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Research Paper

Coenzyme Q deficiency triggers mitochondria degradation by mitophagy

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Abbreviations: CS, citrate synthase; CoQ, coenzyme Q_{10}; CsA, cyclosporin A; 3MeA, 3-methyl adenine; MPT, mitochondrial permeability transition; MRC, mitochondrial respiratory chain; PABA, *para*-aminobenzoic acid; pHB, p-hydroxybenzoic acid; ROS, reactive oxygen species

Key words: autophagy, coenzyme Q, free radicals, mitochondria, mitochondrial disease, mitochondrial permeability transition, mitophagy

Coenzyme Q_{10} (CoQ) is a small lipophilic molecule critical for the transport of electrons from complexes I and II to complex III in the mitochondrial respiratory chain. CoQ deficiency is a rare human genetic condition that has been associated with a variety of clinical phenotypes. With the aim of elucidating how CoQ deficiency affects an organism, we have investigated the pathophysiologic processes present within fibroblasts derived from four patients with CoQ deficiency. Assays of cultured fibroblasts revealed decreased activities of complex II + III, complex III and complex IV, reduced expression of mitochondrial proteins involved in oxidative phosphorylation, decreased mitochondrial membrane potential, increased production of reactive oxygen species (ROS), activation of mitochondrial permeability transition (MPT), and reduced growth rates. These abnormalities were partially restored by CoQ supplementation. Moreover, we demonstrate that CoQ-deficient fibroblasts exhibited increased levels of lysosomal markers (β-galactosidase, cathepsin, LC3 and Lyso Tracker), and enhanced expression of autophagic genes at both transcriptional and translational levels, indicating the presence of autophagy. Electron microscopy studies confirmed a massive degradation of the altered mitochondria by mitophagy. Autophagy in CoQ-deficient fibroblasts was abolished by antioxidants or cyclosporin treatments suggesting that both ROS and MPT participate in this process. Furthermore, prevention of autophagy in CoQ-deficient fibroblasts by 3-methyl adenine or wortmannin, as well as the induction of CoQ deficiency in cells lacking autophagy (by means of genetic knockout of the Atg5 gene in mouse embryonic fibroblasts) resulted in apoptotic cell death, suggesting a protective role of autophagy in CoQ deficiency.

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Introduction

Coenzyme Q_{10} (CoQ) is an essential electron and proton carrier in the mitochondrial respiratory chain (MRC), transferring electrons from complexes I and II to complex III,[1] and contributing to the ATP biosynthesis. Furthermore, CoQ is essential for the stability of complex III in the mitochondrial respiratory chain,[2] functions as an antioxidant in cell membranes,[3] and is involved in multiple aspects of cellular metabolism. CoQ is also required for pyrimidine nucleoside biosynthesis and may modulate apoptosis and mitochondrial uncoupling proteins.[4] CoQ is composed of a benzoquinone ring, synthesized from tyrosine, and a polyprenyl side-chain, generated from acetyl-CoA through the mevalonate pathway.[5] In eukaryotes, the biosynthesis of CoQ is a very complex process involving at least ten genes (COQ genes).[6] Mutations in these genes cause primary CoQ deficiency,[7] a severe and often fatal multisystem disorder. However affected patients respond well to oral CoQ supplementation. To date, mutations have been identified in COQ2, PDSS1 and PDSS2 genes.

The first COQ2 mutation was found in two patients with encephalomyopathy and nephropathy.[8,9] This gene encodes the para-hydroxybenzoate-polyprenyltransferase that condensates the 4-OH benzoate ring with the prenyl side chain. Mutations in the PDSS2 gene, encoding the small subunit of prenyldisphosphate synthase, have also been reported in a patient with Leigh syndrome and nephropathy,[10] as well as a homozygous PDSS1 misssense mutation in two patients with a less severe multisystem disease.[11] Some patients with cerebellar ataxia or isolated myopathies have secondary CoQ deficiencies due to mutations in APTX and ETFDH, genes not directly related to ubiquinone synthesis.[12-15]

The hallmark of CoQ-deficiency syndrome is a decrease in CoQ levels in muscle and/or fibroblasts. Patients exhibit variable degrees of CoQ deficiency in muscle and/or fibroblasts causing decrease of CoQ-dependent MRC activities, such as NADH:citochrome *c* oxidoreductase (complex I + III) and succinate:citochrome *c* oxidoreductase (complex II + III). However, the effects of CoQ deficiency on other MRC aspects, such as ATP synthesis, oxygen consumption, and mitochondrial membrane potential measurements

Table 1 **Biochemical analysis of CoQ levels, incorporation of radiolabeled substrate to CoQ, and fibroblast growth after CoQ supplementation (100 μM) in cultured skin fibroblasts from 4 patients with a CoQ deficiency syndrome**

Patient	CoQ (nmol/g protein)	^{14}C-*p*-hydroxybenzoate (cpm x 10^6/mg protein)	Fibroblast growth upon CoQ supplementation (1 week) (% increase in growth rate)
Case 1	44	(3.20) 22% of control	96%
Case 2	64	(3.90) 26% of controls	87%
Case 3	37	(2.17) 15% of controls	32%
Case 4	21	(1.45) 10% of controls	29%
Control values (n = 20)	107–145 (129)	8.20–18.50 (14.50)	10%

Case 1 and 2 are two patients from the same family who showed a remarkable clinical response to CoQ supplementation. Cases 3 and 4 present a pathogenic mutation in COQ2 gene.

have scarcely been reported. Some authors have reported decreases in oxygen consumption (state-3 respiration) in muscle mitochondria for several substrates except ascorbate/TMDP,[16,17] suggesting a CoQ-level dependent impairment of oxidative phosphorylation. A reduction in the mitochondrial membrane potential of cultured fibroblasts from patients with CoQ deficiency has also been demonstrated.[18,19] These findings suggest that dysfunctional oxidative phosphorylation may be an important factor in CoQ deficiency syndromes. There have been few reports regarding antioxidant status and oxidative stress in CoQ deficiency, and even these few have yielded conflicting results.[20-22] In some studies, no signs of oxidative stress could be found in CoQ-depleted fibroblasts,[23,24] yet in another study, CoQ-deficient fibroblasts incubation with dihydrorodamine (a marker of hydrogen peroxide production) showed a two-fold increase in fluorescence compared with control values.[18] In light of these observations, a more exhaustive and complete assessment of the role of oxidative stress in CoQ deficiency syndrome, appears necessary. In addition, studies examining cell death, in the context of CoQ deficiency, have also been limited and somewhat contradictory. While an increase in apoptotic features has consistently been demonstrated in muscle biopsies of CoQ-deficient patients,[21] no evidence of apoptosis could be established in fibroblasts of CoQ-deficient patients in another study.[20] Therefore, changes in cell death (apoptosis or autophagy), that are closely related to increased free radical damage, merit further investigation with respect to CoQ deficiency syndromes.

Here we aim to assess the pathophysiology of this disease in cultured fibroblasts derived from four patients with an oxidative phosphorylation defect associated with CoQ deficiency.

Results

Biochemical characterization of fibroblasts derived from four patients with CoQ deficiency. First, we confirmed that fibroblasts derived from the patients were deficient for CoQ. Fibroblast analysis results are reported in Table 1. All four cases showed clearly decreased CoQ levels, and a lower rate of CoQ biosynthesis with respect to control fibroblasts. The reduced growth rate of patients was restored by CoQ supplementation.

Mitochondrial dysfunction in CoQ deficiency. As CoQ is an essential component of the mitochondrial respiratory chain, the reduced levels of CoQ in fibroblast would be expected to induce an alteration in the normal mitochondrial electron flow. To determine the presence of mitochondrial dysfunction in CoQ deficient cells, we measured the activities of respiratory enzymes in control and patient fibroblasts (Fig. 1). As expected, patient fibroblasts showed a significant reduction in the activity of those mitochondrial respiratory enzymes whose activities depend directly on CoQ (complex III and complex II + III). In addition, the activity of complex IV was also significantly decreased (Fig. 1). In contrast the activity of complex I was not significantly different from control values.

To further examine mitochondrial dysfunction in CoQ-deficient cells, we compared the expression of mitochondrial proteins in control and patient fibroblasts by western blotting. As shown in Figure 2A, the expression of mitochondrial protein subunits of complexes III (core I subunit), and IV (COX II subunit) was reduced in patient fibroblasts, correlating with their reduced enzyme activities.

To verify that these alterations were directly related to CoQ deficiency, we treated fibroblasts with CoQ for 72 hours and checked for the recovery of mitochondrial functionality. As is shown in Figure 1, enzyme activities were significantly restored following CoQ supplementation. As CoQ is a component of complex III and is required to its stability, proteins from this complex were analyzed (Fig. 2B). CoQ restored partially the composition of complex III.

Mitochondrial membrane potential in CoQ deficiency. To assess the functional consequences of reduced respiratory enzyme activities and protein levels in mitochondria in CoQ deficiency, we determined mitochondrial membrane potential ($\Delta\Psi_m$) in both control and patient fibroblasts by flow cytometry and fluorescence microscopy. As shown in Figure 3A, patient fibroblasts showed a significant reduction in $\Delta\Psi_m$ indicating a lower capacity to produce ATP in CoQ deficient fibroblasts.

ROS production in CoQ deficiency. It is well known that mitochondrial dysfunction is associated with an induction of ROS production in mitochondria. Therefore, we examined ROS levels in control and patient fibroblasts by flow cytometry using the dichlorodihydrofluorescein probe. Figure 3B shows an increased production of ROS in patient fibroblasts compared to control. We also confirmed that CoQ supplementation significantly reduced ROS production.

