Cellular consequences of oxidative stress in riboflavin responsive multiple acyl-CoA dehydrogenation deficiency patient fibroblasts

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Mitochondrial dysfunction and oxidative stress are central to the molecular pathology of many human diseases. Riboflavin responsive multiple acyl-CoA dehydrogenation deficiency (RR-MADD) is in most cases caused by variations in the gene coding for electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO). Currently, patients with RR-MADD are treated with high doses of riboflavin resulting in improvements of the clinical and biochemical profiles. However, in our recent studies of RR-MADD, we have shown that riboflavin treatment cannot fully correct the molecular defect in patient cells producing increased reactive oxygen species (ROS). In the current study, we aim to elucidate the cellular consequences of increased ROS by studying the cellular ROS adaption systems including antioxidant system, mitochondrial dynamics and metabolic reprogramming. We have included fibroblasts from six unrelated RR-MADD patients and two control fibroblasts cultivated under supplemented and depleted riboflavin conditions and with coenzyme Q10 (CoQ10) treatment. We demonstrated inhibition of mitochondrial fusion with increased fractionation and mitophagy in the patient fibroblasts. Furthermore, we indicated a shift in the energy metabolism by decreased protein levels of SIRT3 and decreased expression of fatty acid β-oxidation enzymes in the patient fibroblasts. Finally, we showed that CoQ10 treatment has a positive effect on the mitochondrial dynamic in the patient fibroblasts, indicated by increased mitochondrial fusion marker and reduced mitophagy. In conclusion, our results indicate that RR-MADD patient fibroblasts suffer from a general mitochondria dysfunction, probably initiated as a rescue mechanism for the patient cells to escape apoptosis as a result of the oxidative stress.

INTRODUCTION

Mitochondria produce the majority of cellular energy through oxidative phosphorylation (OXPHOS). The mitochondria also play many other important roles in regulatory mechanisms of the cell, such as cell signaling, proliferation, differentiation and cell death through apoptosis or necrosis (1). Furthermore, mitochondria are known to be one of the major intracellular sources of reactive oxygen species (ROS), mainly originating from the protein complexes (I–IV) involved in OXPHOS (2). In relation to the many important roles of mitochondria in the cell, mitochondrial dysfunction has been linked to a wide range of diseases, and although the molecular mechanisms responsible for the disease development are not fully resolved, it seems clear that oxidative stress play an important role (3–5). ROS is produced continuously during normal cellular function and is known to have an essential function in cellular signaling, by regulating the expression/activity of many genes and enzymes. The positive effect of ROS is highly dependent on the antioxidant system (6). If the level of ROS production exceeds a certain level that overwhelms the antioxidant system, it becomes toxic to the cells resulting in oxidative stress and activation of additional mitochondrial quality control mechanisms to keep mitochondria functional and the cell alive (7,8). However, the exact molecular triggers of...
increased ROS and how mitochondria mechanisms initiate and compensate for physiological and pathological processes remain poorly understood.

Multiple acyl-CoA dehydrogenation deficiency (MADD), also known as Glutaric Aciduria type II, is a rare autosomal recessively inherited disorder of fatty acid, amino acid and choline metabolism (9). In most patients with MADD, the disease is caused by variations in one of two genes encoding electron transfer flavoprotein (ETF) or in the gene encoding electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO), both central to the mitochondrial metabolism (9,10). Some MADD patients respond dramatically to treatment with riboflavin, to such a degree that clinical and biochemical abnormalities are near normalized upon treatment with riboflavin; these patients are referred to as riboflavin responsive multiple acyl-CoA dehydrogenation deficiency (RR-MADD) (11,12). By means of an in vitro cell system overexpressing variant ETF-QO proteins, we have shown that riboflavin, in the form of FAD, functions as a molecular chaperone, stabilizing the ETF-QO proteins. Riboflavin could however not compensate for increased ROS produced in the same cells (13). In a recent study, we confirmed the production of increased ROS, by showing increased mitochondrial superoxide production in fibroblasts from six unrelated RR-MADD patients all carrying amino acid substitutions in the ubiquinone-binding domain of ETF-QO (14). In addition, we also detected decreased coenzyme Q10 (CoQ10) levels and showed that CoQ10 treatment, but not treatment with riboflavin alone, could restore CoQ10 levels and decrease ROS in patient fibroblasts (14). The decreased CoQ10 level was compatible with previous published results by Gempel et al. (15), showing CoQ10 deficiency in muscle biopsies from a group of RR-MADD patients.

