Secondary coenzyme Q10 deficiency and oxidative stress in cultured fibroblasts from patients with riboflavin responsive multiple Acyl-CoA dehydrogenation deficiency

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Coenzyme Q10 (CoQ10) is essential for the energy production of the cells and as an electron transporter in the mitochondrial respiratory chain. CoQ10 links the mitochondrial fatty acid β-oxidation to the respiratory chain by accepting electrons from electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO). Recently, it was shown that a group of patients with the riboflavin responsive form of multiple acyl-CoA dehydrogenation deficiency (RR-MADD) carrying inherited amino acid variations in ETF-QO also had secondary CoQ10 deficiency with beneficial effects of CoQ10 treatment, thus adding RR-MADD to an increasing number of diseases involving secondary CoQ10 deficiency. In this study, we show that moderately decreased CoQ10 levels in fibroblasts from six unrelated RR-MADD patients were associated with increased levels of mitochondrial reactive oxygen species (ROS). Treatment with CoQ10, but not with riboflavin, could normalize the CoQ10 level and decrease the level of ROS in the patient cells. Additionally, riboflavin-depleted control fibroblasts showed moderate CoQ10 deficiency, but not increased mitochondrial ROS, indicating that variant ETF-QO proteins and not CoQ10 deficiency are the causes of mitochondrial ROS production in the patient cells. Accordingly, the corresponding variant Rhodobacter sphaeroides ETF-QO proteins, when overexpressed in vitro, bind a CoQ10 pseudosubstrate, Q10Br, less tightly than the wild-type ETF-QO protein, suggesting that molecular oxygen can get access to the electrons in the misfolded ETF-QO protein, thereby generating superoxide and oxidative stress, which can be reversed by CoQ10 treatment.

INTRODUCTION

Coenzyme Q10 (CoQ10), also known as ubiquinone, is a lipid-soluble molecule localized in the inner mitochondrial membrane, where it acts as a mobile electron and proton transporter from electron transport chain complex I (NADH: ubiquinone reductase) and complex II (succinate:ubiquinone reductase) to complex III (ubiquinone cytochrome c oxidase). In addition, CoQ10 connects mitochondrial fatty acid β-oxidation to the electron transport chain by accepting electrons from electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO) and passing them on to complex III (1–4). Furthermore,
CoQ10 in its reduced form, ubiquinol, is recognized as an antioxidant and free radical scavenger, protecting membrane lipids, proteins and mitochondrial DNA against oxidative damage (5). Intracellular synthesis is the major source of CoQ10, although a small proportion is acquired through the diet (6).

Decreased cellular CoQ10 levels have been identified as either the result of a primary or a secondary deficiency. While a primary CoQ10 deficiency is a known series of autosomal recessive disorders associated with a number of gene variations all directly affecting proteins involved in the biosynthesis of CoQ10 (7–10), decreased CoQ10 levels as the result of a secondary deficiency have been associated with many different disorders. These include mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), cardiac vascular disease, mitochondrial disorders and neurodegenerative disorders. Beneficial effect of CoQ10 treatment or treatment with various CoQ10 analogues has been reported in some of these diseases, both clinically and at a molecular level (7,11–24). In addition, it has been shown that the decreased CoQ10 sometimes correlates with increased oxidative stress (16,17,25). However, in most of these diseases, the causes and consequences and thus the molecular rational for CoQ10 treatment have not yet been clarified.

Recently, it was shown that recessively inherited variations in the Electron Transfer Flavoprotein Dehydrogenase (ETFDH) gene, which codes for the ETF-QO protein, may also be involved in the myopathic form of CoQ10 deficiency (24). In addition to CoQ10 deficiency, these patients showed decreased activities of respiratory chain complexes in skeletal muscle biopsies, and they had clinical and biochemical characteristics of riboflavin responsive multiple acyl-CoA dehydrogenation deficiency (RR-MADD), and showed beneficial effects, of not only riboflavin but also of CoQ10 treatment (24). In contrast, a study by Liang et al. (26) in 2009 could not demonstrate any secondary CoQ10 deficiency or respiratory chain deficiency in two unrelated RR-MADD patients with ETF-QO mutations. Therefore, at present it is disputed whether mitochondrial dysfunction with combined CoQ10 deficiency is a general finding in RR-MADD patients, and consequently if RR-MADD patients should be treated with CoQ10 in addition to riboflavin therapy.

