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Biochimica et Biophysica Acta 1706 (2005) 174–183

BIOCHIMICA ET BIOPHYSICA ACTA BBA

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Specificity of coenzyme Q_{10} for a balanced function of respiratory chain and endogenous ubiquinone biosynthesis in human cells

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Received 9 June 2004; received in revised form 8 October 2004; accepted 25 October 2004
Available online 4 November 2004

Abstract

Coenzyme Q (Q) is an obligatory component of both respiratory chain and uncoupling proteins. Also, Q acts as an antioxidant in cellular membranes. Several neurodegenerative diseases are associated with modifications of Q_{10} levels. For these reasons, therapies based on Q supplementation in the diet are currently studied in order to mitigate the symptoms of these diseases. However, the incorporation of exogenous Q also affects aging process in nematodes probably affecting reactive oxygen species (ROS) production. The aim of the present work is to clarify if supplementation with both Q_{10} and Q_6 isoforms affects mitochondrial Q_{10} content, respiratory chain activity and ROS levels in human cells. Cells incorporated exogenously added Q_{10} and Q_6 isoforms into mitochondria that produced changes in mitochondrial activity depending on the side chain length. Supplementation with Q_{10}, but not with Q_6, increased mitochondrial Q-dependent activities. However, Q_6 affected the mitochondrial membrane potential, ROS production, and increased the protein levels of both catalase and Mn-superoxide dismutase (Mn-SOD). Also, Q_6 induced a transient decrease in endogenous mitochondrial Q_{10} levels by increasing its catabolism. These results show that human cells supplemented with Q_6 undergo a mitochondrial impairment, which is not observed with Q_{10} supplementation.

Keywords: Coenzyme Q; Mitochondria; ROS; Respiratory chain; CoQ uptake

1. Introduction

Ubiquinone (Coenzyme Q, Q) is an essential electron carrier in the mitochondrial respiratory chain [1]. Also, Q is an obligatory factor for uncoupling proteins activation [2], in the opening of the permeability transition pore (PTP) [3], and also acts as antioxidant in the different membranes of the cell [4–7].

Under nonpathological processes, the capability of tissues to synthesise Q apparently decreases during aging [8], but also Q biosynthesis can be affected by dietary supplementation [9]. This uptake further affects diverse functions of the physiology of organisms [10,11]. On the other hand, Q deficiency has been related to severe dysfunctions in muscle and nervous system [12–15]. Modifications of Q levels together with changes in reactive oxygen species (ROS) production appear in mayor neuronal disorders such as Alzheimer's and Parkinson's diseases [16,17]. Supplementation with Q_{10} or analogues has shown benefits in neurodegenerative processes such as Parkinson's [18], Huntington's [19] and Alzheimer's diseases [20], and also in mitochondrial disorders [13,21].

Several studies have demonstrated the incorporation into tissues of exogenous Q administered to animals in the diet [9,22–24]. However, the incorporation of exogenous Q alters different aspects of *C. elegans* phenotype depending on the length of the side chain of Q [24–26].

Due to the variety of effects of Q in organisms [11], and that the mechanisms involved in the uptake by cells and the incorporation of Q into mitochondria are to date

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doi:10.1016/j.bbabio.2004.10.009

not clear, the purpose of the present study was to analyse the incorporation of exogenous Q of different side chain length in HL-60 cells mitochondria. Both Q_6 and Q_{10} (same length than the endogenous Q in human cells) significantly incorporated into mitochondria and they showed a different effect on Q-dependent mitochondrial activities, mitochondrial membrane potential ($\Delta\psi_m$) and ROS production. Also, Q_6 transiently affected endogenous Q_{10} content. We show here the that supplementation of human cells with Q_{10} maintains a balanced respiration, whereas a short side-chain ubiquinone such as Q_6 impairs the respiratory chain activity.