In summary, these results may indicate oxidative stress as an important event in the pathogenesis of RR-MADD. However, the molecular mechanisms related to oxidative stress and the possible resulting molecular and cellular defects are poorly elucidated, since only few studies have investigated parts of these aspects of the disease mechanism in RR-MADD patient (15,16). The current study aims to elucidate the molecular and cellular effect of increased ROS in RR-MADD patient fibroblasts, both under riboflavin-supplemented and -depleted conditions, and when treated with CoQ10.

RESULTS

The mitochondrial antioxidant response in RR-MADD patient fibroblasts

In our previous studies, 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 HEK-293 cells (13). In addition, we showed that the level of ETF-QO protein was increased in patient fibroblasts upon riboflavin treatment (14). Further, we showed, both in the HEK293 cell system and in patient fibroblasts, that variations in the ETFDH gene are related to increased ROS production, compared with controls (13,14). In the same patient fibroblasts, we also showed that treating the cells with CoQ10, but not riboflavin, has beneficial effect on both the CoQ10 level and the ROS level (14). Based on these results, our hypothesis is that patients in riboflavin treatment suffer from chronic oxidative stress, and we want to examine the extent to which the CoQ10 treatment can counteract the oxidative stress. Therefore, we have included both riboflavin supplemented and deficient growth conditions and also CoQ10 treatment conditions in these studies.

In relation to our findings of an increased superoxide levels in the patient fibroblasts, one would expect an increase in the level of mitochondrial manganese superoxide dismutase (MnSOD), the mitochondrial chief ROS scavenging enzyme (17). However, our data obtained by western blotting and quantification of the relevant protein bands showed that all six RR-MADD patient fibroblasts display significantly decreased MnSOD protein levels amounting to 15–60% of the MnSOD level in control fibroblasts and that riboflavin supplementation could not increase the level of MnSOD protein in the control nor in the patient fibroblasts (Fig. 1A). Another relevant protein related to the mitochondrial antioxidant system is Lon, a member of the AAA-proteases, which removes oxidatively damaged proteins and apparently is also involved in mitochondrial DNA (mtDNA) repair (18–21). Investigation of the Lon protein level in the six unrelated RR-MADD patient fibroblasts showed that it was decreased significantly in patient P1, P2, P3, P4 and P6, when compared with controls, cultivated under the same conditions. The decrease in the patient fibroblasts was 20–40% when compared with controls, except one of the patient fibroblasts (P5), which was found to be at control level both under supplemented and deficient riboflavin conditions (Fig. 1B). That P5 showed control level of Lon protein correlates with our previous results, studying mitochondrial superoxide production in patient fibroblasts, where P5 fibroblasts showed the lowest amount of ROS when compared with the other patient cells analyzed (14). Riboflavin supplementation did not affect the protein level of Lon of the control fibroblasts, but the patient fibroblasts showed a significantly riboflavin response.

Finally, we investigated the protein level of DJ-1 (also known as Park7). DJ-1 protects cells against oxidative stress and cell death by eliminating hydrogen peroxide and thereby protects cells against hydrogen peroxide-induced cell death (22). Furthermore, DJ-1 has been shown to be required for correct mitochondrial morphology and function, and for autophagy of dysfunctional mitochondria (23). As represented in Figure 1C, our results showed that, under riboflavin-supplemented conditions, the level of DJ-1 protein is significantly increased by 30–60% in the patient fibroblasts compared with the control fibroblast. Under riboflavin deficient conditions, there are no significant difference between control and patient fibroblasts. However, there was a significant riboflavin response in both the control and patient fibroblasts.

Regulation of mitochondrial dynamics: a cellular mechanism to eliminate oxidative damaged mitochondria

In recent years, there has been increasing focus on mitochondrial dynamics, especially the events involving fusion and fission of mitochondria. Although the details of the two mechanisms are still debated, it has been shown that fusion and fission events are very crucial for the mitochondrial functional maintenance (24). These events enable the mitochondria to remove damaged ‘parts’. Furthermore, it has been shown that the cell regulates the events during oxidative stress by inhibiting the fusion of
Figure 1. Antioxidant response in control and RR-MADD patient fibroblasts. Protein expression of (A) MnSOD, (B) Lon and (C) Park7 in control (n = 2) and patient fibroblasts (n = 6) cultivated for 7 days at supplemented or deficient concentrations of riboflavin, corresponding to 530 and 3.1 nmol/l riboflavin, respectively. The protein amount was determined by western blot analysis of whole-cell protein extract and protein intensities were quantified relative to total protein as described in Materials and Methods. The western blot is a representative blot of three independent experiments. The error bars represent standard error of mean (SEM) of three independent experiments. Significance was calculated by a paired t-test: * P < 0.05, or a two-sampled t-test: ** P < 0.05. × not included.
mitochondria to prevent the damaged mitochondria fusing with the healthy mitochondria. Instead, these damaged mitochondria are targeted for degradation by means of mitophagy (25–27).