Mitocondrial permeability transition (MPT) pore in CoQ deficiency. As a major source of ROS production, mitochondria are especially prone to ROS damage. Such damage can induce the mitochondrial permeability transition (MPT) caused by the opening of nonspecific high conductance permeability transition pores in the mitochondrial inner membrane.[25] To investigate whether CoQ deficiency can induce MPT in patient fibroblasts, we employed the acetoxymethyl (AM) ester of calcein, and $CoCl_2$, a

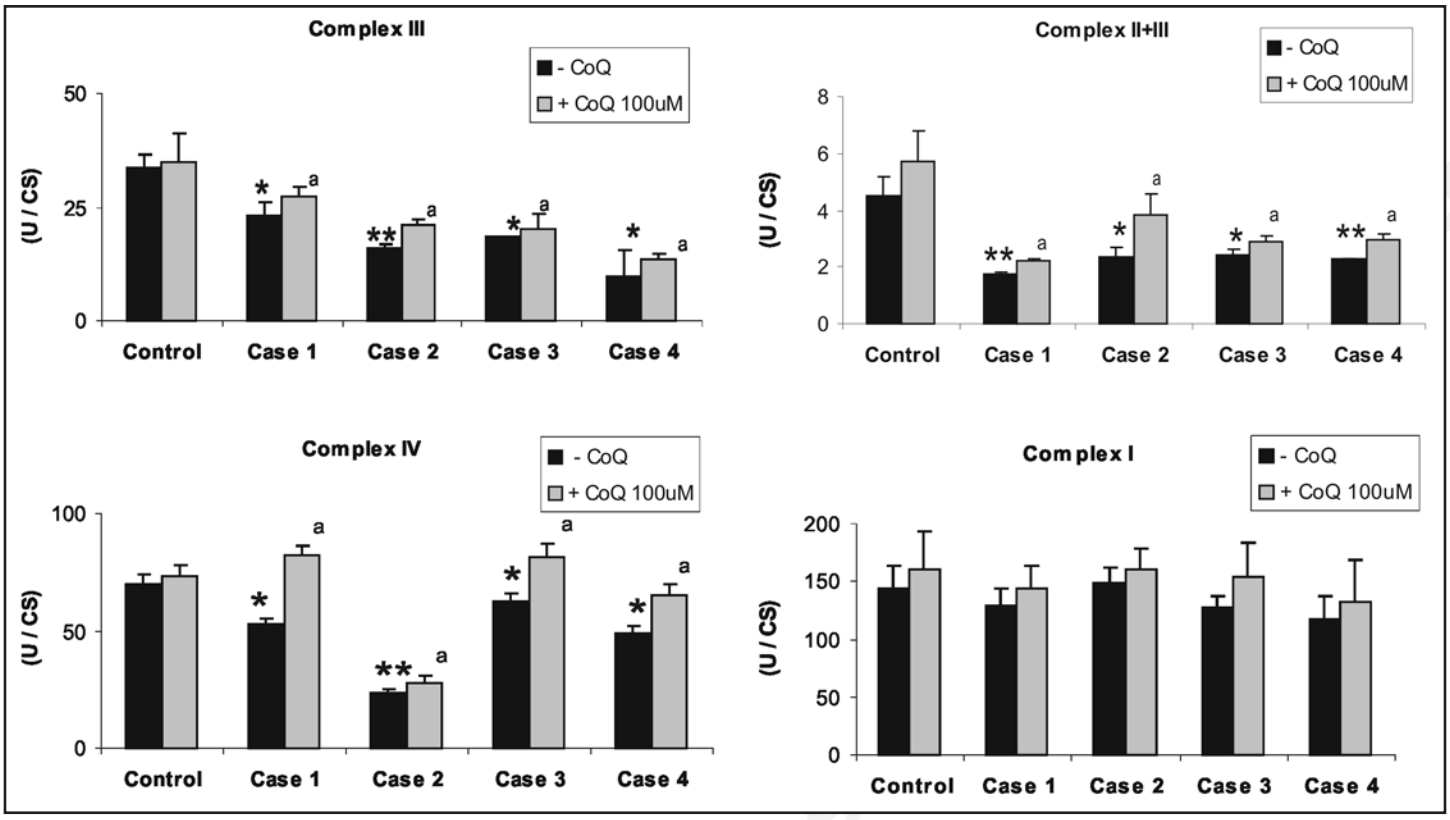

Figure 1. Mitochondrial enzyme activities of respiratory chain complexes in human with CoQ deficiency. NADH:coenzyme Q1 oxidoreductase (complex I), cytochrome *c* oxidase (complex IV), ubiquinol:cytochrome *c* oxidoreductase (complex III), succinate:cytochrome *c* reductase (complex II + complex III) and citrate synthase (CS) were determined as described in Material and Methods. Recovery of enzyme activities in patients fibroblasts under CoQ supplementation (100 μM) for 72 hours, was also assayed. Results (mean ± SD) are expressed in U/CS (units per citrate synthase). *p < 0.05, **p < 0.01 between control and CoQ deficient fibroblasts. ºp < 0.05 between the presence and the absence of CoQ.

quencher of calcein fluorescence, to selectively label mitochondria. In control fibroblasts a distinct mitochondrial label was preserved such that it colocalizes with MitoTracker red reagent signal (Fig. 4A). Calcein fluorescence was lost in mitochondria presenting MPT in CoQ-deficient fibroblasts (Fig. 4A and B). We also confirmed that CoQ supplementation significantly reduced the number of fibroblasts with MPT (Fig. 4B).

Mitochondrial degradation in CoQ deficiency. Recent evidence suggests a possible involvement of ROS and MPT in autophagy.[25,26] In the MPT, the opening of PT pores causes mitochondria to become permeable to all solutes up to a molecular mass of about 1500 Da, an event leading to the activation of the autophagy machinery. To verify the mitochondrial degradation in CoQ deficiency we first analyzed the expression of proteins involved in autophagic processes such as Atg12, Beclin and LC3. Figure 5A and B show that these autophagic proteins (and their corresponding mRNAs) were overexpressed in patient fibroblasts as compared to control fibroblasts. We also investigated the conversion of LC3-I to LC3-II as a marker of autophagic activity. We found a significant increase in LC3-II conversion in patient extracts indicating enhanced autophagosome formation (Fig. 5B). In addition, we found much greater intensity of lysosomal indicators such as β-galactosidase, LC3, cathepsin D, LysoTracker in CoQ-deficient fibroblasts, suggesting increased autophagy in these cells (Fig. 6A). We also quantified levels of acidic vacuoles by using Lysotracker staining and flow cytometry analysis. Acidic vacuoles were significantly increased in patient fibroblasts with respect to controls (Fig. 6B). To confirm the presence of mitochondrial degradation or mitophagy in CoQ deficient cells, we performed electron microscopy on control and patient fibroblasts. Figure 7A–C clearly show the presence of autophagosomes, and laminar bodies in patient fibroblasts indicating extensive autophagy. In early autophagosomes we can clearly see mitochondria that are being degraded. These ultrastructural alterations were markedly alleviated by CoQ supplementation. To verify this hypothesis we performed a double staining immunofluorescence of cathepsin, a lysosomal marker and cytochrome *c*, a mitochondrial marker. Figure 8 clearly shows that cathepsin D colocalized with cytochrome *c*, suggesting that mitochondria were being degraded within autophagosomes. Colocalization was addressed in cells in which no sign of apoptosis was observed.

Role of CoQ, ROS generation and MPT in autophagy. In order to elucidate whether autophagy in CoQ deficient fibroblasts could be mitigated by restoring mitochondrial functionality by CoQ supplementation, we cultured both control and patient fibroblasts in the presence of CoQ (100 μM) for 72 hours and analyzed them by β-galactosidase staining. As is shown in Figure 9, CoQ supplementation drastically reduced the intensity of β-galactosidase staining, indicating a reduction in lysosomal activity following CoQ treatment.

To further examine the role of ROS generation in CoQ-deficiency induced autophagy, we cultured patient fibroblasts in the presence