We have recently demonstrated that for amino acid variations located in the FAD or ubiquinone binding domains of the ETF-QO protein, the beneficial effects of riboflavin are due to the chaperone-like effect of FAD, which stabilizes the structure and activity level of the misfolded variant ETF-QO proteins. This may then allow increased substrate oxidation by the acyl-CoA dehydrogenases that utilize ETF-QO with a consequent increase in ATP production and clinical improvement. In this study, it was shown that such variant proteins when overexpressed in vitro result in increased cellular oxidative stress, which, however, could not be ameliorated by riboflavin treatment (27). Based on these studies, we hypothesize that the beneficial effect of CoQ10 treatment, observed in some RR-MADD patients, is due to the antioxidant effect of CoQ10 which counteracts the superoxide production resulting from the misfolded variant ETF-QO proteins. In the present study, we aim to test this hypothesis by investigating CoQ10 deficiency and reactive oxygen species (ROS) production in cultured fibroblasts from six unrelated patients with RR-MADD and primary gene defects in ETF-QO. To further assess the molecular mechanisms of ROS production from these RR-MADD variant ETF-QO proteins, we investigated the Q10Br binding properties of the corresponding variant Rhodobacter ETF-QO proteins over-expressed in E. coli.

RESULTS

Riboflavin responsive ETF-QO deficiency in fibroblasts from RR-MADD patients

To investigate if there is a molecular rationale for treating RR-MADD patients with CoQ10 in addition to riboflavin, we investigated fibroblasts from six unrelated RR-MADD patients carrying four different ETFDH gene variations known to be associated with RR-MADD. The amino acid variations are all located in the ubiquinone binding domain of ETF-QO (p.Gly170Cys, p.Val451Leu, p.Pro456Leu, p.Pro483Thr) (Fig. 1A).

In our previous study, we showed that riboflavin, when converted to the cofactor FAD, acts as a molecular chaperone, which can improve the in vitro folding and steady state level of misfolded ETF-QO proteins when overexpressed in HEK293 cells (27). Two of the variant ETF-QO proteins studied (p.Pro456Leu and p.Pro483Leu) had amino acid variations in the ubiquinone binding domain.

In accordance with these in vitro findings, western blot analysis of ETF-QO protein amount in fibroblasts from the six RR-MADD patients showed decreased steady state level of ETF-QO protein when compared with controls with a significant increase in ETF-QO protein level after treating the cells with riboflavin (Fig. 2).

Secondary CoQ10 deficiency in RR-MADD

The level of CoQ10 was measured by HPLC in fibroblasts from patients and controls cultured under riboflavin supplemented and deficient conditions as described in Materials and Methods. Figure 3 shows that the mean level of CoQ10 from the six patient fibroblasts is significantly lower when compared with the mean of the control samples under riboflavin supplemented conditions. Under riboflavin depleted conditions, both patient and control fibroblasts show decreased CoQ10 levels.

Oxidative stress and antioxidant effects of short- and long-term CoQ10 treatment

Since it has been shown that moderately decreased levels of CoQ10 can be associated with increased levels of oxidative stress (28), we examined the level of oxidative stress in fibroblasts from RR-MADD patients and control individuals by image cytometry using MitoSOX, which is a red fluorescent mitochondrial superoxide molecular probe. The experiment was performed under riboflavin depleted and supplemented conditions (Fig. 4A), after short-term CoQ10 treatment for 72 h (Fig. 4B), and after long-term CoQ10 treatment, 7 days (Fig. 4C). As seen from Figure 4A, the patient fibroblasts, when compared with the control fibroblasts, showed a higher amount of oxidative stress even in riboflavin supplemented conditions. These results indicate that the mitochondria of these patients have an increased basal level of chronic oxidative...
stress, which cannot be compensated for by riboflavin treatment. After 72 h of CoQ10 treatment, there seemed to be no effect on the oxidative stress in the patient fibroblasts (Fig. 4B), except for fibroblasts carrying the variant ETF-QO p.Val451Leu, which seemed to be shifted towards a larger amount of non-stressed cells after short-term CoQ10 treatment. However, after long-term treatment, 7 days, with CoQ10, there was a clear effect on the percentage of cells in the patient fibroblasts, which were shifted from oxidative stressed cells towards non-oxidative stressed cells. The level of CoQ10 was measured in the cells after CoQ10 treatment and shown to be at or above control levels (data not shown). Taken together, these results show that long-term CoQ10 treatment of these patient fibroblasts has a positive effect upon the level of oxidative stress. Furthermore, the results suggest that in these cells, neither riboflavin deficiency nor secondary CoQ10 deficiency alone is associated with oxidative stress as indicated from the control fibroblasts under riboflavin deficient conditions, thus suggesting the variant ETF-QO proteins to be the source of mitochondrial ROS production.

