Free Rad. Res., Vol. 35, pp. 11–21
Reprints available directly from the publisher
Photocopying permitted by license only

Human Cultured Skin Fibroblasts Survive Profound Inherited Ubiquinone Depletion

VANNA GEROMEL[a], NOMAN KADHOM[a], IRÈNE CEBALLOS-PICOT[b], DOMINIQUE CHRÉTIEN[a], ARNOLD MUNNICH[a], AGNÈS RÖTIG[a] and PIERRE RUSTIN[a,*]

[a]*Unité de Recherches sur les Handicaps Génétiques de l'Enfant (INSERM U393) Hôpital Necker-Enfants Malades, 149, rue de Sèvres, 75743 Paris Cedex 15, France;* [b]*CNRS UMR 8602, Faculté de Médecine Necker, 156, rue de Vaugirard, 75730 Paris Cedex 15, France*

Accepted by Prof. Frank J. Kelly

(Received 28 July 2000; In revised form 2 October 2000)

Beside its role in electron transfer in the mitochondrial respiratory chain, ubiquinone is known to prevent lipid peroxidation and DNA damage by trapping cellular free radicals. Thanks to its antioxidant properties, ubiquinone may represent an important factor controlling both necrotic and apoptotic processes. We have investigated the consequences of a profound inherited ubiquinone depletion on cultured skin fibroblasts of a patient presenting with encephalomyopathy. Interestingly, cell respiration, mitochondrial oxidation of various substrates, and cell growth of ubiquinone-deficient fibroblasts were only partially decreased. Moreover, these cells did not apparently overproduce superoxide anions or lipoperoxides. Finally, apoptosis did not increase as compared to control, even after serum deprivation. These observations suggest that ubiquinone may not play a major role in the antioxidant defenses of cultured fibroblasts and that its role in controlling oxidative stress and apoptosis may greatly vary across cell types, especially as not all tissues were equally affected in the patient despite the widespread ubiquinone depletion *in vivo*.

Keywords: Ubiquinone depletion, apoptosis, free radicals

Abbreviations: CoQ, ubiquinone; ROS, radical oxygen species; SOD, superoxide dismutase

INTRODUCTION

Ubiquinone 50 (2,3-dimethoxy-5-methyl-6-decaprenyl-1,4-benzoquinone), or coenzyme, Q_{10} is an essential lipid component of the respiratory chain (RC) in the mitochondrial inner membrane of all eukaryotes and the plasma membrane of prokaryotes.[1] In the respiratory chain, ubiquinone (CoQ) acts as a mobile carrier transferring reducing equivalents from the various dehydrogenases to the bc_1 complex and participates to the proton-motive Q cycle responsible for proton transfer across the inner membrane.[2,3] CoQ is in large stoichiometric excess as compared to other RC components allowing for a pool function, distributing electrons between the several

*Corresponding author. Tel.: 33 (0)1 44 38 15 84. Fax: 33 (0)1 47 34 85 14. E-mail: rustin@necker.fr.

terminal cytochrome segments.[4] Detailed kinetic analyses, including inhibitor titration, have shown kinetic compartmentation in the CoQ pool between the various dehydrogenases.[5,6]

In mammalian cells, CoQ is not only found in the mitochondrial inner membrane, but is also present in most endomembranes and the plasma membrane.[1] In this latter membrane, CoQ as a partner of a NADH oxidase would be involved in cytosolic NAD(P)H oxidation to acceptors at the cell surface.[7] In addition to its role in electron transfer, CoQ in its reduced form, ubiquinol, acts as a free radical scavenger in the different cell compartments, acting in close connection with α-tocopherol.[8] In particular, CoQ has been claimed to play a key role in controlling apoptosis induced by radical oxygen species (ROS) via its antioxidant action.[9,10] Accordingly, using leukemic cell line HL60, Barroso *et al.* have demonstrated the participation of plasma membrane CoQ in the control of apoptosis induced by serum withdrawal.[11] In addition, several apoptosis-inducing factors, namely UV irradiation,[12] oxidative stress[13] and staurosporine[14] have been reported to induce a decrease of CoQ synthesis.

On the other hand, a decrease in CoQ content has been reported in aging suggesting an inverse correlation between life span and the peroxide-producing potential of mammalian tissues.[15] CoQ may therefore increases organism longevity by maintaining antioxidant defenses. Accordingly, the *Caenorhabditis elegans clock-1* gene, homologous to the yeast coq7 gene possibly involved in CoQ biosynthesis, has been shown to control life span of the worm.[16,17] Finally the length of the hydrophobic isoprenoid side chain of CoQ is regarded as key factor in life span of various species.[15,18]

Conversely, CoQ can be directly or indirectly involved in the generation of $O_2^{\bullet}$, and decreasing the mitochondrial CoQ content has been shown to strongly decrease the rate of $O_2^{\bullet}$ production.[7] Indeed, transition of CoQ to its unstable semiquinone form occurs as an essential intermediate step during electron transfer through the respiring mitochondria and, as such, represents a major source of oxygen reactive species by the respiratory chain.[19]

There has been only few reports of primary ubiquinone biosynthesis deficiency in human so far. They all presented as mitochondrial encephalomyopathies, the CoQ_{10} depletion being detected in skeletal muscle tissue only.[20,21,22] In particular, normal CoQ_{10} content was found in skin fibroblasts and lymphocytes of these patients, precluding a detailed analysis of the mechanisms leading to CoQ_{10} depletion and their potential consequences. We recently reported a case of quinone-responsive mitochondrial encephalomyopathy caused by a defect in CoQ_{10} biosynthesis resulting in widespread severe CoQ_{10} depletion in all tissues tested, including lymphocytes and skin fibroblasts.[23] A defect of the trans-prenyltransferase activity involved in the biosynthesis of CoQ_{10} was inferred from the very low decaprenyl pyrophosphate level detected in patient's skin fibroblasts grown in the presence of labeled mevalonate.[23] This prompted us to investigate the consequences of an inherited CoQ_{10} depletion in human cells. Here, we report on the surprisingly mild impact of CoQ_{10} depletion on human cultured skin fibroblasts, which contrasts with the predictable consequences expected from the role of ubiquinone in numerous aspects of cell life.