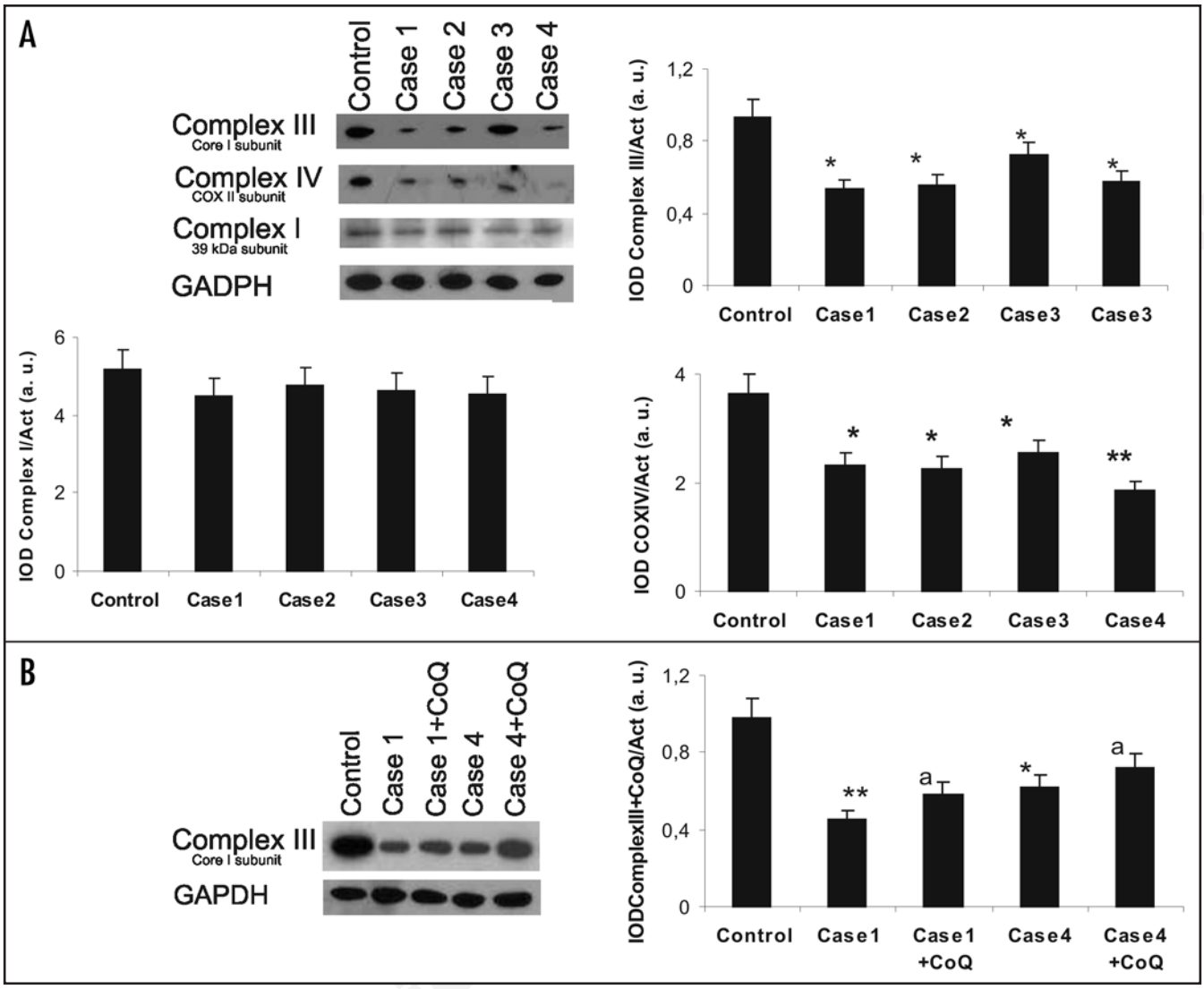

Figure 2. Western blot analysis of mitochondrial proteins in CoQ deficiency. (A) Fibroblast protein extracts (50 μg) were separated on a 12.5% SDS polyacrylamide gel and immunostained with antibodies against complex I (39 KDa subunit), complex III (core 1 subunit), and complex IV (subunit II). (B) Recovery of complex III expression levels in patient fibroblats under CoQ supplementation (100 μM) for 72 hours. Protein levels were determined by densitometric analysis of three different western blots and normalized to GADPH signal. *$p < 0.05$, **$p < 0.01$ between control and CoQ deficient fibroblasts. $^{a}p < 0.05$ between the presence and the absence of CoQ.

of two antioxidants, BHA and N-acetylcisteine, and examined the rate of autophagy. As we have earlier seen for CoQ supplementation, both antioxidants significantly attenuated autophagy (Fig. 9).

To confirm a role for the MPT opening in CoQ deficiency-induced autophagy, we cultured patient fibroblasts in the presence of cyclosporine (5 μM), an immunosuppressant which binds to cyclophilin D and inhibits MPT pore opening. As is shown in Figure 9, the inhibition of MPT by cyclosporine decreased autophagy. In contrast, tacrolimus (5 μM), an immunosuppressant that does not block MPT, had no effect on autophagy (Fig. 9). Together these observations suggest that the ROS production and MPT activation in CoQ deficient cells may be involved in triggering autophagy.

Physiopathology of autophagy in CoQ deficiency. In order to elucidate whether autophagy in CoQ-deficient fibroblasts was a protective or pathological mechanism, we examined the effect of blocking autophagy using 3-MeA (20 mM) or wortmannin (10 μM), well-characterized inhibitors of the early stages of autophagy. First, we confirmed that both inhibitors effectively inhibited autophagy and reduced the β-galactosidase staining of CoQ deficient fibroblats (Fig. 10A). We then examined CoQ deficient and control fibroblasts for viability and apoptosis. Figure 10 clearly shows that inhibiting autophagy in CoQ-deficient fibroblasts significantly reduced cell viability and increased the rate of apoptosis. These observations strongly suggest that autophagy plays a protective role in CoQ-deficient fibroblasts through the recycling/elimination of dysfunctional mitochondria (Fig. 10B and C).

CoQ deficiency in Atg5$^{-/-}$ MEFs sensitized to apoptosis. To verify the role of autophagy in survival of CoQ deficient cells, apoptosis was examined in CoQ deficient wild-type and Atg5$^{-/-}$ MEFs. CoQ deficiency was produced in these cells by treatment with PABA, a specific inhibitor of CoQ biosynthesis.[27] First, we verified that PABA treatment indeed induced CoQ deficiency in wild-type and knockout fibroblasts (Fig. 11A). In the case of MEFs, we determined CoQ_9, which is the predominant form of coenzyme Q in

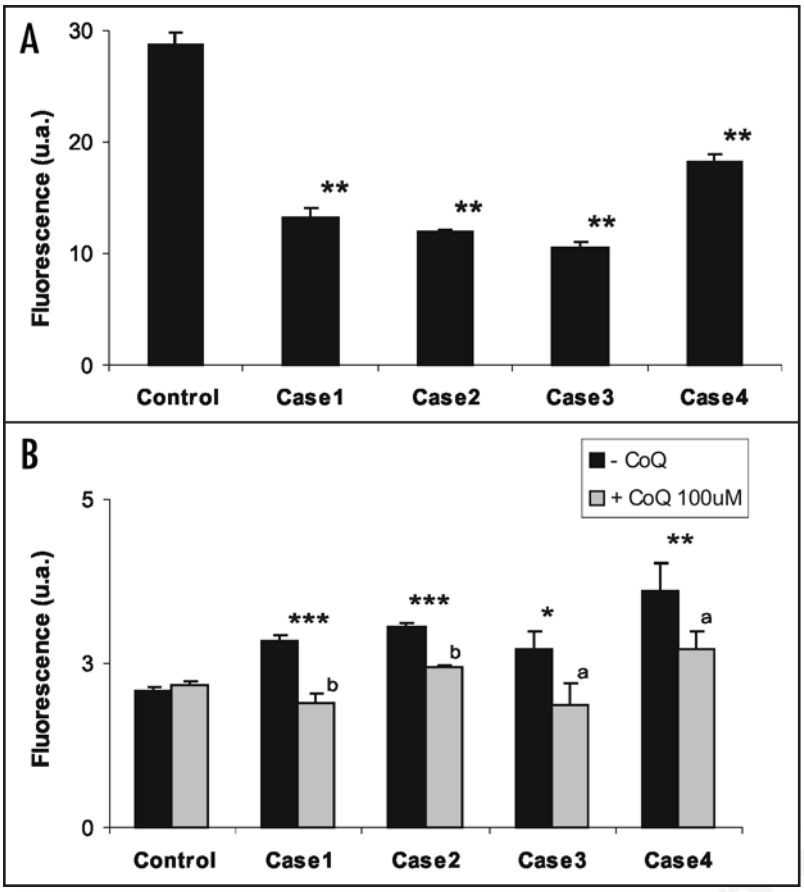

Figure 3. Mitochondrial dysfunction in CoQ deficiency. (A) Mitochondrial depolarization in CoQ-deficient fibroblasts. Membrane potential was assessed by flow cytometry using MitoTracker Red (Molecular Probes, Eugene, OR). A clear decrease of mitochondrial potential was observed in patient fibroblasts. **p < 0.01 between control and CoQ-deficient fibroblasts. (B) ROS generation in CoQ deficiency. Cellular generation of ROS in fibroblasts from control and patients was determined by flow cytometry using dichlorodihydrofluorescein, as described in Materials and Methods. We also tested the effect of CoQ supplementation (100 μM) during 72 hours in ROS generation. Results are expressed as mean ± SD of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 between control and CoQ deficient fibroblasts. ^{a}p < 0.05, ^{b}p < 0.01 between the presence and the absence of CoQ.

mouse cells. We then analyzed cell viability and apoptosis in both CoQ-deficient wild-type and knockout fibroblasts. CoQ deficiency induced a high viability and low level of apoptosis in wild-type MEFs as determined by detecting caspase activation, cytochrome *c* release, and nuclear condensation (Fig. 11B). In contrast, Atg5$^{-/-}$ MEFs were significantly more sensitive to CoQ deficiency induction with a significant decrease in cell viability and a two-fold increase in apoptosis over that in wild-type cells (Fig. 11B). CoQ supplementation of PABA-treated MEFs reduced cell death to control values, confirming the specificity of apoptotic induction by CoQ deficiency.

Discussion

The molecular basis and pathogenic mechanisms of the various primary and secondary forms of CoQ deficiency remain largely unknown. In this work, we studied the pathophysiology of CoQ deficiency in cultured fibroblasts derived from four patients with CoQ deficiency.[8,9,18] First, we assessed mitochondrial function in cultured fibroblasts. As expected, CoQ-deficient fibroblasts showed reduced CoQ-dependent respiratory enzymatic activities (Compex III and Complex II + III). Interestingly, Complex IV also showed reduced activity, while the activity of complex I was not affected. These data correlate with the reduced levels of subunits of complex III and IV seen on immunoblots, while the levels of complex I subunits were not affected. Thus, the expression of proteins of complexes III and IV were reduced, while the expression of proteins of complex I was not affected. These observations suggest that the primary effect of CoQ deficiency might be to affect the activity, organization and assembly of complex III as it has been reported in yeast,[2] with effects on complex IV being a secondary consequence. The respiratory chain consists of interconnected complex I_1-complex III_2-complex IV_4, and III_2IV_4 supercomplexes in a 2:1 ratio.[28] Therefore, complex III disorganization might affect complex IV association with complex III in these large supercomplexes,[29] thus altering complex IV activity and composition.[30]

Deficient mitochondrial protein expression levels and reduced respiratory enzyme activities may impair normal mitochondrial electron flow and proton pumping, inducing a drop in mitochondrial membrane potential. Our data has shown that CoQ-deficient fibroblasts possess reduced mitochondrial potential, which may contribute to impaired mitochondrial protein import and aggravate mitochondrial dysfunction. Similar results in fibroblasts harboring COQ2 or PDSS2 mutations have been recently shown.[19]