Investigation of the mechanisms by which variant ETF-QO proteins produce ROS

Because all patient cells, investigated in this study, carry variant ETF-QO proteins with amino acid substitutions in the ubiquinone binding domain, it could be speculated that the location of the variations affects the binding of CoQ10 to ETF-QO. This may then cause an opening of the ETF-QO structure resulting in the access of oxygen to the electrons of ETF-QO or allowing leakage of electrons out of the misfolded ETF-QO enzyme to oxygen and thereby resulting in increased mitochondrial oxidative stress. To investigate whether the four variant ETF-QO proteins have impaired CoQ10 binding the corresponding variant Rhodobacter ETF-QO proteins were over-expressed in E. coli cells and tryptophan fluorescence quenching studies of each variant ETF-QO protein were conducted using the CoQ10 pseudosubstrate, Q10Br, as described in Materials and Methods. As observed in Table 1, all variant ETF-QO proteins showed decreased Q10Br binding affinity ($K_d$) compared with wild-type ETF-QO. ETF-QO variant p.Pro389Leu (homolog of human p.Pro456Leu) showed the weakest binding of Q10Br, while p.Pro389Thr (homolog of human p.Pro456Thr) showed the lowest effect on the binding, although still almost a triplication of the $K_d$ value when compared with wild-type ETF-QO.

DISCUSSION

In the present investigation, we addressed the question of whether RR-MADD patients in addition to having a primary defect in ETF-QO show secondary CoQ10 deficiency. The question was originally raised by Gempel et al. (24), who described a group of patients with the myopathic form of CoQ10 deficiency, who were diagnosed with RR-MADD based on characteristic urine organic acids and acylcarnitine profiles and the identification of variations in the ETFDH gene. It was suggested that RR-MADD and the myopathic form of CoQ10 deficiency are allelic diseases, and recommended that CoQ10 should be given as a treatment in addition to riboflavin to RR-MADD patients (24). A further study published in 2009 (26) investigated four unrelated RR-MADD patients, all carrying the same variation (p.Ala84Thr) located in the FAD binding domain of ETF-QO. The patients showed a mild response or no response at all following treatment with relatively low concentrations (30 mg/day) of CoQ10. In agreement with these findings, CoQ10 levels and respiratory chain activities comparable with control levels were shown in muscle biopsies from two of the patients, from whom samples were available (26). Until now,
only these two studies (24,26) have investigated secondary CoQ10 deficiency in RR-MADD patients, and have reached contrasting conclusions. Therefore, further investigations to determine evidence and consequences of secondary CoQ10 deficiency in RR-MADD patients are warranted.

CoQ10 has multiple functions in the cell. In addition to its interaction with ETF-QO, in the electron transport chain, CoQ10 function as an electron transporter for complexes I and II, as an antioxidant and free radical scavenger, regulator of cell growth and differentiation, counter actor of apoptosis, cofactor for the uncoupling proteins, and has anti-inflammatory effects (4). Contrary to the many functions of CoQ10, it is uncertain how and why variant ETF-QO protein may be associated with a decrease in CoQ10 status and respiratory chain dysfunction. In the present study, we determined the CoQ10 levels in fibroblasts from six unrelated RR-MADD patients, all carrying amino acid variations located in the ubiquinone binding domain of ETF-QO, and showed that all investigated patient fibroblasts had a moderately decreased amount of CoQ10 compared with control fibroblasts. These results are in agreement with those of the study by Gempel et al. (24).

