al., 2009]. This discrepancy may be due to the fact that in the second study, but not in the first, respiratory chain enzyme activities were

normalized to activity of citrate synthase [Rodríguez-Hernández et al., 2009], a marker of mitochondrial mass [Kirby et al., 2007]. The discrepant results may be also explained by different methodologies used to evaluate mitochondrial bioenergetics after CoQ₁₀ supplementation: López et al. [2010] measured cellular ATP levels and ATP/ADP ratio; in the other 2 studies spectrophotometric methods were used to measure activities of CoQ-dependent complexes. The enzymatic activity assay may produce artifacts due to sonication, freeze-thaw, or detergents that fracture mitochondria and allow access of the CoQ₁₀ accumulated in the cellular membrane to the inner mitochondrial membrane. Additionally, these manipulations disrupt the mitochondrial supercomplexes, minimizing the differences between the experimental groups [Kruse et al., 2008]. Finally, the different sources of the CoQ₁₀, its different formulations and the different solubilization methods may also influence the experimental results.

Because in vitro studies suggested that high lipophilicity of CoQ₁₀ is the major cause of delayed effects of CoQ₁₀ supplementation, the effects of less lipophilic ubiquinone analogs with shorter isoprenoid tail were also tested. However, the 2 short-tail ubiquinone analogs, CoQ₂ and idebenone, did not correct bioenergetics defects [López et al., 2010], which highlights the importance of the decaprenyl tail. Despite the lack of correction of the bioenergetics defect in *COQ2* mutant fibroblasts, all of the CoQ analog tested decreased superoxide anion production and oxidative stress-induced cell death [López et al., 2010]. Thus, the in vitro study of CoQ₁₀-deficient fibroblasts revealed important insights regarding CoQ₁₀ supplementation therapy: (1) the prolonged pharmacokinetics of CoQ₁₀ in restoring respiratory chain activity in CoQ₁₀-deficient cells [Bentinger et al., 2003], a factor that may contribute to the late clinical response to the oral supplementation of CoQ₁₀ [Montini et al., 2008] and suggest that high doses of CoQ₁₀ must be administered; (2) short-tail ubiquinone analogs do not substitute for CoQ₁₀ in the mitochondrial respiratory chain revealing the importance of the decaprenyl tail; and (3) oxidative stress and cell death can be ameliorated by the administration of lipophilic and hydrophilic antioxidants [López et al., 2010].

Concluding Remarks

Studies of CoQ₁₀-deficient fibroblasts indicate that primary CoQ₁₀ deficiencies cause variable defects on ATP synthesis, oxidative stress and cell death, which appear to be related to the specific molecular defect, residual level

of CoQ₁₀, or both (fig. 2). Therapy leads to the amelioration of these effects. Mitochondrial apoptosis has been observed in CoQ₁₀-deficient cells; however, the pathways involved in the induction of this pathway remain to be elucidated. The studies in CoQ₁₀-deficient fibroblasts have been limited by the small number of available cell lines. The clinical heterogeneity and tissue-specificity of CoQ₁₀ deficiency syndrome suggest that differentiated cells may respond to the deficit in ubiquinone differently compared to fibroblasts. In support of this premise, a recent study in a human neuronal model of CoQ₁₀ deficiency has shown different degrees of mitochondrial respiratory chain dysfunction and oxidative stress relative to CoQ₁₀-deficient fibroblasts [Duberly et al., 2013]. Future studies on additional cellular and animal models will help us understand the clinical heterogeneity of CoQ₁₀ deficiency syndrome and may contribute to the development of more effective therapies.

Acknowledgements

This work was partially supported by grants from the Marie Curie International Reintegration Grant Programme (COQMIT-MEL-266691) within the 7th European Community Framework Programme, from Ministerio de Economía y Competitividad, Spain (SAF2009-08315), from the Consejería de Economía, Innovación, Ciencia y Empleo, Junta de Andalucía (P10-CTS-6133) and from the 'CEIBioTic' (20F12/1) and 'Incent' Programs from the Universidad de Granada. L.C.L. is supported by the 'Ramón y Cajal' National Programme, Ministerio de Economía y Competitividad, Spain. M.L.-S. is a predoctoral fellow from the Consejería de Economía, Innovación, Ciencia y Empleo, Junta de Andalucía. C.M.Q. is supported by K23HD065871 from the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD). M.H. is supported by NIH grants 1R01HD057543, R01HD056103 and 1RC1NS070232, a Muscular Dystrophy Association grant, and by the Marriott Mitochondrial Disorder Clinical Research Fund (MMDCRF).

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