To investigate whether this regulation of the fusion/fission events takes place in the investigated RR-MADD patient fibroblasts, as a mechanism to remove mitochondria damaged by oxidative stress, we examined the protein level of mitofusin2 (Mfn2). This protein is one of the key regulators of the fusion event in the mitochondria and has been found to be degraded during inhibition of fusion (28). Our results show that the protein level of Mfn2 is decreased in all, except for P1, the RR-MADD patient fibroblasts when compared with the control fibroblasts, showing a protein level of Mfn2 in the patient fibroblasts of only 40–80% when compared with the control fibroblasts, both under supplemented and deficient riboflavin concentrations. The different Mfn2 protein profile of P1 is surprising; however, in relation to our previous study, it was the variation that was less affected on the CQ10 binding, when compared with the other variations also included in this study (14). There is no significant riboflavin response in the control fibroblasts, but a significant riboflavin response in the patient fibroblasts (Fig. 2A).

Next, we evaluated the mitochondrial morphology in the patient and control fibroblasts by staining mitochondria by means of MitoTracker Red CMXRos labeling, followed by fluorescence microscopy analysis. Images of the MitoTracker-labeled cells showed how the mitochondria surround the nucleus in a thread-like pattern. As shown in Figure 4, three different mitochondrial morphologies were detected in control and RR-MADD patient fibroblasts, denoted normal thread-like, grained/mild fractionated or severely fractionated. The thread-like phenotype represents the normal morphology of the mitochondrial reticulum, generally observed in healthy cells, whereas the grain-like phenotype is a distinct change from the thread-like phenotype to a more punctuated mitochondria, but still with remains of the tread-like structure. Cells displaying the severely fractionated phenotype include mitochondria that show no tread-like structure, only round circular, separated mitochondrial structures. To quantitate the various mitochondrial
Figure 2. Mitochondrial dynamic. Protein expression of Mitofusin 2 (A) and p62 (B) in control and patient fibroblasts cultured for 7 days at supplemented or deficient concentrations of riboflavin, corresponding to 530 and 3.1 nmol/l riboflavin, respectively. The protein amount was analyzed and quantified as described in Figure 1. The error bars represent standard error of mean (SEM) of three independent experiments. Significance was calculated by a paired $t$-test: $^a P < 0.05$, or a two-sampled $t$-test: $^b P < 0.05$. 
phenotypes, a minimum of 300 individual control and patient fibroblasts were selected [by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining] and scored according to the mitochondrial morphology. Categorization of the mitochondria into the three morphology groups may be overlapping, and to avoid biases in evaluating the mitochondria morphology, the nature of the ETFDH genotype was unknown during analysis. The evaluation showed that 90% of the mitochondria in fibroblasts from the two controls displayed the thread-like mitochondrial morphology. In contrast, the patient fibroblasts all displayed a dramatic change in mitochondrial morphology with significantly reduced amount of tread-like structured mitochondria. While fibroblasts carrying the p.Gly170Cys and p.Pro456Leu variant, ETF-QO proteins seem to show the mildest morphology changes of the four genotypes, containing ≈50% tread-like mitochondria, 40% grained mitochondria and only a very small fraction of the severely fractionated mitochondria. Cells expressing the p.Pro456Thr or p.Val451Leu variant proteins seem to be more severely affected with only ≈20% tread-like mitochondria, over 60% grained mitochondria and up to 20% severely fractionated mitochondria (Fig. 4).

Metabolic shifting in RR-MADD patient fibroblasts; a way to regulate ROS production