A connection between low CoQ10 status and increased oxidative stress has been previously demonstrated (11,14–17, 29–33). Therefore, we also measured the level of mitochondrial superoxide in the RR-MADD patient fibroblasts to evaluate if the decreased CoQ10 status of these cells was associated with increased oxidative stress. The results indicated a high percentage of cells with increased oxidative stress in the patient fibroblasts when compared with the controls. This result suggests that RR-MADD patients suffer from basal levels of chronic oxidative stress. Interestingly, our studies also showed that control fibroblasts, in riboflavin depleted conditions, also had CoQ10 deficiency, but that this deficit in CoQ10 did not result in increased oxidative stress. This indicates that in these cells, moderately decreased levels of CoQ10 alone do not result in mitochondrial superoxide production. Rather mitochondrial superoxide production seems to be related to defects in the ETF-QO. These results indicate that superoxide may actually come directly from the variant ETF-QO protein. Since all the amino acid variations included in this study are located in the ubiquinone binding domain of ETF-QO, an explanation for the observed oxidative stress could be a compromised binding of CoQ10 to the variant ETF-QO proteins. This would then allow oxygen to get access to and react directly with electrons in the ETF-QO and/or allow an increased leakage of electrons out from the misfolded enzyme to oxygen producing superoxide. To further understand the molecular mechanisms of ROS production from the variant ETF-QO proteins, we analyzed Q10Br binding of the corresponding variant Rhodobacter ETF-QO proteins over-expressed in E. coli. The fluorescence binding studies showed that the binding of Q10Br, and thus likely also CoQ10, to the variant ETF-QO proteins was compromised, when compared with

Figure 2. Western blot analysis of ETF-QO measured in protein extracts from control and patient fibroblasts grown for 7 days at supplemented or deficient concentrations of riboflavin, corresponding to 530 nmol/l or 3.1 nmol/l riboflavin, respectively. Patient fibroblasts (n = 6) were analyzed for ETF-QO protein expression by western blotting and compared to the expression in control fibroblasts (n = 3), and protein intensities were quantified relative to total protein as described in Materials and Methods. The error bars represent standard error of mean (SEM) of three independent experiments. Significance was measured as a two-paired t-test of the means. *P < 0.05.
wild-type ETF-QO protein suggesting that the observed mitochondrial superoxide in the patient fibroblasts could be produced directly from the misfolded ETF-QO proteins.

A similar mode of action as the one suggested for the variant ETF-QO proteins investigated in the current study was shown for mutations located in close proximity of the ubiquinone binding site of the Succinate-Ubiquinone Oxidoreductase (complex II) which resulted in increased superoxide production (34).

The antioxidant effect of CoQ10 is well known (35–37) and therefore we also treated the fibroblasts with CoQ10 for short and long periods. The results showed that long-term treatment with CoQ10, but not riboflavin, resulted in less oxidative stress in the mitochondria. This result indicates that the decrease in mitochondrial oxidative stress following long-term treatment with CoQ10 may be caused by the antioxidant potential of CoQ10, while riboflavin when converted to FAD most probably function as a chaperone, as reported in our previous study, which showed that riboflavin could improve folding and the amount of active enzyme of certain variant ETF-QO proteins (including p.Pro456Leu), but it could not compensate for increased H2O2 produced in HEK-293 cells over-expressing the variant p.Pro456Leu ETF-QO protein (27).

All amino acid variants investigated in this study are localized in the ubiquinone binding domain of ETF-QO. Two of the six RR-MADD patients, studied by Gempel et al. (24), had amino acid variations localized to the ubiquinone binding domain of ETF-QO (p.Pro456Leu, p.Lys590Glu and p.Pro483Leu), but four of the patients were homozygous for a p.Leu377Pro variation in the FAD binding domain of ETF-QO, and in the patients, in whom CoQ10 treatment was initiated, all showed clinical improvement following CoQ10 treatment. Therefore, the question arises as to whether amino acid variations in the FAD binding domain of the ETF-QO structure might also result in ROS production. One may speculate that the p.Leu377Pro variation may lead to local destabilization of the FAD-binding domain allowing O2 to gain access directly to the flavosemidiquinone generating ROS. Alternatively, variations in the FAD-binding domain may cause long-distance conformational changes that affect ubiquinone binding and result in ROS production by a mechanism similar to the one we suggest for the variant ETF-QO proteins studied in this article. In our previous study, we showed that the effects of amino acid variation in the FAD and ubiquinone binding domain are similar; probably due to the close folding of the two domains in the early stages of the folding process (27). That long-distance conformational changes can affect ubiquinone binding is supported also by Usselman et al. (38) and Beard et al. (39), who showed that amino acid variations in the Fe-S cluster binding domain disturb ubiquinone reductase activity.