PATIENTS AND METHODS

Patients

Patient 1 was a boy who was born after term pregnancy and normal delivery to unrelated healthy parents. His older brother is healthy but two sisters were affected as well. All three patients presented a mitochondrial encephalomyopathy associated with a severe nephrotic

syndrome resulting in kidney failure, which required kidney transplantation. The older sister (by 3 years) died at age 8 years following a rapid neurological deterioration. The second sister (patient 2; older by 1 year) had a milder form of the disease.[23] A severe CoQ_{10} depletion was diagnosed in both patient's cultured skin fibroblasts (patient 1: <5 ng/mg prot; patient 2: 23.4 ng/mg prot; normal: 117–200 ng/mg prot). After diagnosis of CoQ_{10} depletion in these patients, the boy and his sister were given oral CoQ_{10} and the treatment resulted in a spectacular improvement of their general condition.[23]

Cell Culture

Fibroblast strains were established from skin biopsies of controls and the CoQ_{10} deficient patients. Skin fibroblasts were grown in RPMI 1640 (Life Technologies Inc.) with glutamax supplemented with 10% fetal calf serum, 2.5 mM sodium pyruvate, 100 IU penistreptomycin, 200 μM uridine, 2.5 μg/ml fungizone, at 37 °C and 5.0% CO_2.[24] Growth rates were determined on cells plated into 25 cm^2 flasks at 1.5×10^{-5} cells/flask. On days 3, 7, 10, 14, 16, 21, duplicate cell samples were collected and counted.[25]

Enzyme Assays

Spectrophotometric assays of respiratory chain enzymes and polarographic tests were carried out on cultured skin fibroblasts as described.[26] Other enzyme assays were carried out on confluent cells, washed twice with phosphate buffer saline solution and stored in liquid nitrogen. Frozen samples were thawed and diluted with 50 mM KH_2PO_4 buffer (pH 7.8) before enzyme assays at 37 °C. Proteins were measured using the Pierce method. Total superoxide dismutase (EC 1.15.1.1) activity was spectrophotometrically determined by monitoring the autoxidation of pyrogallol at 410 nm.[27] One unit of enzyme activity is defined as the amount of the enzyme required to inhibit the rate of pyrogallol autoxidation by 50%. CuZnSOD (cytosolic) is efficiently inhibited by cyanide whereas MnSOD (mitochondrial) is unaffected. Hence, KCN (2 mM) for 30 minutes at room temperature was used to inhibit CuZnSOD allowing for the specific measurement of the MnSOD activity. Glutathione peroxidase (EC 1.11.1.9) was spectrophotometrically measured at 340 nm using *tert*-butyl hydroperoxide as substrate in a coupled assay with glutathione reductase (EC 1.6.4.2) and NADPH.[28] The reaction mixture (pH 7.0) consisted of 0.5 mM reduced glutathione, 1 mM tert-butyl hydroperoxide, 100 IU/l yeast glutathione reductase (Type III; Sigma Chemical Co., St.Louis, MO), 7.5 μM NADPH, 0.5 mM KCN and 0.1 mM Na_2PO_4. Glutathione reductase activity was spectrophotometrically assayed at 340 nm by following the oxidation of 0.05 mM NADPH in the presence of 1 mM oxidized glutathione in 0.1 M KH_2PO_4 buffer (pH 7.4).[29] The glutathione S-transferase (EC 2.5.1.18) activity was spectrophotometrically measured with 1-chloro-2,4-dinitrobenzene as substrate following the formation of the S-conjugate at 340 nm in 0.1 M Na_2PO_4 buffer (pH 6.5).[30] Enzyme activities were automatically measured in duplicates on a Cobas-Bio centrifugal analyzer (Hoffman-La Roche, 4002 Basel, Switzerland).[31] Catalase (EC 1.11.1.6) was measured using a Clark oxygen electrode by following oxygen evolution in the presence of 10 μM H_2O_2 in 500 μl 0.1 M KH_2PO_4 (pH 7.0).[32] Mitochondrial *plus* cytosolic aconitases (EC 4.2.1.3), citrate synthase (EC 4.1.3.7) and isocitrate dehydrogenase (EC 1.1.1.41) were spectrophotometrically measured on the supernatant (10000 g × 5 min) of Triton X100-treated cells (2% final concentration).[33] Aconitase activity was spectrophotometrically determined at 240 nm following the conversion of 25 mM citrate to cis-aconitate. A subsequent addition of 2.5 mM $MgCl_2$, 0.4 mM NADP and 12.5 mM isocitrate allowed to measure isocitrate dehydrogenase activity in the same cuvette at 340 nm.

Mitochondrial Membrane Potential

Membrane potential was spectrofluorimetrically monitored (LS 50B Spectrofluorimeter, Perkin-Elmer, England) using 0.5 μM rhodamine 123 (excitation: 525 nm, 5 nm band-pass; emission: 558 nm, 5 nm band-pass) in a 37 °C thermostated quartz cell, magnetically stirred, and containing 2 ml of a medium containing 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, 1 mg/ml BSA, 10 mM KH_2PO_4 (pH 7.4) and 0.01% digitonin as to allow exogenous reducing substrate (succinate) to be oxidized by the mitochondria. Alternatively, fibroblasts were plated on a tissue culture-treated chambre slide (Falcon, USA) at a density of approximately 70–80%. After 24 hours, the cells were labeled for 5 min with 2.4 μM rhodamine 123 and examined for fluorescence (Leica microscope).

Lipoperoxidation Measurement

Cis-parinaric acid was used as a fluorescent probe for lipoperoxidation.[34] Measurements (excitation: 318 nm, 5 nm band-pass; emission: 410 nm, 5 nm band-pass) were performed in a 37 °C thermostated quartz cell, magnetically stirred and containing 2 ml of 0.1 M Tris-HCl buffer (pH 7.3).

Measurement of ROS

After 1 h incubation at 37 °C in the presence of 1 μM dihydrorhodamine 123, attached cells were trypsinized and their fluorescence determined (excitation: 485 nm, bandpass: 2.5 nm; emission: 535 nm, bandpass: 10 nm).

Cell Death

Fibroblasts were cultured in serum-free RPMi-1640 medium during 9 hours as to induce cell death. The immunohistochemical detection of apoptosis was achieved according to the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an *in situ* cell death detection kit (Boehringer Mannheim). Fibroblasts were also analyzed for condensed or fragmented chromatin. Cells were dyed with Hoechst 33258 at 15 μM for 15 min at room temperature and their fluorescence examined. DNA fragmentation was studied on cells digested for 18 h at 37 °C in 300 μl of a medium containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.15 M NaCl, 1% SDS, 0.2 mg/ml proteinase K. The aqueous phase was extracted with phenol/chloroform, precipitated with ethanol and digested with DNAse-free RNAse (0.5 mg/ml) in 30 μl 10 mM Tris-HCl (pH 8.0), 1 mM EDTA at 37 °C for 30 min. DNA was analyzed on 1.5% agarose gel.