2. Materials and methods

2.1. Cell culture

HL-60 cells (ATCC, USA), a human promyelocytic cell line, were cultured in RPMI 1640 medium (BioWhittaker, Belgium) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Linus, Spain), and antibiotic/antimycotic solution (Sigma, Spain). Both Q_{10} and Q_6 isoforms (Sigma) were directly dissolved in FCS at a concentration of 20 μM, and a final concentration of 2 μM was used in the experiments. Experiments started by seeding cells at 5×10^5 cells/ml. Cell viability was determined by the trypan blue exclusion method. The radiolabeled precursor of Q, *para*-hydroxybenzoate, [^{14}C]-pHB, was chemically synthesised from L-[U-^{14}C]-tyrosine (Amersham Pharmacia, USA) by alkaline hydrolysis for 5 min at 270 °C as described elsewhere [27]. For anabolism experiments, [^{14}C]-pHB was added at the same time than incorporation of Q forms whereas in catabolism experiments, untreated cells were incubated for 12 h with [^{14}C]-pHB, washed twice with serum-free medium and resuspended in control, Q_{10}- or Q_6-enriched media until harvesting.

2.2. Cell fractionation

Mitochondria were obtained as indicated by Magalhães et al. [28] with some modifications. Cells were pelleted by centrifugation at 500×*g* for 5 min at 4 °C. After two washes with ice-cold PBS, all the subsequent steps were performed at 4 °C. Cells were disrupted with 9 vol. of lysis buffer (HEPES 2 mM, pH 7.4, 0.15 mM $MgCl_2$, 10 mM KCl, 0,5 mM EGTA, 20 μM cytochalasin B, and protease inhibitor cocktail 1:100) (All components from Sigma). Immediately after lysis with a Dounce homogeniser, a concentrated solution of sucrose in lysis buffer was added to reach 0.32 M final sucrose concentration. Undisrupted cells and debris were removed by centrifugation at 1000×*g* for 5 min. Supernatant was considered as the post-nuclear cell extract (PN). Crude mitochondria were obtained by centrifugation of PN at 8000×*g* for 10 min, and a further centrifugation of supernatant at 10,000×*g* for another 10 min. Both pellets were pooled and considered as crude mitochondrial fraction (P_{10}) whereas supernatant (S_{10}) was further centrifuged at 100,000×*g* to obtain the remaining microsomal fraction (P_{100}) and cytosol (S_{100}). To obtain a mitochondria-enriched fraction, crude mitochondrial fraction (P_{10}) was layered onto a discontinuous sucrose gradient performed with 0.8, 1.0, 1.2, 1.4 and 1.6 M sucrose concentrations in 2 mM HEPES pH 7.4 buffer. After centrifugation at 95,000×*g* for 90 min, four fractions were obtained. The fraction located between 1.2 and 1.4 M sucrose layers (fraction 3) contained pure mitochondria as it was shown by its analysis by Western blotting using the following specific antibodies. The low amount of mitoplasts found in the fraction located between the 1.4 and 1.6 M sucrose layers (fraction 4) indicated the preservation of mitochondrial integrity during the purification procedure [29]. Mouse monoclonal anti-cytochrome *c* oxidase subunit I (RDI, USA) (1:1000) and rabbit polyclonal anti-clk-1 (kindly supplied by Dr. Hekimi) (1:1000) antibodies were used a mitochondria markers. Both goat polyclonal anti-ribophorin I (Santa Cruz, USA) (1:1000) and rabbit polyclonal anti-calnexin (StressGen, USA) (1:1000) antibodies were used as endoplasmic reticulum markers. Mouse monoclonal anti-Na^+/K^+-ATPase (ABR, USA) antibody (1:500) was used as plasma membrane marker.