The cause of the CoQ10 deficiency in RR-MADD patients has yet to be established and requires further investigation. In addition to CoQ10 deficiency, RR-MADD patients also show secondary deficiency of the respiratory chain complexes and some fatty acid oxidation enzymes. A possible hypothesis explaining the connection between mutant ETF-QO proteins, oxidative stress and this secondary mitochondrial dysfunction could be either: (1) a transcriptional reprogramming of energy metabolism, also known as Warburg effect, where the energy
metabolism is shifting from oxidative phosphorylation towards aerobic glycolysis. The Warburg mechanism was also suggested for severe MADD (S-MADD) patients in a study by Song et al. (40). In this study, they showed increased aerobic glycolysis both from a zebrafish model, exhibiting a S-MADD genotype/phenotype profile, and from patient fibroblasts (40) or (2) as a possible consequence of an overall mitochondrial loss in these patients as suggested by Wolfe et al. (41). Mitochondrial loss could result from the molecular mechanism of mitophagy. Mitophagy has been shown to be associated with increased oxidative stress and as a ‘helping mechanism’ for the cell to remove oxidative damaged mitochondria hereby mediating protein quality

Figure 4. ROS generation in RR-MADD patient fibroblasts. Fibroblast cultures from controls and patients with RR-MADD were grown in culture media supplemented with or depleted of riboflavin. Oxidative stress levels were determined by image cytometry using the mitochondrial targeted probe, MitoSOX. Results are expressed as stressed cells defined as the MitoSOX intensity above the control level. Cells were treated for 7 days with 530 nmol/l riboflavin alone (A), 72 h with 530 nmol/l riboflavin and 10 μmol/l CoQ10 (B), or 7 days with 530 nmol/l riboflavin and 10 μmol/l CoQ10. Error bars represent standard error of mean (SEM) of three independent experiments. Significance was calculated by a two-sample t-test: *P < 0.05.

Table 1. Q10Br fluorescence quenching studies of variant Rhodobacter ETF-QO proteins overexpressed in E. coli

<table>
<thead>
<tr>
<th>ETF-QO:CoQ10 model</th>
<th>Keq (μM)</th>
<th>fa</th>
<th>N (# x,y patients)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt:Q10Br</td>
<td>6.14(±0.07)</td>
<td>0.39(±0.004)</td>
<td>32</td>
<td>0.9951</td>
</tr>
<tr>
<td>p.Pro389Thr:Q10Br [p.Pro456Thr]</td>
<td>17.6(±1.8)</td>
<td>0.28(±0.03)</td>
<td>30</td>
<td>0.9966</td>
</tr>
<tr>
<td>p.Pro389Leu:Q10Br [p.Pro456Leu]</td>
<td>82.6(±4.0)</td>
<td>0.62(±0.03)</td>
<td>28</td>
<td>0.9984</td>
</tr>
<tr>
<td>p.Gly110Cys:Q10Br [p.Gly170Cys]</td>
<td>28.4(±0.03)</td>
<td>0.234(±0.002)</td>
<td>20</td>
<td>0.9952</td>
</tr>
<tr>
<td>p.Val385Leu:Q10Br [p.Val451Leu]</td>
<td>37.4(±11.4)</td>
<td>0.48(±0.14)</td>
<td>23</td>
<td>0.9966</td>
</tr>
</tbody>
</table>

Tryptophan fluorescence quenching experiments of the purified ETF-QO proteins where performed using a CoQ10 analog, Q10Br, which contains a hydrocarbon chain terminating in a bromine, which quenches the intrinsic fluorescence of ETF-QO and thus can serve as a probe of the ubiquinone binding site of wild-type and variant ETF-QO proteins. Fluorescence data were subjected to a modified Stern–Volmer analysis according to the following equation:

\[
F_0/F = (1/\text{Keq} + 1/[Q]) + 1/\text{fa} \times \text{F}_0/\Delta F \text{ vs. } 1/[Q]
\]

yields \( \text{fa} \) and \( \text{Keq} \) from the y-intercept and the slope, respectively. Where \( \text{Keq} \) is the association constant, \( \text{fa} \) is the fraction of accessible tryptophan residues and \([Q]\) represents the molar concentration of Q10Br in the enzyme solution after each incremental addition. The dissociation constants (\( K_d \)) were calculated as the reciprocal of the \( K_e \). All data in Table 1 were based on Stern–Volmer plots with \( R^2 \) values ≥ 0.995 covering at least 20 contiguous data points. Homologues human ETF-QO amino acid variations are shown in brackets.
control (42). It is also important to note that RR-MADD patients suffer from Ketosis, which could indicate a decreased amount of Acetyl-CoA, which is an important substrate for the synthesis of CoQ10, which could directly result in a decrease in CoQ10 status (3).