ROS production and oxidative stress is another common consequence of dysfunctional mitochondria. Our results show a significant increase in ROS generation in CoQ-deficient fibroblasts that was partially mitigated by CoQ supplementation. CoQ deficiency does not always produce oxidative stress,[23,24] and depends on the severity of CoQ biosynthetic defect.[19] Generated ROS can be released into cytosol and trigger "Reactive Oxygen Species (ROS)-induced ROS-release" (RIRR) in neighboring mitochondria. This mitochondrion-to-mitochondrion ROS-signaling constitutes a positive feedback mechanism for enhanced ROS production, potentially leading to significant mitochondrial injury.[31] Recent studies by a number of groups have demonstrated that ROS can directly modify signaling proteins through different modifications, for example by nitrosylation, carbonylation, disulphide bond formation and glutathionylation. Moreover, redox modification of proteins permits further regulation of cell signaling pathways.[32] Thus, it has been proposed that ROS damage can induce the mitochondrial permeability transition (MPT) by opening of nonspecific high conductance permeability transition (PT) pores in the mitochondrial inner membrane.[25,33,34] This, in turn, leads to a simultaneous collapse of mitochondrial membrane potential. Our results verify this hypothesis, showing the presence of MPT in CoQ deficient fibroblasts. In the MPT, the opening of PT pores causes mitochondria to become permeable to all solutes up to a molecular mass of about 1500 Da.[35,36] After the MPT, mitochondria undergo a dramatic swelling driven by colloid osmotic forces, which culminates in the rupture of the outer membrane and release of proapoptotic mitochondrial intermembrane proteins into the cytosol, such as cytochrome *c*, apoptosis inducing factor, Smac/Diablo and

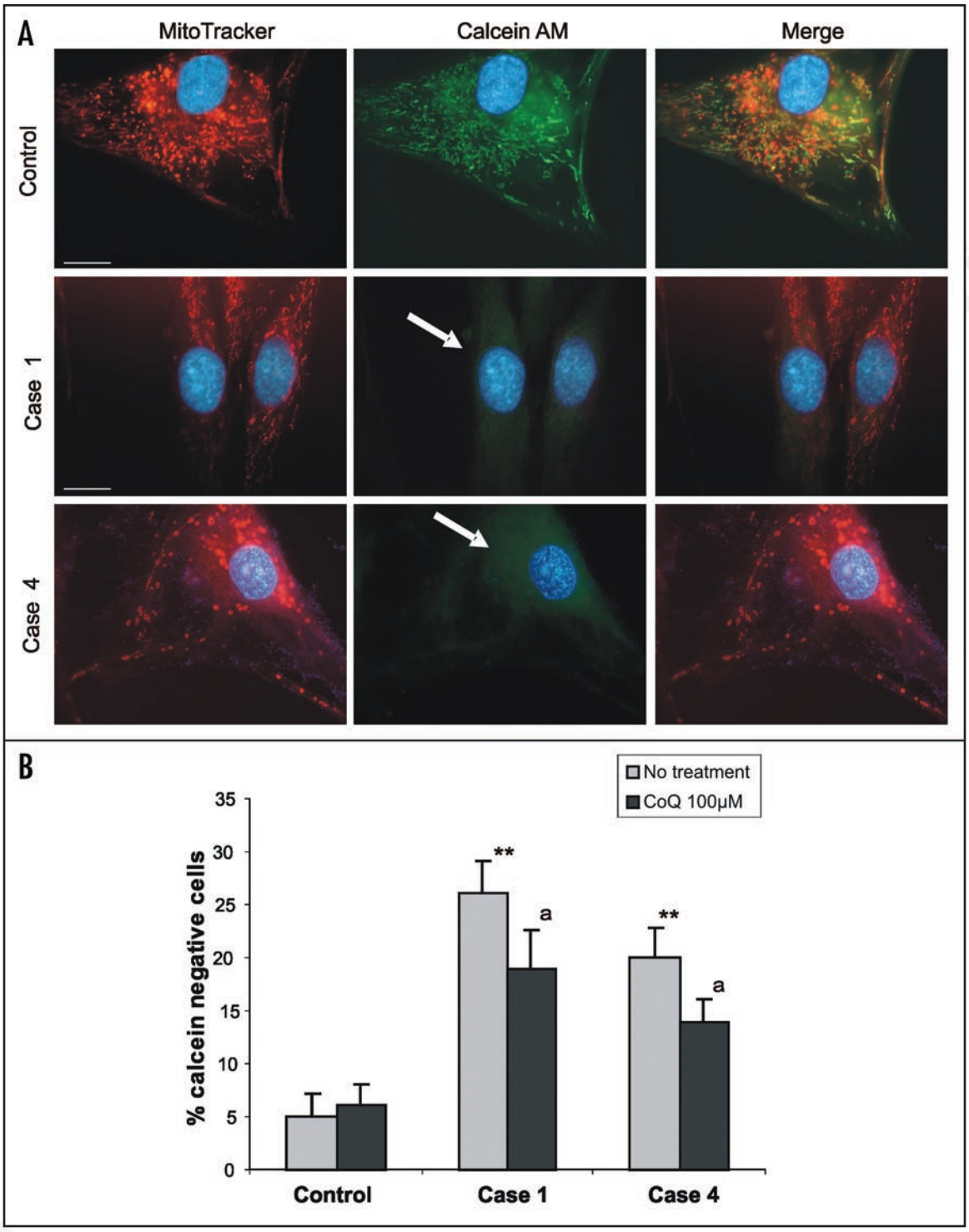

Figure 4. Imaging the Mitochondrial Permeability Transition Pore in control and CoQ deficient fibroblasts. (A) Fibroblasts were loaded with calcein AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including mitochondria. The fluorescence from cytosolic calcein was quenched by the addition of $CoCl_2$, while the fluorescence from the mitochondrial calcein was maintained in normal mitochondria. Arrows: CoQ deficient fibroblasts showing lost of calcein fluorescence, indicating MPT. Bar =15 μm. (B) Quantification of fibroblasts visibly undergoing MPT. **p < 0.01 between control and CoQ deficient fibroblasts. ºp < 0.05, between the presence and the absence of CoQ.

others. Cyclosporin A (CsA), and various of its analogs inhibit the MPT through interaction with cyclophilin D (CypD).[37] PT pores are composed of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator (ANT) in the inner membrane and CypD in the matrix space.[38] It has been reported that ROS can promote MPT by causing oxidation of thiol groups on the adenine nucleotide translocase (ANT), which forms part of the MPT pore.[39] Moreover, a possible involvement of the MPT in the induction of autophagy of altered mitochondria it has been postulated.[25,31,34] Therefore, oxidative stress in CoQ-deficient

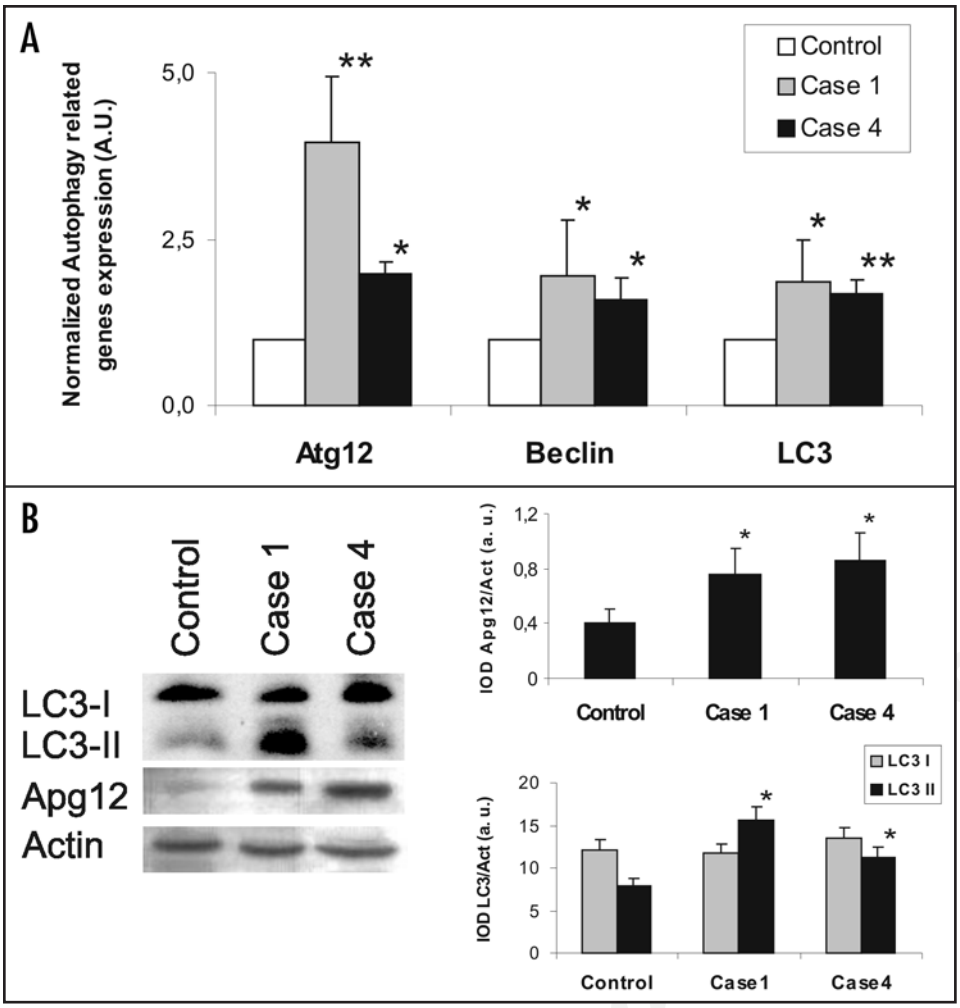

Figure 5. Autophagic transcripts and protein expression. (A) Expression of ATG12, Beclin, and LC3 transcripts in control and patient fibroblasts were assessed by Real Time PCR as described in Material and Methods. (B) Protein expression of Atg12 and LC3 was performed by Western blotting as described in Material and Methods. *p < 0.05, **p < 0.01 between control and CoQ-deficient fibroblasts.

fibroblasts might cause mitochondrial damage, MPT activation and mitochondrial dysfunction. Elimination of these dysfunctional mitochondria would be critical to protect cells from the damage of altered mitochondrial function and release of potentially proapoptotic molecules. Mitochondrial turnover is predominantly autophagic sequestration and delivery to lysosomes for hydrolytic degradation, a process also called mitophagy. Our data suggests mitophagy could take place as a process that specifically targets dysfunctional mitochondria in CoQ deficient fibroblasts. We postulate that the structural composition of mitochondria might be affected by increased ROS production, which in turn determines their self-elimination. An example could be the presence of oxidized mitochondrial unsaturated fatty acids and/or proteins.