Membrane fraction proteins and whole cell extracts were electrophoresed in a 10–15% acrylamide PAGE-SDS and transferred to Immobilon membranes (Amersham Pharmacia). Rabbit polyclonal anti-catalase (Calbiochem, USA) (1:1000) and goat polyclonal anti-Mn-superoxide dismutase (Mn-SOD) (1:500) antibodies (Calbiochem) were used for the determination of these enzymes by Western blotting using whole cell extracts whereas mouse monoclonal anti-tubulin (1:200) (Boehringer Mannheim, Germany) was used as loading control. In all cases, primary antibodies were diluted in 50 mM Tris–HCl pH 7.5/0.85% w/v NaCl (TBS) plus 0.05% v/v Tween 20 (TTBS) supplemented with 5% nonfat milk (TTBSL). Membranes were incubated O/N at 4 °C. After washing with TTBS, membranes were probed with secondary antibodies (1:1000 to 1:10,000) labeled with HRP and diluted in TTBSL for 2 h at room temperature. After washing with TTBS and TBS, detection of specific labeling was carried out by using a chemioluminiscence method (ECL) (Amersham Pharmacia).

Protein in the different membranes was determined by Bradford's method [30].

2.3. Coenzyme Q determination

Q levels in both whole cells and pure mitochondria were determined by HPLC after extraction with hexane as indicated by Gómez-Díaz et al. [31]. Briefly, at least either 1×10^6 cells or 500-μl mitochondria fraction was resuspended in 500 μl of PBS, and after its incubation for 10 min on ice, 500 μl of 2% SDS was added. After mixing by

vortex for 1 min, 2 ml of ethanol/isopropanol (95:5) HPLC grade was added and mixed again for 1 min. Finally, 4-ml hexane was added, mixed and centrifuged at 1000×*g* for 5 min at 4 °C. Extraction of Q with hexane was repeated twice and upper organic phases collected, pooled and dried by vacuum at 37 °C using a rotavapor (Büchi, Switzerland). Dried residue was reconstituted with ethanol HPLC grade. Reconstituted lipids were dried again by using a speed-vac for 2–3 h at 40 °C. Dried residue was kept at −20 °C until final reconstitution with 50–100-μl ethanol. Total Q extracted was analysed by HPLC (Beckman-Coulter, USA) equipped with both an UV/Vis (System Gold ® 168, Beckman-Coulter) and a ECD (Coulochem III; ESA, USA) detectors. Separation was carried out in a C_{18} Kromasil 100 column (Scharlab, Spain) with a mobile phase of *n*-propanol/methanol (35:65) containing 13.9 mM lithium perclorate at a flow-rate of 1 ml/min. Decylubiquinone (DQ) (Sigma) was used as internal standard.

To determine newly synthesised Q, cells were incubated in the presence of 4.5 nM [^{14}C]-pHB directly dissolved in culture media. [^{14}C]-Q_{10} was analysed with a HPLC Beckman-Coulter 126 System Gold® connected to a radio-flow detector LB 509 with a solid cell YG 150 μl U4D (Berthold Technologies, Germany).

The reduction of Q was carried out incubating pure mitochondria (50 μg) in 40 mM phosphate buffer pH 7.5 in the presence of antimycin A (20 μg/ml), KCN (0.5 mM), and 0.5 mM β-mercaptoethanol with either 0.2 mM NADH or 5 mM succinate for 30 min at 37 °C. β-Mercaptoethanol itself does not reduce Q but protect the reduced form against reoxidation during extraction [32]. After incubation, Q was extracted by adding 165-μl HPLC-grade *n*-propanol (Panreac, Spain) and after vortexing for 2×30 s at maximum speed, sample was centrifuged for 5 min at 4 °C in microfugue at maximum speed and 100 μl of supernatant was directly injected to the HPLC system. The QH_2/Q ratio was determined by using a Coulochem III ECD.