RESULTS

CoQ_{10} Depletion and Mitochondrial Respiratory Chain Activities

Severe CoQ_{10} depletion caused the deficiency of several quinone-dependent respiratory chain activities namely succinate cytochrome *c* reductase, glycerol-3-phosphate cytochrome *c* reductase and dihydro-orotate cytochrome *c* reductase in both patient's fibroblasts (Table I). According to the slightly higher residual amounts of ubiquinone detected in patient 2 cultured fibroblasts,[23] enzyme activities were less severely decreased in these latter fibroblasts. CoQ_{10} depletion also resulted in the decrease of intact cell respiration to less than 40% of control (Patient 1: 3.7 nmol/min/mg prot; controls: 7.2 ± 2.0 nmol/min/mg prot; $n = 85$). Residual respiration was fully cyanide- and antimycin-sensitive (not shown). The preserved sensitivity to inhibitors indicates that electrons fed to the respiratory chain by the dehydrogenases did not quantitatively escape the chain to directly react with molecular oxygen. The analysis of the enzyme activity ratios to complex IV also revealed, that CoQ_{10} depletion variously altered respiratory pathways (dihydro-

TABLE I Respiratory chain enzyme activities in CoQ_{10}^- and control fibroblasts

	Activities (nmol/min/mg prot)				Activity ratios		
	P1	P2	Control ($n=85$)		P1	P2	Control ($n=85$)
CII	17	20	12–42	CIV/CII	5.5	4.8	4.7 ± 0.7
CIII	91	92	33–187	CIV/CIII	1.0	1.1	1.0 ± 0.2
CIV	94	96	47–182	CIV/G3Pdh	8.5	6.0	7.4 ± 0.8
G3Pdh	11	16	6–26	CIV/CS	1.1	0.8	1.5 ± 0.3
CS	85	113	51–111				
CII+CIII	**15**	**22**	16–68	CIV/CII+CIII	**6.3**	**4.4**	3.0 ± 0.3
G3Pdh+CIII	**4**	**12**	9–38	CIV/G3Pdh+CIII	**23.5**	8.0	5.8 ± 0.9
DHOdh+CIII	**1**	–	4–8 ($n=5$)	CIV/DHOdh+CIII	**94**	–	16.4 ± 3.2 ($n=5$)

The values of respiratory chain activities of patient fibroblasts represent mean values of triplicates. The extreme absolute control values are indicated. The absence of a normal distribution of absolute control values precluded the use of standard deviations. As control activity ratios follow a Gaussian distribution,[43] these values are presented as mean ± SD. CI-CIV: respiratory chain complexes; G3Pdh: glycerol-3-phosphate dehydrogenase; CS: citrate synthase; DHOdh: dihydro-orotate dehydrogenase.

orotate cytochrome *c* reductase > glycerol-3-phosphate cytochrome *c* reductase > succinate cytochrome *c* reductase), suggesting different CoQ_{10} requirements (Table I).

As expected, none of the individual enzyme activities was affected and normal complex III activity suggested that decylubiquinol, used as a reductant in the decylubiquinol cytochrome *c* reductase assay, was able to feed electrons to this complex despite severe ubiquinone depletion. Similarly, decylubiquinone, acting as an electron acceptor in the succinate decylubiquinone DCPIP reductase assay measuring complex II activity, appeared to readily accept electrons from this complex. Ubiquinone might therefore be facultative for measurement of these activities, the synthetic quinone being able to directly interact with donor and acceptor sites of the complex III and complex II, respectively. Alternatively, the residual amount of bound CoQ_{10} might be sufficient to warrant normal electron flow from and to the synthetic quinone acceptor.

CoQ_{10} Depletion, Fibroblast Morphology and Growth Rates

Cultured skin fibroblasts from patient 1 (CoQ_{10}^- fibroblasts) and control were tested for morphological changes under phase-contrast microscope. The size of patient 1 fibroblasts was roughly

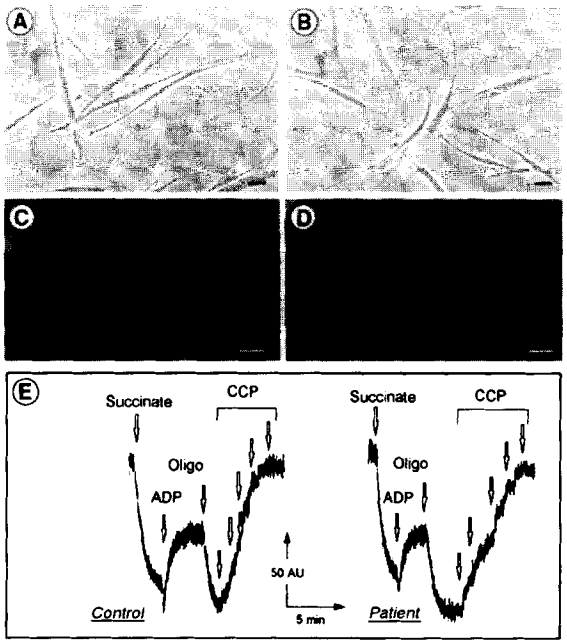
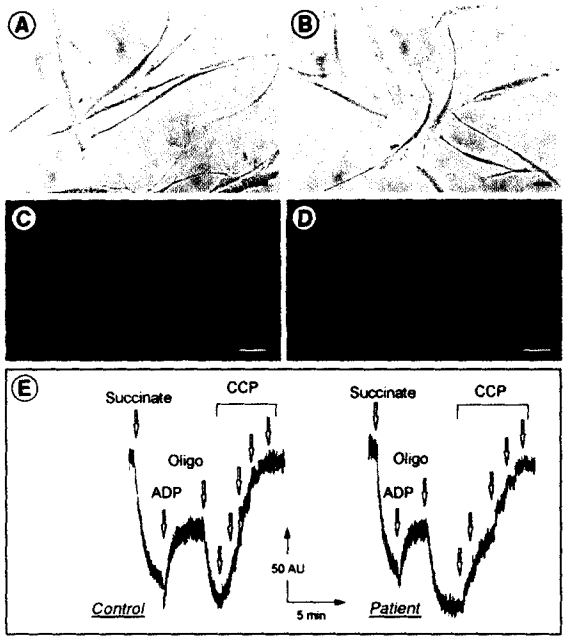
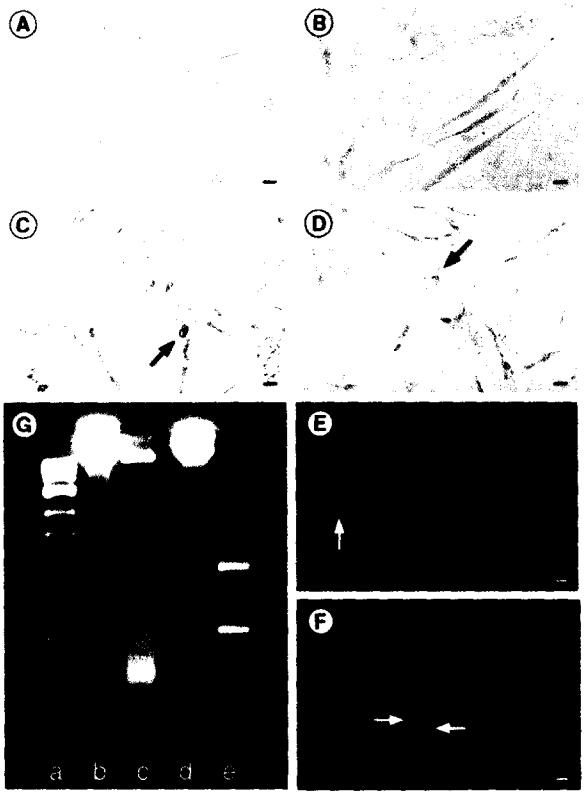