Autophagy is a process by which cytosol and organelles are sequestered within double-membrane vesicles that deliver the contents to the lysosome/vacuole for degradation and recycling of the resulting macromolecules.[40] Autophagy is typically activated by fasting and nutrient deprivation. During fasting, autophagy is important for generating amino acids and metabolic intermediates to maintain ATP production. Both insufficient and excess autophagy can promote cell injury.[41] Appropriate regulation of autophagy is thus essential for cellular homeostasis. The molecular mechanism underlying autophagy has been extensively researched in the past decade, and the genes participating in this process, denoted ATGs,[42] were found to be conserved in yeast and humans.[43,44] In CoQ-deficient fibroblasts, we demonstrated here increased expression of autophagic genes and proteins suggesting the activation of the autophagic machinery in these patients. Moreover, lysosomal/autophagic markers (β-galactosidase, LC3, cathepsin, LysoTracker) were enhanced in CoQ-deficient fibroblasts indicating lysosomal proliferation. Such findings led to the assumption that autophagy was activated in CoQ-deficient fibroblasts. We confirmed these results by electron microscopy that clearly showed the presence of laminar bodies and autophagosomes engulfing mitochondria. Immunofluorescence studies also verified that typical lysosomal enzymes, such as cathepsin D, colocalized with a mitochondrial marker, such as cytochrome *c*. These results suggest that autophagy in CoQ-deficient fibroblasts is characterized by a selective degradation of dysfunctional mitochondria or mitophagy.

But are ROS and MPT essential for autophagy in CoQ deficiency? To address this question, we tested the effect of N-acetyl-cysteine (NAC), and BHA, two general antioxidants, and the MPT-inhibitor cyclosporin on the formation of autophagosomes in CoQ-deficient fibroblasts using β-galactosidase as a marker. Results showed that both antioxidants and MPT inhibitors reduced β-galactosidase staining. These data suggest that ROS generation and MPT are involved in CoQ deficiency-induced autophagy. Increasing evidence corroborates the fact that autophagy of mitochondria may occur selectively. For example in yeast, the mitochondrial proteins Uth1p and Aup1, are required for efficient mitochondrial autophagy, but corresponding mammalian proteins have yet to be identified.[45,46] Moreover, recent studies report that ROS plays a key role in selenite-induced mitochondrial damage and mitophagy.[47,48] Our work also strongly indicates that ROS/MPT induced autophagy can show selectivity for mitochondria in CoQ deficiency.

There is a controversy to consider if autophagy promotes or prevents cell death.[41,49,50] If autophagy removes damaged mitochondria that would otherwise activate caspases and apoptosis, then autophagy could be protective. In agreement with this hypothesis, we demonstrated that disruption of autophagic processing in CoQ-deficient fibroblasts by 3-MA or wortmannin promoted caspase-dependent cell death. To verify this hypothesis, we examined the effects of CoQ deficiency in a well-established genetic knock-out of autophagy, $Atg5^{-/-}$ mouse embryonic fibroblasts (MEFs).[19,20] The

absence of autophagy in Atg5$^{-/-}$ cells sensitized them to apoptosis when coenzyme Q deficiency was induced by treatment with a specific inhibitor of CoQ biosynthesis (PABA). All together, these findings indicate that inhibition of autophagy in CoQ deficiency can promote cell death, suggesting the protective role of autophagy in this disease.

Material and Methods

Reagents. Monoclonal Anti-Actin antibody, butylated hydroxyanisole (BHA), N-acetylcisteine, 3-methyl adenine (3MeA), propidium iodide (PI), 4-aminobenzoate (4AB), and trypsin were purchased from Sigma Chemical Co., (St. Louis, Missouri). Monoclonal Antibodies specific for oxidative phosphorylation (Anti-human Complex I (NADH-ubiquinol Oxidoreductase, 39 kDa subunit), Complex III (ubiquinol-cytochrome *c* oxidoreductase, Core 1 subunit) and Complex IV (cytochrome *c* oxidase; COX II), Carboxy-2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), and Hoechst 3342, were purchased from Invitrogen/Molecular Probes (Eugene, Oregon). Cyclosporine A was acquired from Fluka BioChemika (Buchs SG, Switzerland). Anti-cytochrome *c* antiobodies were purchased from PharMingen (BD Bioscience, San Jose, California). Anti-GAPDH monoclonal antibody, clone 6C5, was purchased from Research Diagnostic, Inc., (Flanders, New Jersey). Anti-hAtg12 was acquired from Biosensis (South Australia, Australia). Anti-MAP LC3 (N-20) and Anti-Cathepsin D were purchased from Santa Cruz Biotechnology (Santa Cruz, California).

Anti-active caspase-3 was obtained from Cell Signalling Technology (Beverly, Massachusetts). A cocktail of protease inhibitors (complete cocktail) was purchased from Boehringer Mannheim (Indianapolis, Indiana). The Immun Star HRP substrate kit was from Bio-Rad Laboratories Inc., (Hercules, California).

Fibroblasts cultures. Cultured fibroblasts were derived from four patients with a CoQ deficiency. Cases 1 and 2 are patients from the same family (daughter and father) with a remarkable clinical response to CoQ supplementation.[18] Cases 3 and 4 presented a pathogenic mutation in the COQ2 gene;[8,51] Control fibroblasts were human primary fibroblasts from healthy volunteers. Mouse embryonic fibroblasts (MEFs) derived from wild-type and Atg5$^{-/-}$ mouse embryos were a kind gift of Noboru Mizushima, Tokyo Medical and Dental University, Japan.[52,53] Ethical issues: The study was approved by the Ethical Committee of the Hospital San Joan de Deu, and samples from patients and controls were obtained according to the Helsinki Declarations of 1964, as revised in 2001.

Fibroblasts from CoQ deficient patients and controls were cultured at 37°C using DMEM 4.5 g/L Glucose, L-Glutamine, Pyruvate (Invitrogen, Prat de Llobregat, Barcelona) supplemented with an antibiotic/antimycotic solution (Sigma Chemical Co., St. Louis, Missouri) and 20% fetal bovine serum (FBS, Linus). When required, supplemental CoQ pre-diluted in FBS was added to the plates at a final concentration of 100 μM (Coenzyme Q10, Synthetic Minimun 98%, HPLC, Sigma).

Fibroblasts growth rate. Two hundred thousand fibroblasts were cultured in the absence or presence of CoQ (100 μM), for 72 h. After removing the supernatant along with any dead cells, cell counting was performed from three high power fields with an inverted microscope and a 40X objective.

Mitochondrial enzyme activities. Activities of NADH:coenzyme Q1 oxidoreductase (complex I), succinate deshydrogenase (complex II), cytochrome *c* oxidase (complex IV), ubiquinol:cytochrome *c* oxidoreductase (complex III), succinate:cytochrome *c* reductase (complex II + complex III) and citrate synthase (CS) were determined in cell extracts using previously described spectrophotometric Methods.[8,54] Results are expressed as Units/CS (mean ± SD). Proteins of fibroblasts homogenates were analyzed by the Lowry procedure.[55]