2.4. Mitochondrial activities and ROS determination

Mitochondria-enriched fractions were used to determine complexes I+III and II+III activities. In both cases, mitochondria were incubated in 40 mM sodium phosphate buffer pH 7.5 plus 0.25 mM KCN and containing either 0.2 mM NADH or 5 mM succinate, and reduction of beef-heart cytochrome *c* (0.5 mM) was measured at 550 nm for 5 min. To discriminate the rotenone-sensitive NADH–cytochrome *c* reductase activity (Complex I+III) from the rotenone-insensitive NADH–cytochrome *c* reductase activity due to the NADH–cytochrome b_5 reductase located in the outer mitochondrial membrane, 5 μM rotenone was added for the last 2 min of incubation as indicated by Birch-Machin and Turnbull [33]. An extinction coefficient (ε) of 27.8 mM^{-1} cm^{-1} was used to calculate the specific activity that was expressed as nmol/min/mg protein.

Mitochondrial membrane potential ($\Delta\psi_m$) was determined by using the specific dyes 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidalolylcarbocyanine iodide (JC1), rhodamine 123 (rhod123), and dihexyloxacarbocyanine ($DiOC_6(3)$) (Molecular Probes, USA). Cells were cultured as indicated above and stained with either 5 μg/ml JC1, 10 μg/ml rhod123 or 20 nM $DiOC_6(3)$ for 30 min at the end of the treatment, washed twice with PBS and fluorescence determined by flow cytometry by using a cytofluorimeter EPICS XL (Coulter, USA). In the case of JC1, $\Delta\psi_m$ was determined by analysing the ratiometric analysis of the fluorescence emitted by the aggregated form (FL2, 590 nm) and the free form of the dye (FL1, 527 nm). In the case of rhodamine 123, $\Delta\psi_m$ was determined by the analysis of the green fluorescence emitted by the dye (FL1, 525 nm).

ROS were determined cytofluorometrically by using hydroethidium (HE) (Sigma) for superoxide anion, and carboxy-2′,7′-dichlorodihydrofluorescein diacetate (cDCFDA) (Molecular Probes) and dihydrorhodamine 123 (dH-rhod123) (Molecular Probes) for hydrogen peroxide and other peroxides. Cells were incubated with 4 μM HE, 10 μg/ml cDCFDA or 10 μg/ml (dH-rhod123) during the last 30 min of treatment, and fluorescence emitted by their respective oxidised forms, ethidium (FL3, 610 nm), dichlorofluorescein (FL1, 525 nm) or rhodamine 123 (FL1, 525 nm), was analysed using an EPICS XL (Coulter) flow cytometer.

2.5. Statistical analysis

Statistical comparison among groups was carried out by the Student's *t* test. A P value of <0.05 was considered as statistically significant. Values are expressed as the mean±S.D.

3. Results

3.1. Purity of mitochondrial fractions

Membranes isolated in the layer between 1.2 and 1.4 M sucrose fractions (fraction 3) showed a higher concentration of the mitochondrial markers COX and Clk-1/Coq7 determined by immunoblotting (Fig. 1). Densitometric analysis demonstrated that about 40% of mitochondria were included in this fraction of the gradient. The plasma membrane marker Na^+/K^+ ATPase was almost absent in fraction 3, and the content of endoplasmic reticulum markers, which mainly sedimented at higher speed than mitochondria (P_{100}), was clearly low. We used in this work this fraction 3 as mitochondria-enriched fraction. The 1.4/1.6 M sucrose layer (fraction 4) was almost free or mitochondrial markers indicating that the integrity of the outer membrane was preserver during isolation procedure.