It is well documented that RR-MADD patients in addition to ETF-QO deficiency have secondary deficiency of components of the electron transport chain sometimes accompanied by downregulation of enzymes of the fatty acid β-oxidation (14,15,33–35). Interestingly, investigations of a Zebrafish model of MADD and of fibroblasts from MADD patients have shown that decreased OXPHOS in MADD could be part of a metabolic reprogramming, including an upregulation of genes and proteins assigned to aerobic glycolysis, indicating an initiation of a Warburg effect (36,37). The Warburg effect is an essential metabolic change, which occurs in cancer cells to sustain biomolecule production and proliferation, but it also protects the cells from increasing ROS production by shuttling metabolites away from OXPHOS (17,38). We therefore investigated whether the RR-MADD patient fibroblasts showed indication of a Warburg effect. As a main regulator of mitochondrial ROS, MnSOD has recently been shown to play a major role in the initiation and regulation of the Warburg effect in cancer cells by controlling ROS-mediated activation of hypoxia-inducible factor 1α (HIF-1α), which controls the expression of a number of target genes that drive the metabolic reprogramming. As in RR-MADD cells (Fig. 1A), MnSOD expression is low in the initiation of the Warburg effect and early cancer development (17,38). In addition to the protein amount analysis showing reduced level of MnSOD (Fig. 1A), the expression (mRNA level) of SOD2, the gene expressing MnSOD, was also analyzed in order to investigate if the significant decreased protein level of MnSOD might be caused by a transcriptional regulation and not just a posttranscriptional mechanism. Our results showed that the mRNA level of SOD2 was significantly decreased in patients 1, 4, 5 and 6 under both riboflavin-supplemented and deficient conditions, as that there was no significant riboflavin response. Whereas cells from patients 2 and 3 showed control level of SOD2 mRNA, both under riboflavin-supplemented and deficient conditions (Fig. 5A). To get further indications of possible activation of a metabolic shift in the RR-MADD patient fibroblasts, we analyzed the protein level of SIRT3. SIRT3 has many roles in the cell.
Mainly SIRT3 functions as a mitochondrial deacetylase, which controls the activity of mitochondrial respiration by activating enzymes of fatty acid oxidation, amino acid metabolism and the electron transport chain. Recently, SIRT3 has also been recognized as an important tumor suppressor, as it inhibits ROS production through deacetylation and activation of MnSOD, which then inhibits the initiation of the Warburg effect by regulating ROS levels and the activation of HIF1α (17,39–41). A significant decrease (40–80%) in the level of SIRT3 protein (Fig. 5B) was identified in the patient fibroblasts when compared with the control fibroblasts under riboflavin-supplemented conditions, supporting the hypothesis that a metabolic regulation is initiated in these patient fibroblasts. Due to a significant riboflavin response in the SIRT3 level in the control fibroblasts, but not in the patient fibroblasts, a reduction in the protein level of up to 50% was found upon riboflavin depletion, resulting in the same level of SIRT3 protein in control and patient fibroblasts under these conditions. To support that metabolic reprogramming is taking place in the patient fibroblasts, we analyze the protein level of short-chain acyl-CoA dehydrogenase (SCAD) and medium-chain acyl-CoA dehydrogenase (MCAD), as representatives of the mitochondrial fatty acid β-oxidation. SCAD protein amount was found to be significantly different between the patient and the control fibroblasts, both under riboflavin-supplemented and -deficient conditions, and a significant riboflavin response was seen for both controls and patients (Fig. 5C). MCAD protein seems more riboflavin sensitive in control fibroblasts, as a significantly decrease was found between control and patient fibroblasts only under riboflavin-supplemented conditions (Fig. 5D).
Figure 5. Metabolic regulations in the RR-MADD patient fibroblasts. (A) mRNA level of SOD2, measured relative to POP4 and measured by RT-qPCR as described in Materials and Methods, (B) protein level of SIRT3, (C) protein level of SCAD and (D) protein level of MCAD. Control and patient fibroblasts cultivated for 7 days at supplemented or deficient concentrations of riboflavin, corresponding to 530 and 3.1 nmol/l riboflavin, respectively. The error bars represent standard error of mean (SEM) of three experiments. Significance was calculated by a paired $t$-test: $^* P < 0.05$ or a two-sampled $t$-test: $^{**} P < 0.05$. 
Figure 5. Continued
The molecular effect of CoQ10 treatment in RR-MADD patient fibroblasts

Based on our previous study, where we showed that CoQ10 treatment of the patient fibroblasts could reduce the amount of ROS, most likely produced from misfolded variant ETF-QO proteins with impaired CoQ10-binding affinity (14), we wanted to investigate if treatment with CoQ10 can reverse or inhibit some of the effects seen in the mitochondria of the RR-MADD patient fibroblasts. This would indicate that these events are initiated by the increased ROS produced from misfolded ETF-QO proteins in the patient fibroblasts.