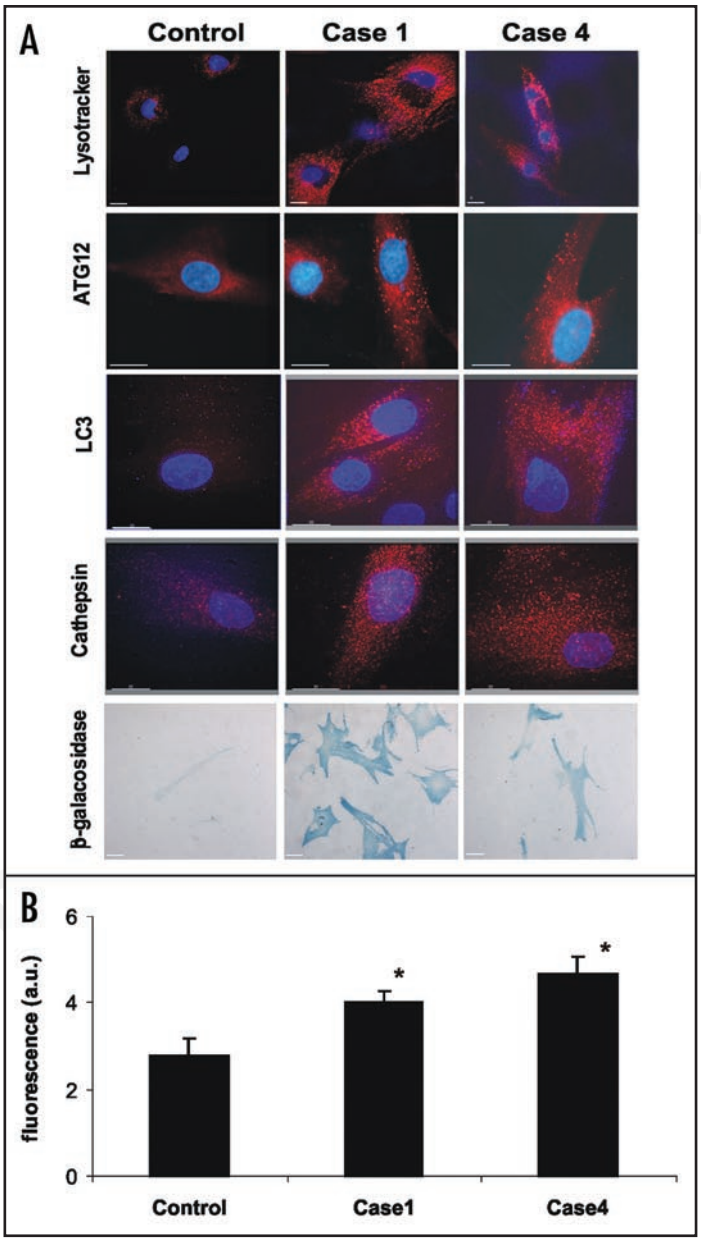

Figure 6. Autophagic markers in CoQ deficient fibroblasts. (A) Representative images of autophagic markers (Lysotracker, ATG12, LC3, cathepsin D) in control and patients' fibroblasts that were visualized by immunofluorescence, as described in Material and Methods. β-galactosidase staining was examined by light microscopy as described in Material and Methods. Bar = 15 μm. (B) Quantification of acidic vacuoles in control and patient fibroblast by Lysotracker staining and flow cytometry analysis.*p < 0.01 between control and CoQ-deficient fibroblasts.

CoQ determination. Cell samples were lysed with 1% SDS and vortexed for 1 min. A mixture of ethanol:isopropanol (95:5) was added and the samples were vortexed for 1 min. To recover CoQ, 5

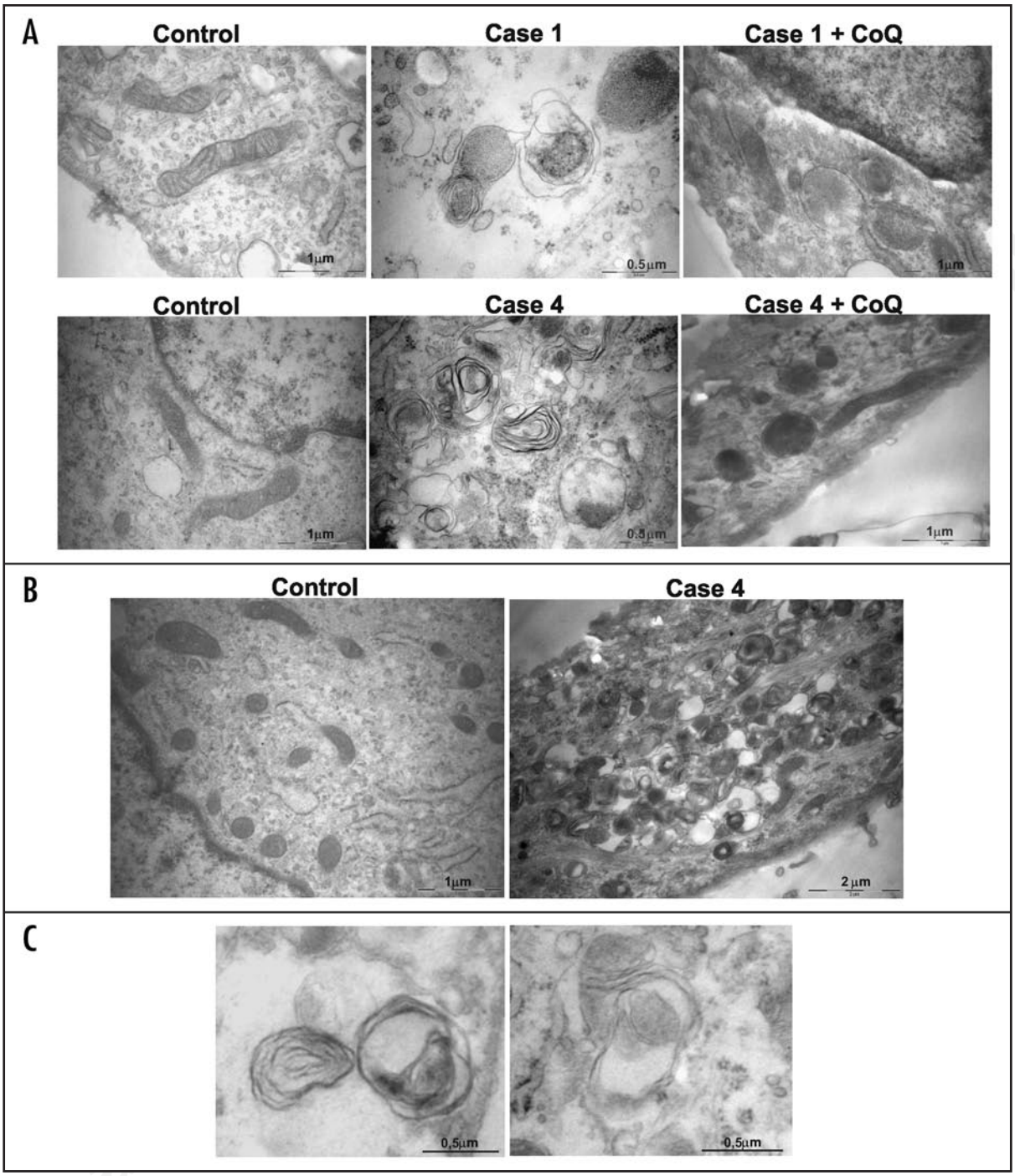

Figure 7. Ultrastructure of CoQ-deficient fibroblasts. (A) Control fibroblasts are showing mitochondria with typical ultrastructure. Bar = 1 μm. Laminar bodies and autophagosome with mitochondria were present in CoQ-deficient fibroblast (case 1 and 4); Bar = 0.5 μm. Mitochondria of fibroblasts from patients supplemented with CoQ (100 μm) for 72 h. Bar = 1μm. (B) Control (Bar = 1 μm) and case 4 (Bar = 2 μm) fibroblasts at low magnification. (C) Case 1 and 4 fibroblasts at high magnification (Bar = 0.5 μm).

ml of hexane was added and the samples were centrifuged at 1000 xg for 5 min at 4°C. The upper phases from three extractions were recovered and dried using a rotary evaporator. Lipid extracts were suspended in 1 ml of ethanol, dried in a speed-vac and stored at -20°C. Samples were suspended in the suitable volume of ethanol prior to HPLC injection. Lipid components were separated by a Beckmann 166–126 HPLC system equipped with a 15-cm Kromasil C-18 column in a column oven set to 40°C, with a flow rate of 1 ml/min and a mobile phase containing 65:35 methanol/n-propanol and 1.42 mM lithium perchlorate. CoQ levels were analyzed with ultraviolet (System Gold 168), electrochemical (Coulochem III ESA) or radioactivity (Radioflow Detector LB 509, Berthod Technologies) based detectors as necessary. Coenzyme Q_9 (CoQ_9) was used as internal standard. In the case of MEFs, CoQ_9 levels were analyzed using CoQ_6 as internal standard. CoQ content was determined as nmol/g protein.

CoQ biosynthesis rate. The CoQ biosynthesis rate in these cells was determined by the incorporation of the radiolabelled p-hydroxybenzoic acid ([^{14}C]-pHB), a precursor of the quinone ring of CoQ, in the presence of 100-fold excess of non radiolabeled pHB.[18] CoQ synthesized by cells was extracted and analyzed with a radioflow detector after HPLC separation as described previously. Results are expressed as cpm x 10^6/mg protein.

Measurement of intracellular generation of ROS. Flow-cytometric analysis of the intracellular generation of ROS was performed using dichlorodihydrofluorescein, as well as dihydrorhodamine 123, as a probe. Cells were cultured in six-well plates (35 mm diameter well) and, at confluence, incubated with dichlorodihydrofluorescein (1 μM) or dihydrorhodamine 123 (1 μM) for 30 min. at 37°C. Once the incubation was finished, cells were harvested, washed, centrifuged, resuspended in D-MEM medium and analyzed by flow cytometry.

Mitochondrial membrane potential ($\Delta\Psi_m$). Cells were cultured in six-well 35 mm plates, and at confluence, cells were incubated for 30 min with 100 nM Mitotracker (Mitotracker Red CMXRos, Molecular Probes). Cells were subsequently harvested, washed with fresh medium, washed, centrifuged (500 xg), resuspended in RPMI medium and analyzed by flow cytometry.

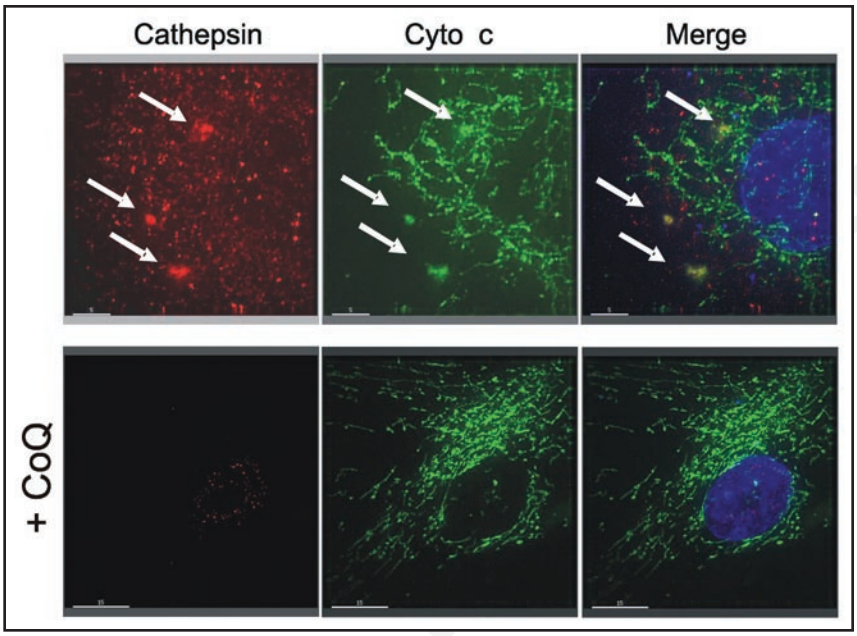

Figure 8. Mitochondrial degradation during autophagy. Cultured fibroblasts from case 4 were cultured in the presence or absence of CoQ (100 μM) for 72 hours. Then, cells were harvested, fixed, and immunostained with anticathepsin (lysosomal marker) and cytochrome *c* (mitochondrial marker) and examined in a fluorescence microscope as described in Material and Methods. Colocalization of both markers was assessed by the DeltaVision software. Bar = 5 μm.