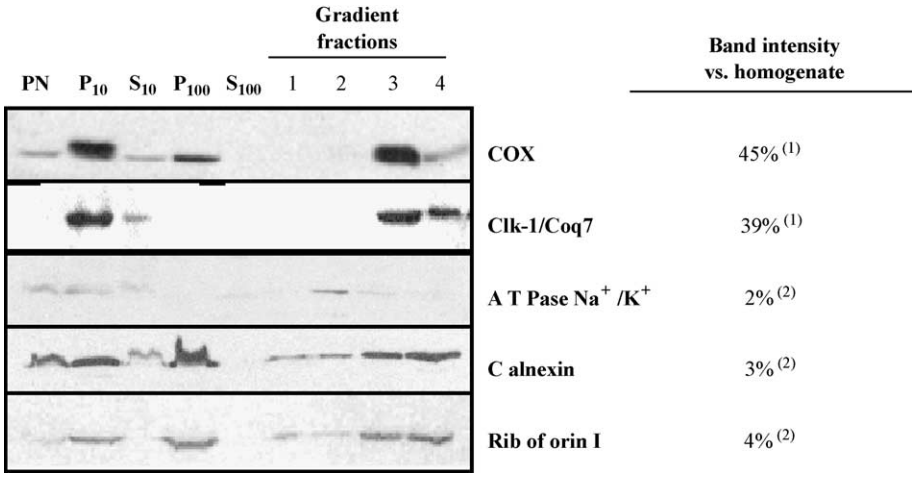

Fig. 1. Determination of mitochondria purity after sucrose gradient purification. Fractions obtained from centrifugation were electrophoresed in a 10% SDS-PAGE gel. PN: post-nuclear fraction, P_{10}: crude mitochondrial fraction, S_{10}: supernatant after 10,000×*g* centrifugation; P_{100}: microsomal fraction obtained after 100,000×*g* centrifugation; S_{100}: cytosol; Gradient fractions: fractions obtained after sucrose-gradient centrifugation of P_{10}. After electrophoresis, proteins were transferred to Immobilon membranes and probed with antibodies anti-cytochrome *c* oxidase (COX) and Clk-1/Coq7 (mitochondria), ribophorin I and calnexin (endoplasmic reticulum), Na^+/K^+ ATPase (plasma membrane). Blots were analysed and quantification of the whole amount of specific protein (blot intensity×total volume of sample) recovered in gradient fraction 3 compared to the amount found in the whole homogenate. Data represent a Western blot from one purification procedure. (1)Percentage of protein recovered in fraction 3 vs. whole homogenate. (2)Percentage of contamination in fraction 3.

3.2. Incorporation of Q isoforms

The incorporation of Q isoforms in mitochondria of HL-60 cells was analysed by HPLC in mitochondria-enriched fractions after the incubation of cells with 2 μM of either Q_{10} or Q_6. Q isoforms accumulated through time in mitochondria reaching 400 pmol/mg protein at 12-h incubation independently of the side-chain length (Fig. 2). Q_{10} intake was calculated by subtracting Q_{10} content of mitochondria in control cells.

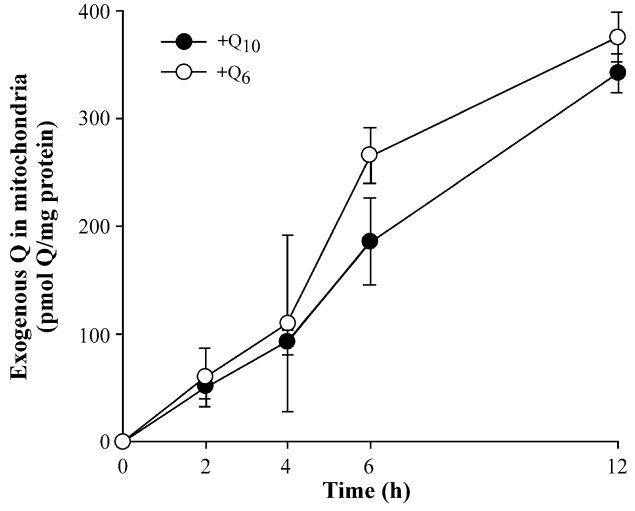

Fig. 2. Exogenous Q is incorporated into mitochondria. Quantification of exogenous Q_{10} and Q_6 incorporated into pure mitochondria isolated from fraction 3. The net amount of Q_{10} incorporation into mitochondria was estimated by subtracting the amount of Q_{10} obtained in mitochondria from controls to the total amount of Q_{10} found in Q_{10}-supplemented cultures. Data represent the mean±S.D. from three different experiments indicated as pmol exogenous Q/mg protein.