FIGURE 1 *Cellular and mitochondrial morphology of patient 1 and control fibroblasts.* (A) Phase contrast examination of control and (B) patient 1 fibroblasts (×25 objective); (C) Fluorescent staining of mitochondria in control and (D) patient 1 fibroblasts with the membrane potential-sensitive fluorescent dye rhodamine 123 (×40 objective) (E): measurement of membrane potential with rhodamine 123 in control and patient (CoQ_{10}^-) fibroblasts. Experimental conditions as described under Materials and Methods. (See Color plate I at the end of this issue.)



Color Plate I (see page 15, figure 1) *Cellular and mitochondrial morphology of patient 1 and control fibroblasts.* (A) Phase contrast examination of control and (B) patient 1 fibroblasts (×25 objective); (C) Fluorescent staining of mitochondria in control and (D) patient 1 fibroblasts with the membrane potential-sensitive fluorescent dye rhodamine 123 (×40 objective) (E): measurement of membrane potential with rhodamine 123 in control and patient (CoQ_{10}^{-}) fibroblasts. Experimental conditions as described under Materials and Methods.



Color Plate II (see page 17, figure 3) *Cell death in control and patient 1 fibroblasts. A, B*: TUNEL staining of control (A) and patient 1 (B) fibroblasts. (C, D) TUNEL staining of control (C) and patient 1 (D) fibroblasts after 9 h of fetal calf serum deprivation. (E, F) Hoechst staining of control (E) and patient 1 (F) fibroblasts after 9 h of fetal calf serum deprivation. (G) analysis of DNA fragmentation in fibroblasts; lines a, e: molecular weight markers; line b: control fibroblasts; line c: control fibroblast treated with 100 nM staurosporine; line d: Patient 1 fibroblasts. Experiments were carried out as described under Materials and Methods.

similar to that of control, with typical spindle-shape (Figure 1A,B). Mitochondria from patient 1 cells accumulated a membrane potential-sensitive fluorescent dye (rhodamine 123) showing a filamentous network similar to that of control cells (Figure 1C,D). Accordingly, normal membrane potential was measured in mitochondria from digitonin-permeabilized patient 1 fibroblasts when using rhodamine 123 in the presence of succinate (Figure 1E). Noteworthy, a similar amount of uncoupler (*m*-Cl-CCP) was required to fully collapse membrane potential from both patient 1 and control fibroblasts (Figure 1E).

Subsequently, we compared the growth rate of CoQ_{10} and control fibroblasts (Figure 2). Noticeably, patient 1 and 2 cultured fibroblasts presented quite similar growth rates (not shown). Control cells exhibited an usual growth pattern: a short lag phase, followed by an exponential phase, ending with a plateau. At day 16, cell monolayer began to detach. We repeatedly noted that patient 1 cells more slowly reached the exponential phase, resulting in a slightly lower initial growth rate ($g = 0.57$). However the growth rate of patient 1 cells rapidly increased to a maximal value similar to that of controls (about 1.8; Table II). Growth rate represents the instant balance between cell multiplication and cell death. Thus, slight initial decrease in growth rate might stem from increased cell death. For this reason, and because CoQ_{10} has been regarded as a key factor controlling apoptosis,[10] we subsequently measured various parameters associated with cell death and apoptosis.

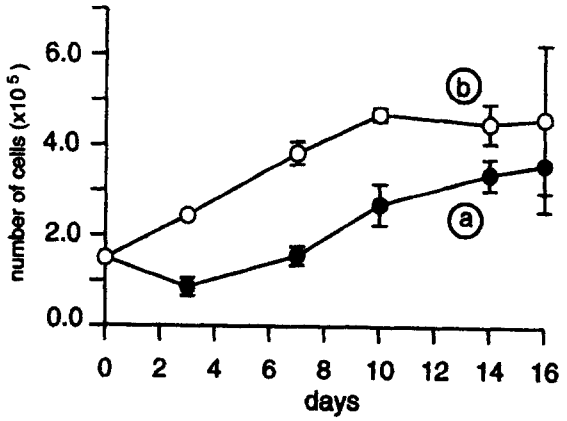


FIGURE 2 *Growth of control and patient 1 fibroblasts.* Patient 1 (a) and control (b) fibroblasts were cultured in RPMI medium supplemented by uridine and pyruvate as to allow respiratory deficient cells to grow and the curves were recorded as described under Materials and Methods.

TABLE II Growth rate of patient 1 and control fibroblasts

Fibroblasts	Growth rate				
	day 3	day 7	day 10	day 14	day 16
Control	1.84	1.40	1.17	0.88	0.90
Patient 1	0.57	1.80	1.74	1.25	1.06

Growth rate (g) of control and patient 1 fibroblasts was calculated from triplicates for each corresponding interval of time as the following ratio: g = cell number at a given time (t)/ cell number at time ($t - t_{-x}$), x representing the number of days between two cell counting.