Immunoblotting. Western blotting was performed using standard methods. After protein transfer, the membrane was incubated with various primary antibodies diluted 1:1000, and then with the corresponding secondary antibody coupled to horseradish peroxidase at a 1:10000 dilution. Specific protein complexes were identified using the Immun Star HRP substrate kit (Biorad Laboratories Inc., Hercules, California).

β-galactosidase test.[56] Cultured cells were washed in PBS (pH 7.4), fixed with 3.7% formaldehyde, and incubated overnight at 37°C in freshly prepared staining buffer [1 mg mL-1 X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside), 5 mM $K_3Fe[CN]_6$, 5 mM $K_4Fe\ [CN]_6$, and 2 mM $MgCl_2$ in PBS, pH 6.0, or in citrate-buffered saline, pH 4.5]. At the end of the incubation, cells were washed with PBS, examined and photographed using a Leica CTR 5000 microscope. β-galactosidase staining was quantified using ImageJ software.

Loading of lysotracker red. LysoTracker (100 nM) was added to cultured fibroblasts. After 30 min, each well was washed two times with fresh DMEM, and fixed with 2% paraformaldehyde in PBS for 10 min at 4°C. The red fluorescence of LysoTracker was visualized and measured using a DeltaVision system (Applied Precision; Issaquah, Washington) with an Olympus IX-71 microscope.

Real time PCR. The fibroblast expression of Atg12, MAP-LC3, and Beclin1 genes in fibroblasts was analyzed by SYBR Green quantitative PCR using mRNA extracts and primers hCOQ-RQ-F and hCOQ-RQ-R. Real Time Beclin 1 primers 5'-GGA TGG ATG TGG AGA AAG GCA AG-3' (Forward primer) and 5'-TGA GGA CAC CCA AGC AAG ACC-3' (Reverse primer) amplify a sequence of 152 nucleotides. Human Atg12 primers 5' ATT GCT GCT GGA GGG GAA GG 3' (Forward primer) and 5' GGT TCG TGT TCG CTC TAC TGC 3' (Reverse primer) amplify a sequence of 198 nucleotides. Human MAP-LC3 primers 5'-GCC TTC TTC CTG CTG GTG AAC-3' (Forward primer) and 5'-AGC CGT CCT CGT CTT TCT CC-3' (Reverse primer) amplify a sequence of 91 nucleotides. Actin was used as a housekeeping control gene.

Mitochondrial permeability transition (MPT). MPT was evaluated using the the MitoProbe Transition Pore Assay Kit (Invitrogen/Molecular Probes). Cells are loaded with calcein-AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, calcein AM is cleaved by intracellular esterases to liberate calcein (a very polar fluorescent dye), which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time. The fluorescence from cytosolic calcein can be quenched by the addition of cobalt ($CoCl_{2)}$, while the fluorescence from the mitochondrial calcein is maintained in normal conditions. Activation and inhibition of MPT pore formation in control and patient fibroblasts in vivo were visualized through simultaneous loading with calcein AM and MitoTracker Red dyes. Green calcein fluorescence is visible throughout the cell; orange fluorescence indicates the colocalization of calcein and MitoTracker Red indicators. Presence of MPT allows cobalt to enter the mitochondria, which quenches the calcein fluorescence but leaves the red fluorescence of the MitoTracker Red dye unaffected. Residual calcein fluorescence would be expected in mitochondria where the MPT had not occurred.

Immunofluorescence microscopy. Fibroblasts were grown on 1mm width (Goldseal No. 1) glass coverslips for 24–48 h in DMEM containing 20% FBS. Cells were rinsed once with PBS, fixed in 3.8% paraformaldehyde for 5 min at room temperature, and

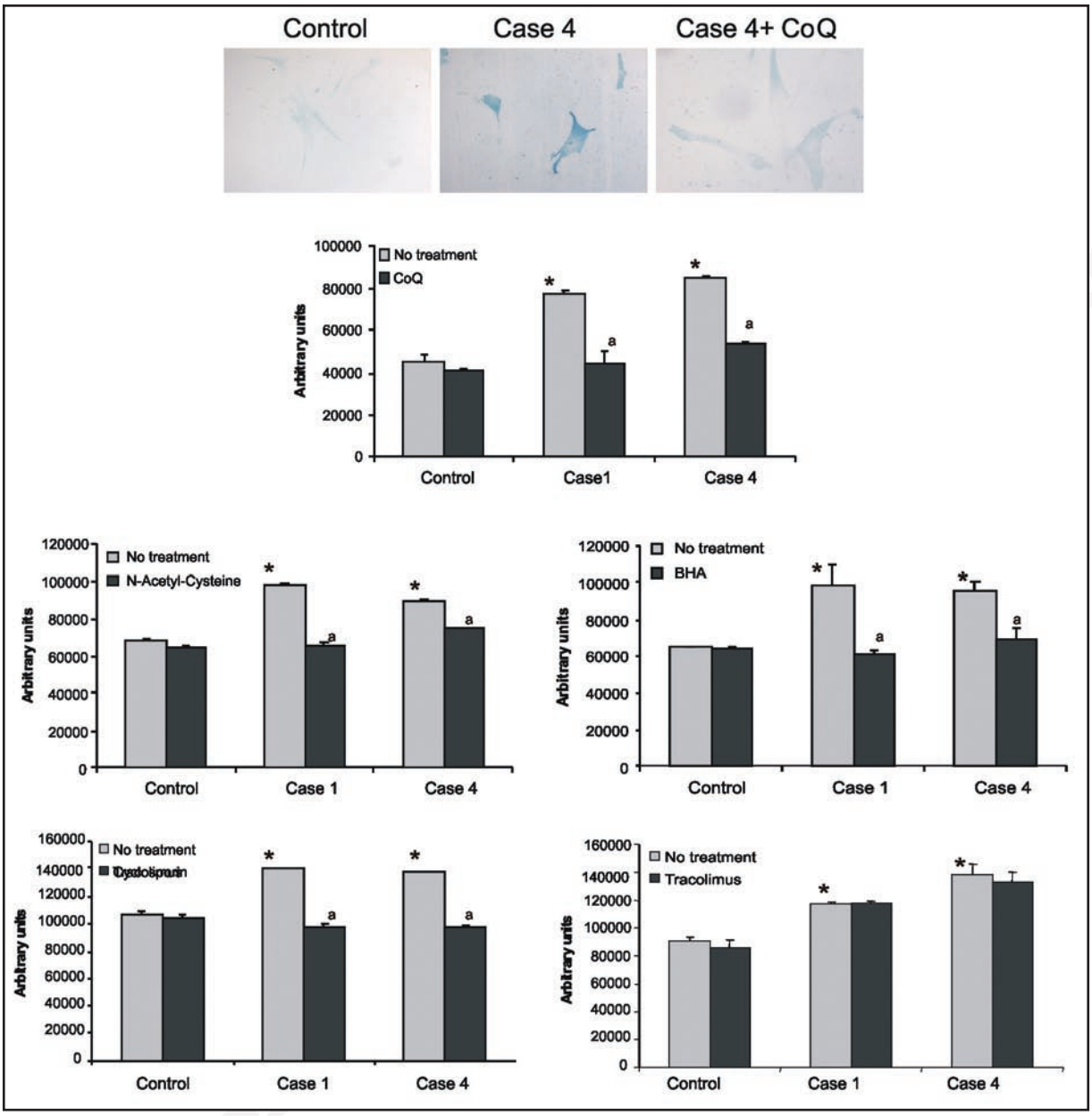

Figure 9. Role of ROS and MPT in CoQ deficiency mitophagy. β-galactosidase staining was performed on cultured fibroblasts from control and patients (case 1 and 4) treated with two antioxidants BHA (40 μM), and N-acetylcisteine (10 mM), and an inhibitor of MPT, cyclosporine (5 μM). Tacrolimus (5 μM), an immunosuppressant which has no effect in MPT, was used a negative control. As positive control we treated fibroblasts with CoQ (100 μM), which restores the deficiency and reduces β-galactosidase staining. β-galactosidase staining was quantified using the ImageJ software. Results are expressed as mean ± SD of three independent experiments. *p < 0.05 between control and patients cells. °p < 0.05 between the presence and the absence of the drugs.

permeabilized in 0.1% saponin for 5 min. For immunostaining, glass coverslips were incubated with primary antibodies diluted 1:100 in PBS, and were incubated 1–2 h at 37°C in a humidified chamber. Unbound antibodies were removed by washing the coverslips with PBS (three times, 5 min). The secondary antibody, a FITC-labeled goat antimouse antibody or a tetramethyl rhodamine goat antirabbit (Molecular Probes), diluted 1:100 in PBS, were added and incubated for 1 h 37°C. Coverslips were then rinsed with PBS for 3 min, incubated for 1 min with PBS containing Hoechst 33342 (1 μg/ml) or TOPRO (1 μM) and washed with PBS (three 5 min washes). Finally, the coverslips were mounted onto microscope slides using Vectashield Mounting Medium (Vector Laboratories, Burlingame, California) and analyzed using an upright fluorescence microscope (Leica DMRE, Leica Microsystems GmbH, Wetzlar, Germany). Deconvolution studies and 3D projections were performed using a DeltaVision system (Applied Precision; Issaquah, Washington) with an Olympus IX-71 microscope. The deconvolved images were derived from optical sections taken at 30-nm intervals using a 60x PLAPON objective with a 1.42 numerical aperture.