To confirm that exogenous Q was functionally incorporated into the mitochondrial inner membrane, we studied the reduction of Q by either adding NADH (Fig. 3A) or succinate (Fig. 3B) in mitochondria-enriched fractions. Both NADH and succinate reduced both Q_{10} and Q_6 in supplemented mitochondria. This reduction was partially prevented by either rotenone or malonate that are inhibitors of complex I and II, respectively.

3.3. Effect of Q supplementation on mitochondria

The Q-dependent activities of the respiratory chain were analysed in mitochondria-enriched fractions isolated from HL-60 cells after being cultured in the presence of either 2 μM Q_{10} or Q_6 for 6 h. Q_{10} supplementation induced a significant increase of both complex I+III and complex II+III (23.3% and 18.1%, respectively) (Fig. 4). However, Q_6 supplementation only induced a nonsignificant increase in complex I+III activity, and a slight and nonsignificant decrease of complex II+III activity compared to non-supplemented mitochondria (Fig. 4B). Mitochondrial membrane potential ($\Delta\psi_m$) was significantly lower in those cells supplemented with Q_6 but it was not affected in cells supplemented with Q_{10}. The $\Delta\psi_m$ was determined by the ratiometric analysis of the fluorescence emitted by the aggregated and free forms of JC1 (Fig. 5A) or by rhodamine123 (Fig. 5B), both compounds known to accumulate into active mitochondria. Similar results were found with $DiOC_6(3)$ whose fluorescence signal was 36.5±4.5% lower than in controls and was not affected in Q_{10}-supplemented cultures.

The production of ROS in the mitochondria of HL-60 cells supplemented with either Q_{10} or Q_6 was also determined by flow cytometry. Hydrogen peroxide levels