In conclusion, the present study shows that in fibroblasts from RR-MADD patients, CoQ10 treatment can compensate for an increased mitochondrial oxidative stress, most likely produced from misfolded variant ETF-QO proteins with decreased CoQ10 binding. Our results thus apply a molecular rational for at least some of the beneficial effect of CoQ10 treatment originally documented in RR-MADD patients by Gempel et al. (24), and suggest that CoQ10 together with riboflavin should be considered in the treatment regimes of RR-MADD patients.

MATERIALS AND METHODS

Patient and control samples

Human skin fibroblasts from six unrelated RR-MADD patients and three healthy controls (Camprex #CC-2509 annotated Control 1, Promocell #c-12300 #2090402.2 annotated Control 2, ATCC # CRL-2450 annotated control 3) were included in this study. The patients were diagnosed with RR-MADD based on clinical symptoms and characteristic plasma acylcarnitines and/or urine organic acids profiles, all of which were corrected or ameliorated by riboflavin treatment. In vitro oxidation of long-chain fatty acids in fibroblasts from the six patients were within heterozygous or wild-type levels when cultured in standard riboflavin supplemented media consistent with the patients being compound heterozygous or homozygous for ETFDH gene variations previously reported to be associated with RR-MADD (24,26,41,43,44). The patient fibroblasts were deidentified according to approval (m-20090126) and regulations from the Danish Ethical Committee. Further, to prevent any possibility of patient identification, only the genotype information with respect to riboflavin responsiveness is given: P1: p.[Pro456Thr]+[FS], P2: p.[Gly170Cys]+[FS], P3: p.[Pro456Leu]+[MS], P4: p.[Pro456Leu]+[FS], P5: p.[Gly170Cys]+[FS] and P6: p.[Val451Leu]+[MS], where MS is the missense variation and FS the frameshift variation.

Cell culturing

The fibroblasts were cultured in RPMI-1640 (Roswell Park Memorial Institute) medium (Lonza) supplemented with 2 mmol/l of 1-glutamine (Leo Pharmaceutical), 10% fetal calf serum (Biological Industries) and 1% penicillin/streptomycin (Leo Pharmaceutical) at 37°C, 5% (v/v) CO2. The fibroblasts were cultured until 60–70% confluence and then media were replaced at 37°C. After 3 days. After 7 days, the cells were harvested. The high concentration of riboflavin 530 nmol/l was chosen on the basis that previous studies provided evidence that a concentration of 530 nmol/l riboflavin in culture medium is sufficient to prevent riboflavin deficiency in HepG2 cells. This concentration is above the level of riboflavin observed in plasma from individuals given riboflavin treatment (45–47). The low concentration of riboflavin, 3.1 nmol/l, was chosen on the basis that previous studies provided evidence that HepG2 cells develop severe riboflavin deficiency if cultured in medium containing 3.1 nmol/l riboflavin and that this concentration represents the level of riboflavin observed in plasma from moderately deficient pregnant women (45,48).

Western blotting

Protein was extracted in a lysis buffer [50 mM Tris–HCl, pH 7.8, 5 mM EDTA, pH 8.0, 1 mmol/l DTT, 10 μg/ml aprotinin (Sigma-Aldrich), 1 mg/ml trypsin inhibitor (Bie & Berntsen), one tablet of protease inhibitors (Roche) in 10 ml and 1% Triton X-100], according to Schmidt et al. (49). Following centrifugation, 25 μg of the protein was analyzed by SDS–PAGE on 12.5% Tris–HCl Criterion Gels (Bio-Rad). Western blot analysis was performed according to Schmidt et al. (49) with monoclonal anti-ETF-QO (Ab126576) (Abcam) antibody, followed by incubation with secondary HRP antibody (Dako) using ECL plus western blotting Detection System (Amersham Biosciences). Detection was done using the ChemiDoc-It® Imaging System (UVP). The intensities of bands were quantified using VisionWorksLS Image Acquisition (UVP). All data were related to total protein.