CoQ_{10} Depletion and Fibroblast Death

Immunohistochemical (TUNEL) reaction was performed on both patient 1 and control cells so as to detect increased DNA fragmentation and potential build up in apoptotic body number (Figure 3A,B). In parallel, nucleus morphology was examined using Hoechst dye (Figure 3A,F). Neither qualitative nor quantitative differences between patient 1 and control cells could be observed. Accordingly, no difference in DNA fragmentation was observed when DNA was analyzed on 1.5% agarose gel (Figure 3G). Known to promote cell apoptosis,[9] removal of the serum from the culture medium of patient 1 and control fibroblasts induced apoptosis in a significant but similar number of cells (Figure 3C,D). Finally, 3-(4,5-dimetylthiazol-2)-2,5-diphenyl tetrazolium bromide (MTT) reduction was not significantly different between patient 1 and control cells (not shown). Taken together, these experiments indicated that severe CoQ_{10} depletion in cultured skin fibroblasts causes no

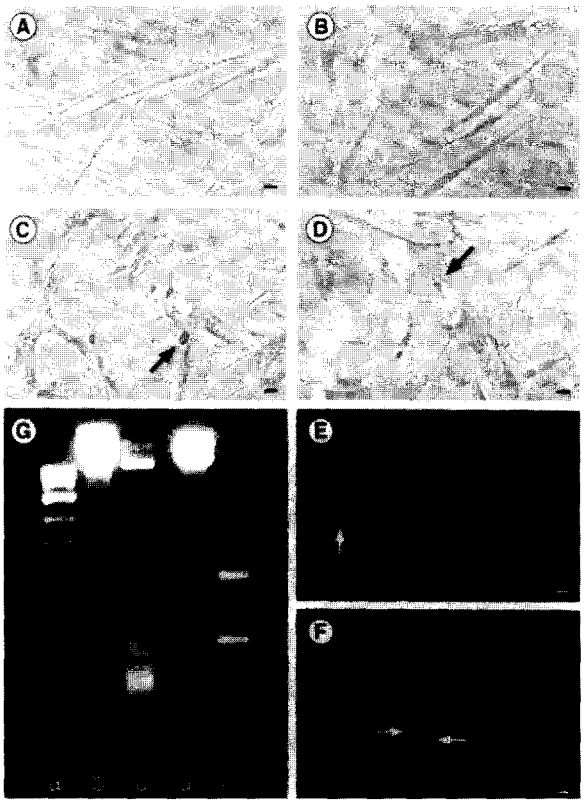


FIGURE 3 *Cell death in control and patient 1 fibroblasts. A, B*: TUNEL staining of control (A) and patient 1 (B) fibroblasts. (C, D) TUNEL staining of control (C) and patient 1 (D) fibroblasts after 9 h of fetal calf serum deprivation. (E, F) Hoechst staining of control (E) and patient 1 (F) fibroblasts after 9 h of fetal calf serum deprivation. (G) analysis of DNA fragmentation in fibroblasts; lines a, e: molecular weight markers; line b: control fibroblasts; line c: control fibroblast treated with 100 nM staurosporine; line d: Patient 1 fibroblasts. Experiments were carried out as described under Materials and Methods. (See Color plate II at the end of this issue.)

significant increase in cell death or susceptibility to apoptosis.

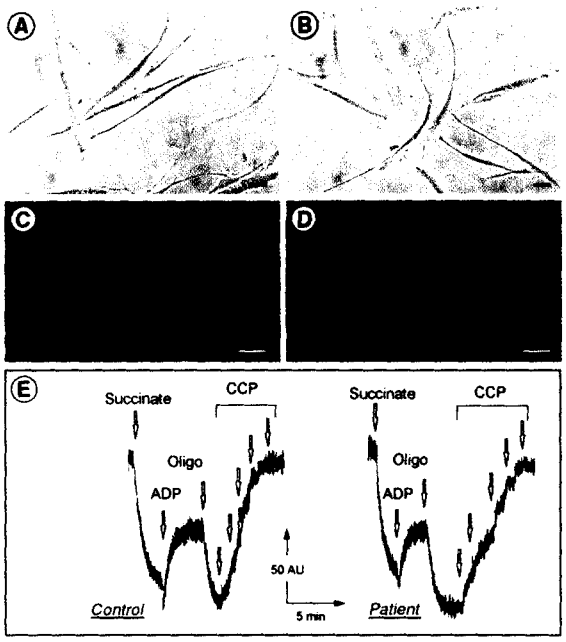
CoQ_{10} Depletion and Antioxidant Defenses

Because the putative anti-apoptotic activity of CoQ_{10} is believed to be related to its antioxidant activity, we hypothesized that a decreased antioxidant capacity caused by CoQ_{10} depletion should be balanced by an increase activity of alternative antioxidant defenses. We therefore measured activity of several key antioxidant enzymes and the glutathione reservoir (Table III). Glutathione peroxidase activity, known to control lipoperoxidation, was the only enzyme activity found to slightly increase. This observation suggests that patient 1 cells should undergo a very limited oxidative stress related to the CoQ_{10} depletion. However, we found that privileged targets of ROS in both cell membranes (lipids) and soluble phase (iron-sulfur containing aconitases, cytosolic *plus* mitochondrial) were not hit in patient 1 cells as compared to control (Table III). In order to directly detect an increased ROS production, we used the non-fluorescent dihydrorhodamine 123 (which reacts with H_2O_2 and possibly with superoxide anions giving rise to fluorescent oxidized rhodamine 123). The largely similar fluorescence increase detected in the patient 1 (+6.4 AU) and the control (+7.7 AU) cells suggests that no increased ROS production occurred in CoQ_{10}^- cells. Supplementing culture medium with a controlled amount of H_2O_2 as to induce an exogenous oxidative stress similarly altered growth rate of patient 1 and control cells (Figure 4A).

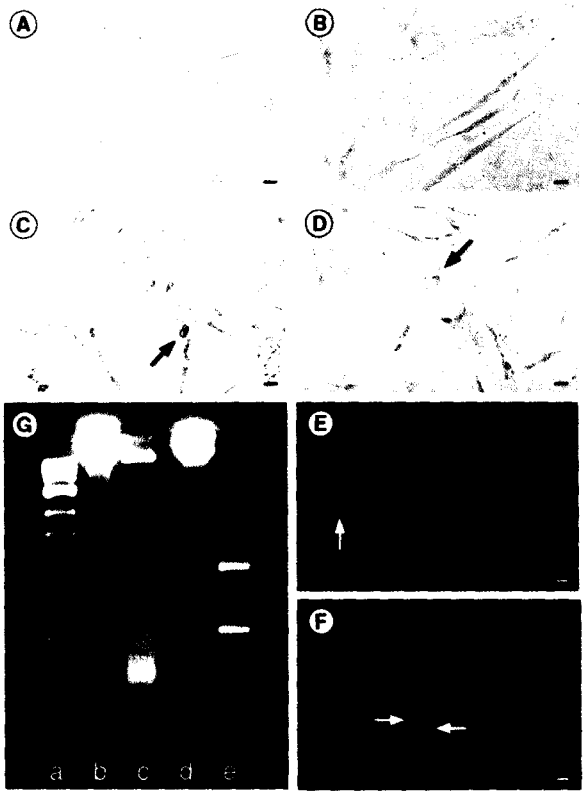
TABLE III Measurement of antioxidant and marker enzymes in patient 1 and control fibroblasts