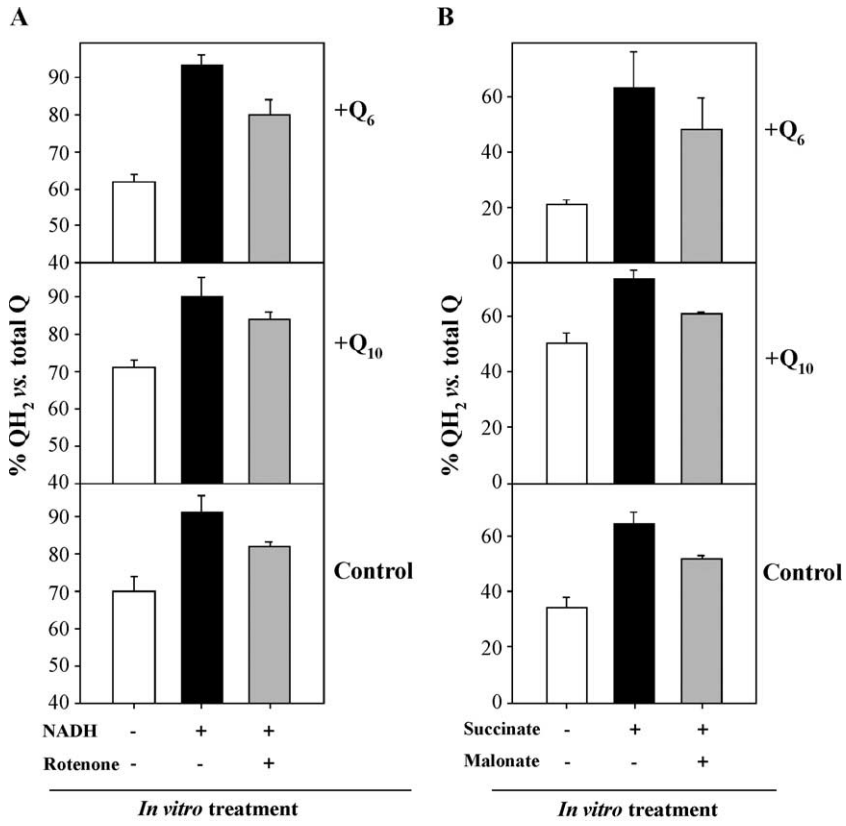

Fig. 3. In vitro reduction of exogenous Q isoforms by mitochondrial complex I and II. Percentage of the reduced Q form (QH_2) vs. total Q content in pure mitochondria from control, Q_{10}- and Q_6-supplemented cultured cells after the incubation with 0.2 mM NADH in the presence or absence of the complex I-specific inhibitor rotenone (5 μM) (A) or with 5 mM succinate in the presence or absence of the complex II-specific inhibitor malonate (10 mM) (B). Data represent the mean±S.D. of the percentage of QH_2 vs. respective total Q from three experiments performed in duplicated.

did not significantly changed in Q_{10}-supplemented cells but Q_6 did induce a significant time-dependent increase (Fig. 6A). Superoxide anion levels were also increased in Q_6-supplemented HL-60 cells after 6 h of treatment but did not change in cells supplemented with Q_{10} (Fig. 6B).

The expression of both catalase and Mn-SOD was analysed by Western blotting in Q supplemented HL-60 cells (Fig. 7). Clearly, Q_6 incorporation increased both catalase and Mn-SOD that were unchanged in HL-60 cells supplemented with Q_{10}.

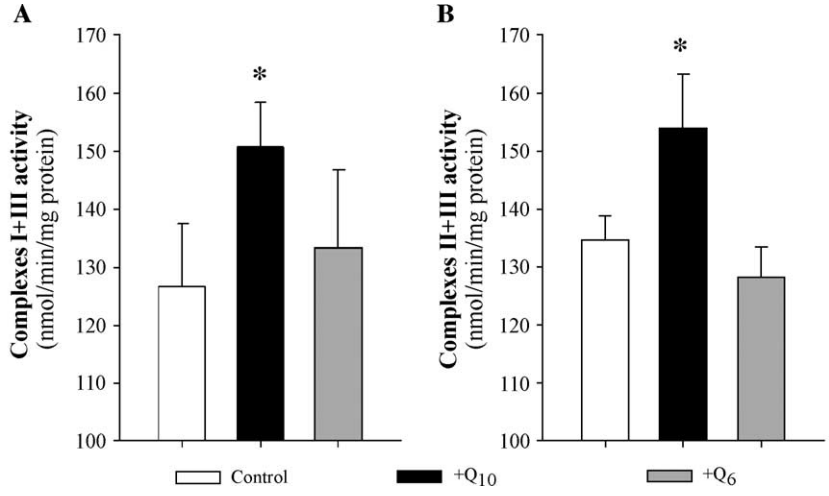

Fig. 4. Respiratory chain activities in pure mitochondria isolated from HL-60 cells supplemented with Q isoforms. (A) Rotenone-sensitive NADH–cytochrome *c* reductase activity (Complex I+III). Data are represented as the mean±S.D. from three different experiments performed in triplicate. Activity is indicated as nmol/min/mg protein. (B) Succinate–cytochrome *c* reductase activity (Complex II+III). Data are represented as the mean±S.D. from three different experiments performed in triplicate. Activity is indicated as nmol/min/mg protein. *Significant differences vs. control, $P \leq 0.05$.