Electron microscopy. Fibroblasts were fixed for 15 min in the culture plates with 2% glutaraldehyde in culture medium, then for 30 min in 2% glutaraldehyde-0.1 M NaCacodylate/HCl, pH 7.4. They were then washed three times in 0.2 M NaCacodylate/HCl, pH 7.4 for 10 min and then post-fixed with 1% OsO_4-0.15

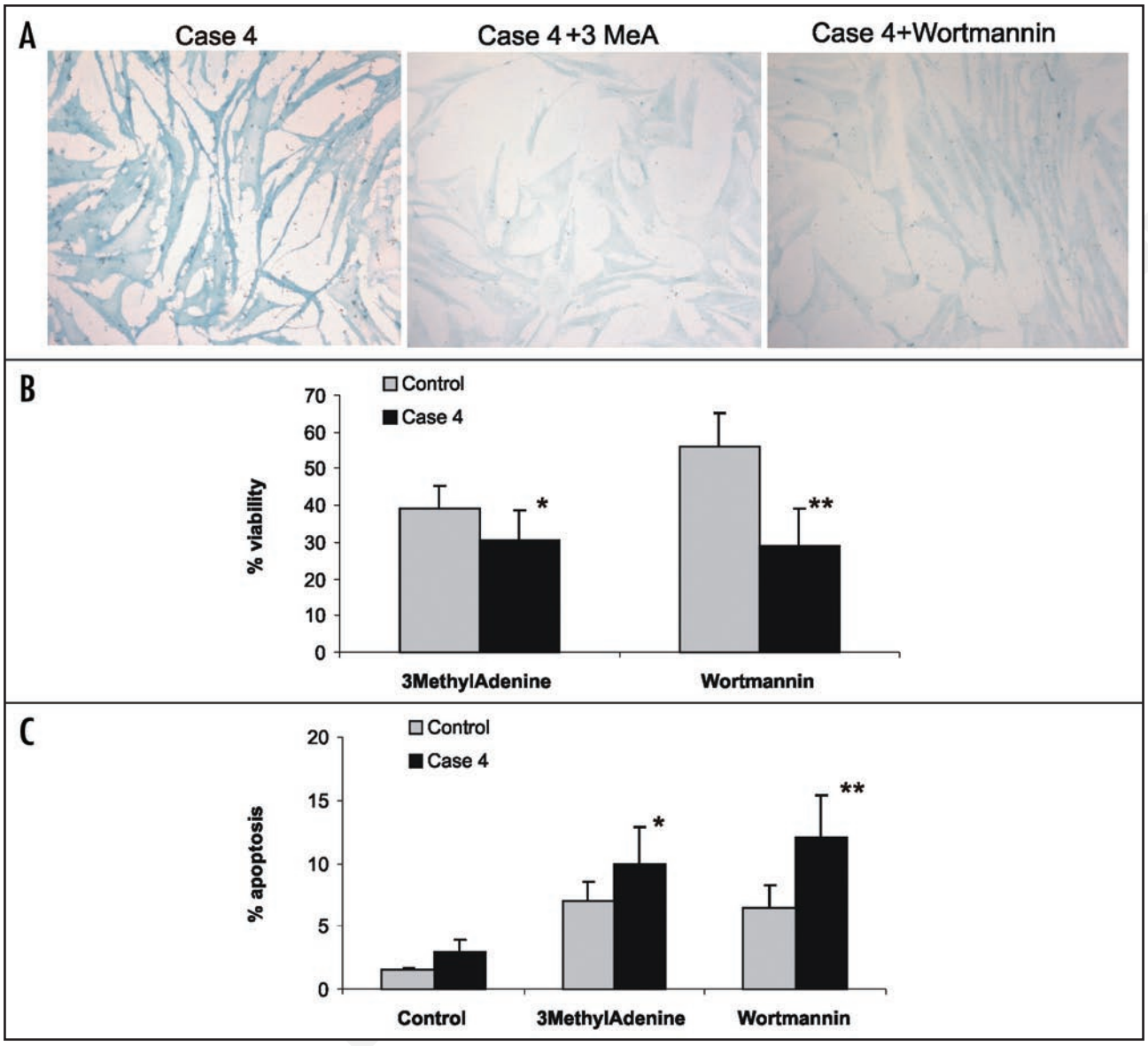

Figure 10. Autophagy is required for optimal survival of CoQ deficient fibroblasts. Control and CoQ-deficient fibroblasts (case 4) were treated for 48 hours with 3-MA (20 mM) or wortmannin (10 μM), and we determined β-galactosidase staining (A), cell viability (B), and the rate of apoptotic cells (C) as described in Material and Methods. Results are expressed as mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ between control and CoQ-deficient fibroblasts.

M NaCacodylate/HCl, pH 7.4 for 30 min. After dehydration in increasing concentrations of ethanol, 5 min for each step: 30, 50, 70 and 95%, impregnation steps and inclusion were performed in Epon and finally polymerized at 60°C for 48 h. 60–80 nm sections were obtained using an ultramicrotome RMC-MTX (Tucson, Arizona), and contrasted with uranyl acetate and lead citrate. Observations were performed on a Philips CM-10 transmission electron microscope.

Analysis of apoptosis and viable cells. Apoptosis was assessed by observing nuclei fragmentation by Hoechst staining, cytochrome *c* release, and caspase 3 activation. Viable cells were determined from their normal nuclear morphology and exclusion of propidium iodide. In each case 10 random fields and more of 500 cells were counted.

Statistical analysis. All results are expressed as mean ± SD of three independent experiments. The measurements were statistically analyzed using Student t-test for comparing two groups and ANOVA for more than two groups. The level of significance was set at $p < 0.05$.

Conclusion

In normal physiology, cells utilize autophagy to eliminate damaged, dysfunctional, and superfluous cytoplasmic components to maintain cellular homeostasis and adjust to changing physiological demands. In this respect, mitochondrial degradation by autophagy (mitophagy) may play an essential role in maintaining cellular homeostasis in CoQ deficiency. We suggest that autophagy is triggered in our model as a "stress-induced" mechanism involved in the degradation of dysfunctional CoQ-deficient mitochondria. This work is the first direct demonstration of the role of autophagy in the pathophysiology of CoQ deficiency.

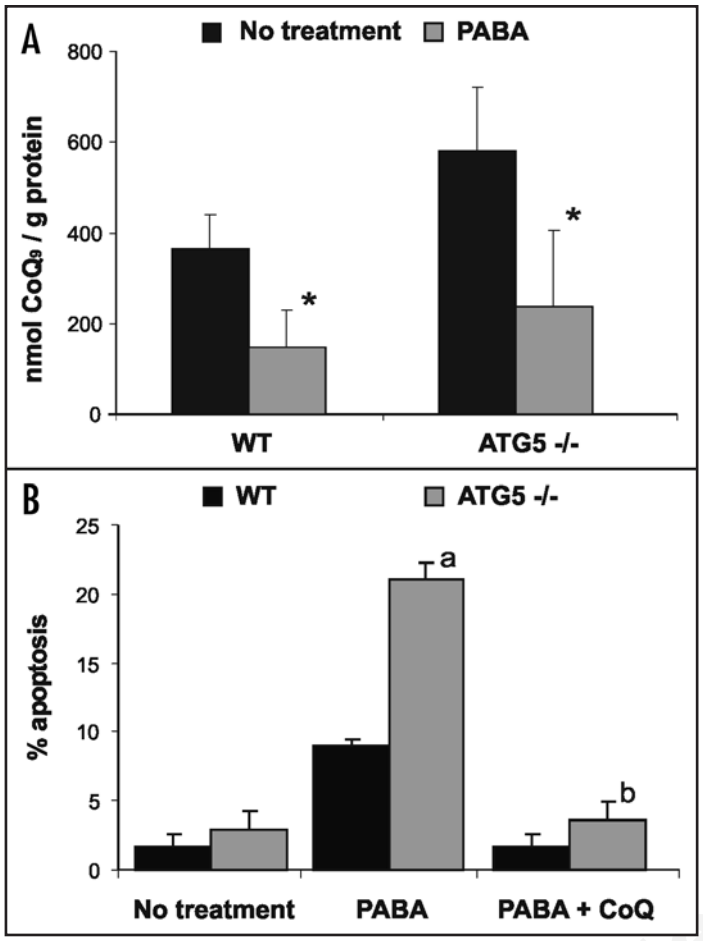

Figure 11. Apoptosis is increased in CoQ-deficient Atg5$^{-/-}$ cells. CoQ deficiency in wild-type and Atg5$^{-/-}$ MEFs was induced by treatment with PABA 100 μM for 72 hours. (A) CoQ_9 levels in wild-type and knockout mouse fibroblasts treated with PABA. (B) Apoptosis was assessed in both autophagy-proficient (wild-type), and autophagy-deficient cells (Atg5$^{-/-}$) treated with PABA as described in Material and Methods. PABA-treated MEFs were also supplemented with 100 μM CoQ for 72 hours to verify the specificity of apoptosis induction by coenzyme Q deficiency. Results are expressed as mean ± SD of three independent experiments.*$p < 0.01$ between the presence and the absence of PABA. $^{a}p < 0.01$ between Atg5$^{-/-}$ and wild-type MEFs. $^{b}p < 0.01$ between Atg5$^{-/-}$ in the presence or absence of CoQ.

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