Activities	Fibroblasts	
	Patient 1	Control ($n=5$)
(units/mg prot)		
Superoxide dismutase		
Total	23.3	20.93 ± 8.6
Mn-dependent	18.9	14.77 ± 5.8
Cu/Zn-dependent	4.4	6.17 ± 2.5
(μmol/min/mg prot)		
Glutathione peroxidase	12.9	8.75 ± 3.16
Glutathione synthetase	0.47	0.45 ± 0.20
Glutathione reductase	5.7	5.97 ± 1.50
Reduced Glutathione	0.024	0.029 ± 0.013
(nmol/min/mg prot)		
Catalase	101	77 ± 21
Citrate synthase	69.6	53.9 ± 12.9
Aconitase	10.3	8.0 ± 2.9
Isocitrate dehydrogenase	69.3	65 ± 19

Activities were measured as described under Materials and Methods. One unit of enzyme activity is defined as the amount of enzyme required to inhibit the rate of pyrogallol autoxidation by 50%.



Color Plate I (see page 15, figure 1) *Cellular and mitochondrial morphology of patient 1 and control fibroblasts.* (A) Phase contrast examination of control and (B) patient 1 fibroblasts (×25 objective); (C) Fluorescent staining of mitochondria in control and (D) patient 1 fibroblasts with the membrane potential-sensitive fluorescent dye rhodamine 123 (×40 objective) (E): measurement of membrane potential with rhodamine 123 in control and patient (CoQ_{10}^{-}) fibroblasts. Experimental conditions as described under Materials and Methods.



Color Plate II (see page 17, figure 3) *Cell death in control and patient 1 fibroblasts. A, B*: TUNEL staining of control (A) and patient 1 (B) fibroblasts. (C, D) TUNEL staining of control (C) and patient 1 (D) fibroblasts after 9 h of fetal calf serum deprivation. (E, F) Hoechst staining of control (E) and patient 1 (F) fibroblasts after 9 h of fetal calf serum deprivation. (G) analysis of DNA fragmentation in fibroblasts; lines a, e: molecular weight markers; line b: control fibroblasts; line c: control fibroblast treated with 100 nM staurosporine; line d: Patient 1 fibroblasts. Experiments were carried out as described under Materials and Methods.

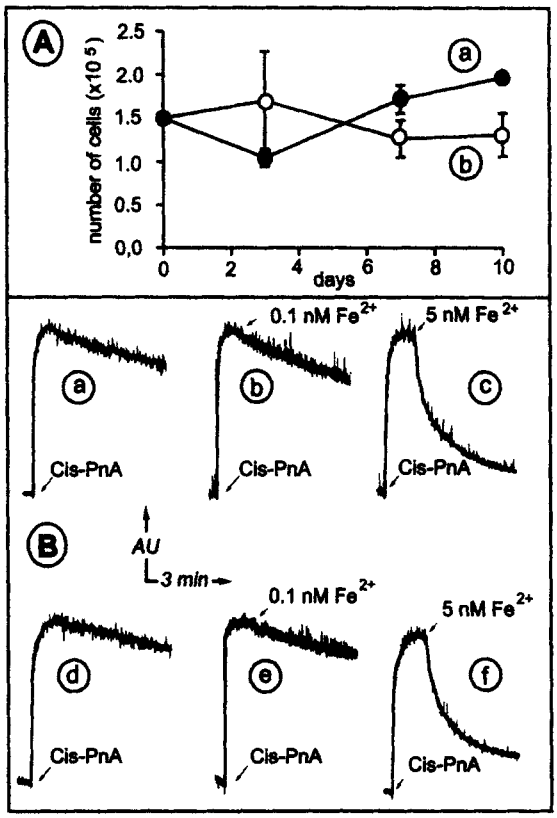


FIGURE 4 *Effect of hydrogen peroxide and reduced iron on patient 1 and control fibroblasts.* (A) Growth of patient 1 (a) and control (b) fibroblasts in the presence of 20 μM of H_2O_2. (B) Peroxidation of *cis*-parinaric acid in control (a, b, c) and patient 1 (d, e, f) fibroblasts membrane in the absence (a, d) or presence (b, c, e, f) of reduced iron. Experimental conditions as described under Materials and Methods.

CoQ_{10} Depletion and Lipoperoxidation

Considering the change in glutathione peroxidase activity, we tested for an increase of the level of lipid peroxidation in patient 1 fibroblasts. Upon addition of *cis*-parinaric acid, a rapid increase in fluorescence intensity, due to the incorporation of the probe in the membranes, was observed in both CoQ_{10}^- and control cells (Figure 4B a, d). A similar slow decay of fluorescence was observed, indicative of a similar peroxidation rate under this experimental condition. Finally, addition of low (0.1 nM; Figure 4B traces b,e) or high amounts of Fe^{2+} (5 nM, Figure 4B traces c,f), an active promoter of lipoperoxidation, similarly affected both patient 1 and control cells.

DISCUSSION

Primary widespread CoQ_{10} biosynthesis deficiency is a rare condition and only one case has been hitherto reported.[23] The skin fibroblasts of the patients reported were unable to synthesize decaprenylpyrophosphate, the precursor of the CoQ_{10} side chain, resulting in a profound CoQ_{10} depletion.[23] This provided us the unique opportunity to study the consequence of CoQ_{10} depletion on human cells.

Surprisingly, despite the decrease of the electron flow rate from several dehydrogenases to the bc_1 complex, a severe CoQ_{10} depletion was consistent with a reduced but still significant cell respiration (30 to 40% of controls). Along these lines, it is worth remembering that the extraction of lipids, including CoQ_{10}, by hexane-treatment of isolated mitochondria, did not result in a complete blockade of electron flow from dehydrogenases to the bc_1 complex.[35] By contrast the yeast coq_3 mutant strain, lacking 3,4-dihydroxy-5-hexaprenyl benzoate methyltransferase activity, with no residual CoQ_{10}, was unable to grow on non-fermentable carbon sources such as glycerol, suggesting that these cells were strongly respiratory deficiency.[36] Respiratory deficiency in patient 1 skin fibroblasts was however sufficient to hamper a normal growth rate as indicated by the delay in cell growth (see Figure 2).