Treatment with CoQ10

Cells treated with CoQ10 were incubated with a final concentration of 10 μmol/l CoQ10 (Sigma-Aldrich) for 72 h or 7 days before harvest/measurements.

Determination of CoQ10 levels in cultured skin fibroblasts

After lipid extraction from homogenized cultured skin fibroblasts, total cellular CoQ10 status was determined by reversed-phase HPLC coupled to UV detection at 275 nm using the method of Duncan et al. (50). The CoQ10 levels were expressed as nmol/mg of protein.

Mitochondrial ROS levels by MitoSOX measurements

Mitochondrial superoxide levels were measured using the MitoSOX™ Red Mitochondrial Superoxide Indicator (MitoSOX) (Invitrogen, USA), which is a live-cell permeant and is rapidly and selectively targeted to mitochondria. Once inside the mitochondria, MitoSOX is oxidized by superoxide anion; neither by other ROS nor by reactive nitrogen species, and exhibits red fluorescence (with excitation at 510 nm and emission at 580 nm). After treatment for the indicated time periods, cells were incubated in Hank’s balanced salt solution (HBSS) (Sigma) containing MitoSOX at 5 μmol/l for 20 min at 37°C. After incubation, cells were washed twice with PBS, then trypsinized, re-suspended in Hoechst 33342 at 10 g/ml and incubated for 15 min at 37°C for nuclear staining. Measurements of cellular fluorescence were performed with the Nucleo-Counter® NC-3000 image cytomter (NC-3000) (Chemometec, Denmark). The configuration of the NC-3000 used in this study consists of two different LEDs having peak wavelengths at 365 and 530 nm. The NC-3000 software was used for fluorescent
image acquisition, image analysis, subpopulation definition and quantification, and data visualization.

**Q10Br binding measurements of RR-MADD variant Rhodobacter ETF-QO proteins**

*Overexpression and purification of Rhodobacter ETF-QO proteins*

The *Rhodobacter sphaeroides ETFDH* gene (67% sequence homology with the human ETF-QO) was cloned into the pET21a expression vector and protein was expressed in *E. coli* C43 as previously described (38). Site-directed mutagenesis of the pET21a-ETFQD expression vector was done using the Stratagene QuikChange® II XL site-directed mutagenesis kit allowing production of the variant *Rhodobacter sphaeroides ETF-QO* proteins p.Pro389Thr, p.Gly110Cys, p.Pro389Leu or p.Val385Leu, corresponding to the variant human proteins; p.Pro456Thr, p.Gly170Cys, p.Pro456Leu and p.Val451Leu, respectively (Fig. 1A). Gene variations were confirmed by sequencing. Variant and wild-type plasmids were transformed into *E. coli* C43 cells. Cells were grown and protein purified as described previously (38).

**Synthesis and purification of Q10Br**

The ubiquinone pseudosubstrate Q10Br (Fig. 1B) was synthesized and purified based on the methods of Yu and Yu (51) and Watmough et al. (52), where the Florisil column chromatography technique used for purification of the final product was replaced by preparative thin layer chromatography. Plate layers containing the product were scraped from the plate, dissolved in methanol and filtered. The methanol was removed and the product was either redissolved in CDCl₃ prior to assessing purity via NMR analysis (Varian, 300 MHz) or redissolved in ethanol prior to use in fluorescence quenching studies.

**Fluorescence quenching studies**

Similarly to the method of Watmough et al. (52), a Gilford Fluoro IV fluorometer was used to measure the fluorescence of 2 ml of a 2 μmol/l solution of *R. sphaeroides* ETF-QO in 20 mM HEPES, pH 7.5, 25°C, with 0.2 mg/ml (0.4 mM) dodecyl maltoside added. The excitation wavelength was 295 nm and the emission wavelength was 334 nm. Increments (0.5–3.0 μl) of 1 mM stock solutions of Q10Br in ethanol were added. Stabilized fluorescence measurements were recorded after each added increment.

**Data analysis**

The fluorescence measurement data were subjected to a modified Stern–Volmer analysis according to Lakowicz and Watmough et al. (52,53).

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**Conflict of Interest statement.** None declared.

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**REFERENCES**