As seen in Figure 6A, CoQ10 treatment increased the amount of p62 protein in the patient fibroblasts up to 40%. Fibroblasts from patient P6 (p.Val451Leu) showed a very small increase in the p62 protein level. In parallel, CoQ10 treatment also increased the protein level of Mfn2 between 20 and 40%, except for P6 (p.Val451Leu), which did not show increased levels of Mfn2 after CoQ10 treatment (Fig. 6B). The missing effect of CoQ10 treatment on the p62 and Mfn2 protein level, in P6 cells, might be explained by the morphology studies (Fig. 4A and B), where the mitochondrial morphology in fibroblasts from patient P6 was clearly more affected towards fractionation than any of the other patient fibroblasts, indicating that there is more damage to these mitochondria, and therefore the oxidative stress might be too severe for CoQ10 to reverse the effect. These results also correlate with our previous studies showing the highest amount of ROS production in cells from P6 when compared with the other patients (14). The protein level of the AAA-protease Lon was also increased upon CoQ10 treatment for some of the investigated patient fibroblasts. In patient 1, the Lon protein level was raised to control levels, and for patient 2 to ~80% of control level, whereas for patients 3–6 there were no effect of the CoQ10 treatment (Fig. 6C). For the protein level of MnSOD, we did only see an effect of CoQ10 treatment for patient 1, in which we see an ~30% increase in MnSOD protein upon CoQ10 treatment (Fig. 6D). We did not see any effect on the protein level of SIRT3 (data not shown), and also the mitochondrial mass did not change upon CoQ10 treatment (Fig. 3).

DISCUSSION

In 2007, others and we showed that variations in the ETFDH gene are associated with secondary dysfunction of components of the electron transport chain (15,35), and we speculated that this mitochondrial dysfunction could be initiated and propagated by ROS (35,42–44). Recently, we then investigated and confirmed that increased mitochondrial ROS are produced in fibroblasts from patients suffering from RR-MADD and showed that ROS most likely originate from a leakage of electrons from the misfolded ETF-QO proteins (13,14). Based on these finding, we have recently suggested that a series of defense mechanisms are initiated by the cell to cope with the damaging effects of increased ROS (45). Upon increased ROS, the cell tries to remove the overproduced ROS to prevent the damaging effect on proteins, lipids and DNA (46). To decrease ROS, the cell initiates upregulation of the antioxidant system. An essential mitochondrial antioxidant is MnSOD, which is in the first line of defense when mitochondria experiencing oxidative stress. MnSOD is capable of detoxifying superoxide radical ions (45). Our results on the antioxidant response seem more complex than first expected. While an increase in both MnSOD and Lon protein levels was expected, as a cause of the increased oxidative stress detected in these fibroblasts, the opposite result was obtained. Reduction in the MnSOD protein level has however been detected in other diseases with increased oxidative stress, especially various forms of cancer (17), but also in diseases like SCAD deficiency; another disorder of mitochondrial fatty acid β-oxidation (47). Why MnSOD protein level is decreased in these diseases is still obscure and needs to be further investigated, but a suggested mechanism could be MnSOD’s involvement in the regulation of the metabolic reprogramming as discussed above and in (38). Interestingly, the protein level of MnSOD was found to be increased in a proteomic study of fibroblasts from a patient with severe ETF-QO misfolding (S-MADD) and lack of ETF-QO protein (37). The biphasic MnSOD levels in RR-MADD and S-MADD could reflect different levels of ROS produced as a consequence of mild versus severe ETF-QO misfolding and these results may underline MnSOD’s critical role in buffering cellular ROS levels and bringing them to a level at which the cell can minimize damage by initiating signaling stress responses. The abovementioned downregulation of MnSOD in a proteomic study of fibroblasts from patients carrying mild misfolding SCAD enzymes (47) is another example supporting the regulatory effect of MnSOD. In another study of cells overexpressing a severe variant of the SCAD enzyme, we have shown increased MnSOD expression. These cells also produced increased mitochondrial superoxide and had a fractionated mitochondrial morphology that could be mitigated by MitoQ treatment (48). Thus, these results from patients with fatty acid oxidation disorders show cellular adaptation to chronic oxidative stress by activating cellular survival pathways (49).

Mitochondrial Lon protease has previously been shown to be sensitive to oxidative stress and elevated levels of Lon proteins are found in cells experiencing increased oxidative stress (18,19,50). The role of Lon during oxidative stress is to specifically remove, by degradation, oxidative damaged proteins, thereby preventing aggregation, which may give rise to additional ROS production and thus initiation of a potential vicious circle of oxidative stress. Reduced levels of Lon have previously been identified in patients with neurodegenerative spastic paraplegia (51). In addition, it has been shown that especially oxidative stress due to electron transport chain dysfunction affects the Lon protein level negatively (52). Additionally, it has been shown that Lon regulates and maintains the mtDNA, where reduced levels of Lon initiate the transcription of mtDNA and reduce the amount of mtDNA lesions (20,21). A suggestion to the role of Lon could therefore be that Lon under normal cellular conditions binds to mtDNA, but upon oxidative stress the binding of Lon to the mtDNA is changed, allowing proteins of the DNA repair and replication systems to bind to mtDNA (53).