The residual respiratory capacity of patient mitochondria accounts at least in part for the survival of the patients lacking CoQ_{10}. It is worth remembering that life-threatening organ dysfunction (renal failure) only occurred after several years of life in the patients. Similar clinical course has been previously observed in the patients presenting with other respiratory chain deficiency.[37,38] In these cases, survival and organ functioning presumably depend on the

residual level of respiratory chain activity. Along the same lines, it is worth remembering that an almost complete deficiency of key oxidative metabolism enzymes including the Krebs cycle enzymes, may be consistent with life.[39]

A major issue of this study is the putative role of CoQ_{10} in controlling free radical generation and associated cell death. This role has been inferred from several elegant studies, which gave strong support to this view. The *Clock-1* gene in *C. elegans*, has been shown to control respiration, behavior and life span of the worm and the function of *clock-1* gene homologue in yeast (coq7) seems to be closely related to CoQ_{10} biosynthesis.[40] On the other hand, ubiquinone-deficient mutant yeast showed increased sensitivity to polyunsaturated fatty acid peroxidation product.[41] However, despite the use of several approaches to detect even subtle changes in ROS metabolism in CoQ_{10} depleted fibroblasts, we only detected a slight increase in glutathione peroxidase activity. While this increase is likely to correspond to an increased lipoperoxidation, it is apparently sufficient to cope with this condition as no increased peroxidation products were detected in patient fibroblasts. Why CoQ_{10} depletion may have such a minor impact on ROS metabolism in cultured human fibroblasts is questionable. CoQ_{10} is a double face compound, possibly acting both as a potent anti-oxidant and as a pro-oxidant when semi-reduced. Depletion may therefore result in a balanced decrease of both pro- and antioxidant activities, with negligible consequences.

It is also interesting to note that, while perturbation of CoQ_{10} synthesis leads to short life span in animal models, patients with widespread severe CoQ_{10} depletion did not present any feature of premature aging. They may die early from organ dysfunction (kidney failure at age 8–10, rapid neurological deterioration) when not orally given CoQ_{10}. On the other hand, cataract, which is often associated with oxidative stress,[42] was observed in the patient 1, an observation that supports the view that CoQ_{10} depletion may alter antioxidant defenses in particular cell types. Most interestingly, cataract completely disappeared upon CoQ_{10} oral supplementation in the patient.[23]

Based on our results on cultured skin fibroblasts it is difficult to assign to CoQ_{10} a major role in antioxidant defenses of skin fibroblasts and the control of CoQ_{10} on the electron transfer should be less stringent than previously thought. On the other hand, based on the clinical pattern of our patient, it is conceivable that the role of CoQ_{10} should markedly differ among cell types.

Acknowledgements

We thank Drs. A. Tzagoloff and A. Barrientos for critical reading of the manuscript. This work was supported in part by the Association Française contre les Myopathies (AFM). VG is recipient of a grant from the AFM.

References

[1] L. Ernester and G. Dallner (1995) Biochemical, physiological and medical aspects of ubiquinone function. *Biochemica Biophysica Acta*, **1271**, 195–204.
[2] B. Chance, A. Azzi, I.Y. Lee, C.P. Lee and B. Mela (1966) Proton movements in mitochondrial membranes. *Nature*, **212**, 372–376.
[3] P. Mitchell (1976) Possible molecular mechanisms of the protonmotive function of cytochrome systems. *Journal of Theoretical Biology*, **62**, 327–367.
[4] A. Kröger and M. Klingenberg (1973) Further evidence for the pool function of ubiquinone as derived from the inhibition of the electron transport by antimycin. *European Journal of Biochemistry*, **39**, 313–323.
[5] M. Gutman and N. Silman (1977) Mutual inhibition between NADH oxidase and succinoxidase activities in respiring submitochondrial particles. *FEBS Letters*, **26**, 207–210.
[6] P. Rustin, F. Moreau and C. Lance (1980) Malate oxidation in plant mitochondria via malic enzyme and the cyanide-insensitive electron transport pathway. *Plant Physiology*, **66**, 457–463.
[7] L. Sun, E.E. Sun, F.L. Crane, D.J. Morre, D. Lindgren and H. Low (1992) Requirement for coenzyme Q in plasma membrane electron transport. *Proceedings of the National Academy of Sciences (USA)*, **89**, 11126–11130.
[8] A. Lass and R.S. Sohal (2000) Effet of coenzyme Q_{10} and α-tocopherol content of mitochondria on the production of superoxide anion radicals. *FASEB Journal*, **14**, 87–94.

[9] M.D. Jacobson (1996) Reactive oxygen species and programmed cell death. *Trends in Biochemical Sciences*, **21**, 83–86.

[10] T. Kagan, C. Davis, L. Lin, Z. Zakeri (1999) Coenzyme Q_{10} can in some circumstances block apoptosis, and this effect is mediated through mitochondria. *Annals of the New York Academy of Sciences*, **887**, 31, 47.

[11] M.P. Barroso, C. Gomez-Diaz, J.M. Villalba, M.I. Buron, G. Lopez-Lluch and P. Navas (1997) Antioxidant ascorbate is stabilized by NADH-coenzyme Q_{10} reductase in the plasma membrane. *Journal of Bioenergetics and Biomemembranes*, **29**, 259–267.

[12] M.L. Lu, M. Sato, B. Cao and J.P. Richie (1996) UV irradiation-induced apoptosis leads to activation of a 36-kDa myelin basic protein kinase in HL-60 cells. *Proceedings of the National Academy of Sciences (USA)*, **93**, 8977–8982.

[13] W.H. Chan, J.S. Yu and S.D. Yang (1999) PAK2 is cleaved and activated during hyperosmotic shock-induced apoptosis via a caspase-dependent mechanism: evidence for the involvement of oxidative stress. *Journal of Cell Physiology*, **178**, 397–408.

[14] Ahlemeyer and B. Krieglstein (2000) Inhibition of glutathione depletion by retinoic acid and tocopherol protects cultured neurons from staurosporine-induced oxidative stress and apoptosis. *Journal Neurochemistry International*, **36**, 1–5.

[15] A. Lass, S. Agarwal and R.S. Sohal (1997) Mitochondrial ubiquinone homologues, superoxide radical generation, and longevity in different mammalian species. *Journal of Biological Chemistry*, **272**, 19199–19204.

[16] A. Wong, P. Boutis and S. Hekimi (1995) Mutations in the clk-1 gene of Caenorhabditis elegans affect developmental and behavioral timing. *Nature Genetics*, **139**, 1247–1259.

[17] B. Lakowski and S. Hekimi (1996) Determination of life-span in Caenorhabditis elegans by four clock genes. *Science*, **272**, 1010–1013.

[18] R.G. Cutler (1985) Peroxide-producing potential of tissues: inverse correlation with longevity of mammalian species. *Proceedings of the National Academy of Sciences (USA)*, **14**, 4798–4802.