If the antioxidant system cannot eliminate the produced ROS, our data from the present study suggest that two mechanisms are initiated to prevent oxidative stress mediated damage; (1) inhibition of fusion events resulting in fractionation of the mitochondria and initiation of mitophagy, taking place as a process that specifically remove dysfunctional mitochondria in RR-MADD patient fibroblasts. The overall distribution of the mitochondrial
morphological phenotypes from control fibroblasts and RR-MADD patient fibroblasts was clearly different (Fig. 4). Thus, these results support the results showing decreased levels of Mfn2 and p62 in the patient cells (Fig. 2A and B). Taken together, the results suggest that one mechanism for the RR-MADD patient fibroblasts to cope with the increased oxidative stress is to initiate fractionation of the mitochondria and inhibit the fusion in order to isolate the damaged mitochondria, which then can be removed by mitophagy. This mechanism is thought as a survival mechanism for the mitochondria to

Figure 6. Effect of CoQ10 treatment. Protein level of (A) p62 (B) Mitofusin 2 (C) Lon and (D) MnSOD for control and patient fibroblasts cultivated for 7 days in standard riboflavin-supplemented RPMI-1640 media with or without 10 μM CoQ10 treatment. Significance was calculated by a paired t-test: *P < 0.05.
Figure 6. Continued
remove parts of the damaged mitochondria, instead of entire mitochondria, thereby minimizing the risk of losing so many mitochondria that the cell would be targeted for apoptosis/necrosis. Our results also show that CoQ10 treatment can counteract decrease in both Mfn2 and p62 protein levels, which is in accordance with our recent findings that CoQ10 can decrease ROS production in the patient fibroblasts (14). (2) A metabolic shift in RR-MADD patient fibroblasts might also serve as a defense mechanism. The secondary decrease in fatty acid oxidation enzymes and electron transport chain components in RR-MADD patients (14,15,33–35) may be an escape from an additional increase of ROS, originating from electron leakage from a damaged electron transport chain and, as recently suggested, the variant ETF-QO proteins (14). A further advantage of a shift away from mitochondrial OXPHOS in RR-MADD patients would be that this may force the cells to shift towards glycolysis as an alternative source of energy and biosynthetic intermediates to ensure survival. Our results from CoQ10 treatment showed that while CoQ10 could inhibit the initiation of autophagy and increase the protein level of Mfn2 and p62, it had no significant effect on MnSOD and SIRT3 expression in the RR-MADD patient cells. These results could support the hypothesis that an initiation of a metabolic shift is an early-oxidative stress response event, while regulation on the mitochondrial dynamic is an event activated later and maybe only when the ROS have reached a higher level (Fig. 7).

Elimination of dysfunctional mitochondria, caused by oxidative stress, is critical to protect the cells from the damage caused by an altered mitochondrial function and the possible release of proapoptotic factors. The increase in mitochondrial mass that was found in the patient fibroblasts when compared with the control fibroblasts may be caused by two regulations: (i) it might simply indicate that the patient fibroblasts upregulate the biogenesis of mitochondria to cope with the increased removal of damaged mitochondria and (ii) it is important to emphasize that RR-MADD patients suffer from both mitochondrial fatty acid β-oxidation and electron transport chain deficiency resulting in a decreased ATP level in these patients (36). Therefore, it could be speculated if the upregulated biogenesis of mitochondrial mass, in the patient fibroblasts, is a way for the patient fibroblasts to compensate for the low ATP. Wen et al. also found increased mitochondria biogenesis in RR-MADD patients (54). These results are furthermore supported by Kim et al. showing upregulation of the mechanistic/mammalian target of rapamycin complex 1 in RR-MADD (55), a serine/threonine kinase which has been shown to be a regulator of the mitochondrial metabolism and biogenesis (56).

In recent years, there has been an increasing focus on mitochondrial pharmacology due to increasing number of diseases found to be associated with defects in the mitochondria. Pharmacological compounds like resveratrol and bezafibrate have been shown to have beneficial effects in animal models of Parkinson’s

**Figure 7.** Cause of events during oxidative stress. Results from this study support a hypothesis where different regulatory mechanisms are regulated in relation to the oxidative stress amount. While oxidative stress is at a minimum, we will have healthy cells with some active antioxidant level, which would be expected to exist in a form of positive balance, keeping the oxidative stress level within minimum levels. The initiation of a metabolic shift, switching away from OXPHOS and towards glycolysis in an attempt to reduce the main source of oxidative stress, is an early-oxidative stress response event, while regulation on the mitochondrial dynamic is an event activated later and maybe only when the ROS have reached a higher level, supported by our results from CoQ10 treatment that can only reduce the top of the mitochondrial stress level resulting in regulation of the markers of mitochondrial dynamics and mitophagy. At one point, the oxidative stress will reach the level of oxidative stress which will initiate apoptosis, the ultimate endpoint. Proteins/genes investigated in this study are noted in black under the mechanism in which it plays a role.
disease (57), Huntington’s disease (58,59), Alzheimer’s disease (60) and also in mouse models of mitochondrial myopathy (61). The beneficial effects on these diseases, all being related to both neurodegeneration and mitochondrial dysfunction, indicate that such treatments might also be beneficial for RR-MADD patients. Especially interesting in relation to treatment of RR-MADD patients is resveratrol because it has been shown to stimulate sirtuin (SIRT) activity and thereby transcription of nuclear genes involved in the energy metabolism (62,63). Furthermore, a recent study has shown resveratrol to decrease oxidative stress (64). Furthermore, resveratrol treatment has been shown to result in induction of the antioxidant system of the cell itself, such as MnSOD and glutathione reductase (65–68). Because RR-MADD patients have secondary respiratory chain deficiency, and in relation to our results also decreased SIRT3 and MnSOD expression it could be interesting to investigate the effects of resveratrol treatment in RR-MADD fibroblasts. The effect of treatment with bezafibrate has been shown to be associated with mitochondrial proliferation and enhanced OXPHOS capacity per muscle mass (61). Bezafibrate treatment of carnitine palmitoyltransferase II deficient patients fully restored fatty acid oxidation (69,70). Since RR-MADD patients have been shown to suffer from electron transport and fatty acid oxidation deficiency, due to missense gene variations, bezafibrate treatment of these patients would be interesting. However, long-term effects of regulation on transcription factors must be cautiously considered. Taken into consideration that RR-MADD patients have primary defects in ETF-QO, enhancing the electron transport capacity and fatty acid oxidation might give a positive short term effect, but may also result in long-term accumulation of substrates or increased electron leak and ROS, due to defective ETF-QO function. It could be speculated that the regulations of the different mechanisms we find in the present study, such as inhibition of mitochondrial fusion and regulation of SIRT3 and MnSOD, are probably initiated to ensure survival of the cell and, therefore, reversing these events might result in apoptosis.

In conclusion, the present study shows that as a result of increased ROS in RR-MADD patient fibroblasts, the cells initiate a number of mitochondrial rescue mechanisms that (i) removes the damaged mitochondria, resulting from the oxidative stress, (ii) activates the mitochondrial biogenesis to cope with the loss of mitochondria from mitophagy and (iii) activates a metabolic reprogramming, possibly to prevent further ROS to accumulate. Our data suggest that patients under riboflavin treatment have mild chronic oxidative stress and therefore most likely are more sensitive to additional stress factors, as discussed in our recent review (49). CoQ10 can in this system only partially correct for the cellular damage, perhaps due to the known obstacle to cross the mitochondrial membrane (71). This also correlates with our previous result showing that CoQ10 could only partially correct the increase in ROS production in the patient fibroblasts (14). Clearly, further studies using synthetic analogs and/or different doses/duration of CoQ10 treatment are needed. However, the fact that CoQ10 has shown therapeutic benefit in other patients with CoQ10 deficiency (72,73) together with results from this and our previous study (14) strongly suggest that CoQ10 treatment in supplement 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 two healthy controls (Camprex #CC-2509 annotated Control 1, Promocell #c-12300 #2090402.2 annotated Control 2) were included in this study. The six patient samples **were** the same as were utilized in our previous study (14). The third control from the recent study was excluded from the present study based on very low/lacking MnSOD protein, combined with a very low SOD2 gene expression. All three controls and one patient samples (P6) were investigated for variations in the 5-UTR, 3-UTR, part of the promoter and the coding exons of SOD2. However, the sequence data could not explain the variable SOD2 expression in the controls (data now shown). The patients were diagnosed with RR-MADD based on clinical symptoms and characteristic plasma acylcarnitines and/or urine organic acids, 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 having at least one ETFDH gene missense variation, previously reported to be associated with RR-MADD (15,16,33–35). The patient fibroblasts were de-identified according to approval (m-20090126) and the Act on Research Ethics Review of Health Research Projects. Further, to prevent any possibility of patient identification, only the genotype information essential with respect to riboflavin responsiveness is given: P1: p.[Pro456Thr]+[MS], P2: p.[Gly170Cys]+[FS], P3: p.[Pro456Leu]+[MS], P4: p.[Pro456Leu]+[FS], P5: p.[Gly170Cys]+[FS] and P6: p.[Val451Leu]+[MS], where MS, missense variation; FS, frame shift variation. The same group of patients has previously been studied in Cornelius et al. (14).