[19] H. Nohl, L. Gille, K. Schönheit and Y. Liu (1996) Conditions allowing redox-cycling ubisemiquinone in mitochondria to establish a direct redox couple with molecular oxygen. *Free Radical Biological and Medicine*, **20**, 207–213.

[20] S. Ogasahara, A.G. Engel, D. Frens and D. Mack (1989) Muscle coenzyme Q_{10} deficiency in familial mitochondrial encephalomyopathy. *Proceedings of the National Academy of Sciences (USA)*, **86**, 2379–2382.

[21] C. Sobreira, M. Hirano, S. Shanske, R.K. Keller, R.G. Haller, E. Davidson, F.M. Santorelli, A.F. Miranda, E. Bonilla, D.S. Mojon, A.A. Barreira, M.P. King and S. DiMauro (1997) Mitochondrial encephalomyopathy with coenzyme Q_{10} deficiency. *Neurology*, **48**, 1238–1243.

[22] E. Boitier, F. Degoul, F. Desguerre, C. Charpentier, D. François, G. Ponsot, M. Diry, P. Rustin and C. Marsac (1998) A case of mitochondrial encephalomyopathy associated with a muscle coenzyme Q_{10} deficiency. *Neurological Science*, **156**, 41–46.

[23] A. Rötig, A.L. Appelkvist, V. Geromel, D. Chrétien, N. Kadhom, P. Edery, M. Lebideau, G. Dallner, A. Munnich, L. Ernester and P. Rustin (2000) Quinone-responsive multiple respiratory chain disfunction due to widespread coenzyme Q_{10} deficiency. *Lancet*, **356**, 391–395.

[24] T. Bourgeron, D. Chrétien, P. Amati, A. Rötig, A. Munnich and P. Rustin (1993) Expression of respiratory chain deficiencies in human cultured cells. *Neuromuscular Disorders*, **3**, 605–608.

[25] C. Wolfrom, N. Kadhom, G. Polini, J. Poggi, N. Moatti and M. Gautier (1989) Glutamine dependency of human skin fibroblasts: modulation by hexoses. *Experimental Cell Research*, **183**, 303–318.

[26] P. Rustin, D. Chrétien, B. Gerard, T. Bourgeron, A. Rötig, J.M. Saudubray, A. Munnich (1994) Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta*, **228**, 35–51.

[27] S. Marklund and G. Marklund (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, **47**, 469–474.

[28] D.E. Paglia and W.N. Valentine (1967) Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Clinical Laboratory Medicine*, **70**, 158–159.

[29] I. Calberg and B. Mannervik (1985) Glutathione reductase. *Methods in Enzymology*, **113**, 484–490.

[30] W.H. Habig, M.J. Pabst and W.B. Jacoby (1974) Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, **249**, 7130–7139.

[31] R.H. Jaskot, E.G. Charlet, E.C. Grose, M.A. Grady and J.H. Roycroft (1983) An automated analysis of glutathione peroxidase, S-transferase, and reductase activity in animal tissue. *Journal of Analytical Toxicology*, **7**, 86–88.

[32] P. Rustin and M.F. Alin (1984) Purification and spectral properties of Kalanchoe blossfeldiana leaf mitochondria. *Physiologie Végétale*, **22**, 93–101.

[33] H. Nishida, M. Shibata, K. Koseki, Y. Nakao, Y. Kawashim, Yoshida and K. Tagawa (1987) Peroxidative injury of the mitochondrial respiratory chain during reperfusion of hypothermic rat liver. *Biochemical and Biophysical Acta*, **890**, 82–88.

[34] C. Garcia-Riuz, A. Collel, A. Morales, N. Kaplowitz and J.C. Fernandez-Checa (1995) Role of oxidative stress generated from the mitochondrial electron transport chain and mitochondrial glutathione status in loss of mitochondrial function and activation of transcription factor nuclear factor-kappa B: studies with isolated mitochondria and rat hepatocytes. *Molecular Pharmacology*, **48**, 825–835.

[35] P. Pasquali, L. Landi, L. Cabrini and G. Lenaz (1981) Effect of ubiquinone extraction on ubiquinol-1 oxidase activity in beef heart mitochondria. *Journal of Bioenergetic and Biomembranes*, **13**, 142–148.

[36] B.N. Marbois, Y.R. Xia, A.L. Lusis and C. Clarke (1994) Ubiquinone biosynthesis in eukaryotic cells: tissue distribution of mRNA encoding 3,4-dihydroxy-5-polyprenylbenzoate methyltransferase in the rat and mapping of the CoQ_3 gene to mouse chromosome 4. *Archives of Biochemistry and Biophysics*, **313**, 83–88.

[37] V. Cormier-Daire, D. Chrétien, P. Rustin, A. Rötig, C. Dubuisson, E. Jacquemin, M. Bernard and A. Munnich (1997) Neonatal and delayed-onset liver involvement in

disorders of oxidative phosphorylation. *Journal of Pediatrics*, **130**, 817–822.

[38] A. Munnich, P. Rustin, A. Rötig, D. Chrétien, J.P. Bonnefont, C. Nuttin, V. Cormier, A. Vassault, P. Parvy, J. Bardet, C. Charpentier, D. Rabier and J.M. Saudubray (1992) Clinical aspects of mitochondrial disorders. *Journal of Inherited Metabolic Disease*, **15**, 448–455.

[39] T. Bourgeron, D. Chrétien, J. Poggi-Bach, S. Doonan, D. Rabier, A. Munnich, A. Rötig, P. Landrieu and P. Rustin, (1994) Mutation of the fumarase gene in two siblings with progressive encephalopathy and fumarase deficiency. *Journal of Clinical Investigation*, **93**, 2514–2518.

[40] T. Jonassen, M. Proft, F. Randez-Gil, J. Shultz, N.B. Marbois, K.D. Entian and F.C. Clarke (1998) Yeast Clk-1 homologue (Coq7/Cat5) is a mitochondrial protein in coenzyme Q synthesis. *Journal of Biological Chemistry*, **273**, 3351–3357.

[41] B.N. Marbois and C.F. Clarke (1996) The CoQ_7 gene encodes a protein in saccharomyces cerevisiae necessary for ubiquinone biosynthesis. *Journal of Biological Chemistry*, **271**, 2995–3004.

[42] C.M. Hutnik and B.D. Nichols, (1999) Cataracts in systemic diseases and syndromes. *Current Opinion in Ophthalmology*, **10**, 22–28.

[43] D. Chrétien, J. Gallego, A. Barrientos, J. Casademont, F. Cardellach, A. Munnich, A. Rötig and P. Rustin (1998) Biochemical parameters for the diagnosis of mitochondrial respiratory chain deficiency in humans, and their lack of age-related changes. *Biochemical Journal*, **329**, 249–254.