#### Cells culture

The fibroblasts were cultured in RPMI-1640 (Roswell Park Memorial Institute) medium (Lonza) supplemented with 2 mmol/l of L-glutamine (Leo Pharmaceutical), 10% fetal calf serum (FCS) (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 was replaced by fresh RPMI-1640 medium containing 530 nmol/l riboflavin or by riboflavin-depleted RPMI-1640 medium (custom made by PromoCell) with addition of riboflavin to a final concentration of 3.1 nmol/l. The media was changed 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 (74). This concentration is above the level of riboflavin observed in plasma from individuals given riboflavin treatment (74–76). The low concentration of riboflavin, 3.1 nmol/l was chosen on the basis that human liver cells (HepG2) developed severe riboflavin deficiency if cultured in medium containing 3.1 nmol/l riboflavin (unpublished data). In support of this notion, the concentration of 3.1 nmol/l represents the level of riboflavin observed in plasma from moderately deficient pregnant women (74,76).
Treatment with CoQ10
Cells treated with CoQ10 were incubated in standard medium containing 10 μmol/l CoQ10 (Sigma–Aldrich) for 7 days before harvest/measurements. The choice of 10 μmol/l CoQ10 is based on our previous published paper in which we found this concentration to have a positive effect on the oxidative stress in the patient fibroblasts (14).

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. (48). 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. (48) using MnSOD (Stressgen, SOD-111), Lon (generated previously (51)), DJ-1 (Park7) (Abcam, Ab4150), p62 (Abcam, Ab56416), Mfn2 (Abcam, Ab56889), SCAD (Abcam, Ab72767–50), MCAD (Mitoscience, Ms726), SIRT3 (Abcam, Ab86671) antibodies, followed by incubation with secondary HRP antibody (Dako). Protein bands were visualized 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.

Quantitative reverse transcription-PCR
Total RNA was isolated from fibroblasts using TRIzol® Reagent (Invitrogen) and isolated RNA was treated with DNase free (Ambion) to remove any remaining DNA contamination. Complementary DNA (cDNA) was synthesized from 0.5 μg RNA using iScript cDNA synthesis kit (Bio-Rad) with oligo(dT) primers. SOD2 (Hs001670309_m1) mRNA amount was measured relative to Ribonuclease P protein subunit p29 (POP4) (Hs00198357_m1) and measured in triplicates by quantitative reverse transcription-PCR (qRT-PCR). SOD2 (Hs001670309_m1) mRNA amount was measured relative to Ribonuclease P protein subunit p29 (POP4) (Hs00198357_m1) and measured in triplicates by quantitative real-time PCR using the ABI Step One Plus Detection System (Applied Biosystems) and TaqMan® probe chemistry (Applied Biosystems), as previously described (51). All probes used was conjugated to a 6-FAM™ dye at the 5′ end and a non-fluorescent quencher group in the 3′ end.

Visualization of the mitochondrial morphology
Control and patient fibroblasts were incubated 20 min at 37°C with 100 mmol/l MitoTracker Red CMXROs probe (Molecular Probes) in 10 cm² slideflasks (Nunc). MitoTracker stains mitochondria in living cells in a membrane-dependent manner. Fibroblasts were fixed in 4% (w/v) neutral buffered formalin (CellPath). Cells were permeabilized in freshly made, cold 70% ethanol. One microgram per microliter of DAPI (Sigma–Aldrich) was applied as nuclear DNA labeling. Imaging was done by using a 563 nm line of a helium–neon laser to detect the MitoTracker Red CMXROS and the 405 nm line of a 405–30 nm diode laser to detect the DAPI, on a confocal laser scanning microscope (LSM 710, Zeiss) using a ×63 oil-immersion objective with a numerical aperture of 1.4. Sequential imaging was done by normal fluorescence microscopy (Leica DM RB).

Mitochondrial mass
Patient and control fibroblasts were washed with PBS and incubated at 37°C for 30 min with 100 nm MitoTracker Green FM (Molecular probes). Cells were harvested using PBS/EDTA containing 1% trypsin and resuspended in PBS. Samples were analyzed using a FACSARiaIII (BD Biosciences). Mitotracker fluorescence was detected using a 488 nm laser and a 502 nm long pass filter followed by a 530/30 nm band pass filter. Dead cells were detected using a 561 nm laser and fluorescence from propidium iodide positive cells were detected in a 610/20 nm band pass filter. Relative mitochondrial mass is shown as mean intensity per 15 000 cells counted. Data were analyzed using DIVA software (v.6.1.3, BD Biosciences).

Statistics
Upon analysis of treatment response a paired t-test (P < 0.05) was used and upon comparison of control versus patient groups a two-sample t-test was used (P < 0.05).

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