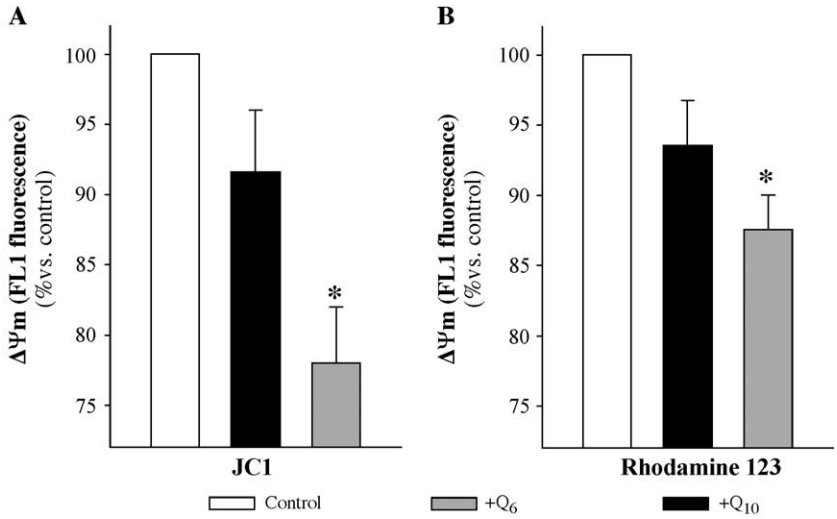

Fig. 5. Mitochondrial transmembrane potential in HL-60 cells after exogenous Q incorporation. Cells were incubated under normal conditions (control) or with 2 μM Q_{10}- or Q_6-supplemented FCS for 6 h and $\Delta\psi_m$ was determined by flow cytometry using the potential-dependent specific mitochondrial dyes JC1 (A) or rhodamine 123 (B). For determination of $\Delta\psi_m$ with JC1, the ratiometric analysis of orange fluorescence (FL2, 575 nm) emitted by aggregated JC1 and the green fluorescence (FL1, 525 nm) emitted by free JC1 was performed. In the case of rhodamine 123, $\Delta\psi_m$ was determined by analysing the mean fluorescence intensity at 525 nm (FL2/FL1 fluorescence). Data represent the mean±S.D. of the percentage of signal vs. signal found in control cells. *Significant differences vs. control, $P \leq 0.05$.

3.4. Q supplementation affects endogenous Q_{10}

To study the influence of exogenous Q isoforms in endogenous Q_{10} metabolism, we analysed the time course of Q_{10} levels in mitochondria after the incubation of HL-60 cells with 2 μM of either Q_{10} or Q_6 (Fig. 8A). Q_{10}-supplemented cells showed a significant increase in Q_{10} compared to controls. However, the incubation of cells with Q_6 induced a transient decrease (4–6 h) of endogenous Q_{10}.

To determine the cause of the decrease of endogenous Q_{10} in Q_6-supplemented HL-60 cells, we studied the catabolism rate preincubating cells with radiolabeled [^{14}C]-pHB for 12 h. The concentration of [^{14}C]-Q_{10} synthesised was then measured after 6 h of supplementation with either Q_6 or Q_{10}. Incubation with Q_{10} did not modify the levels of [^{14}C]-Q_{10} whereas Q_6 addition certainly induced a significant decrease of [^{14}C]-Q_{10} (Fig. 8B). On the other hand, biosynthesis of [^{14}C]-Q_{10}, studied by incubation of cells with [^{14}C]-pHB at the same time than exogenous Q_{10} or Q_6, was not affected during the first 12 h of incubation (Fig. 8C).

4. Discussion

Mitochondria are the main source of ROS in cells inducing the oxidation of macromolecules such as DNA and proteins that accumulate in their oxidised forms during aging [34]. This is in agreement with the free radical theory of aging, which indicates an inverse relationship between ROS production and life span length in different species [35,36]. Different complexes of the respiratory chain are responsible for ROS production in mitochondria [37–41], but the unstable semiquinone isoform of Q also contributes to this radical production [42,43].

The incorporation of lipids into membranes depends on the availability of space and it is well known that the inner mitochondrial membrane Q-levels are currently at non-saturating concentrations for electron transport [44,45]. In this study, we show that HL-60 cells can take up different Q isoforms from the media, which are significantly incorporated into mitochondrial membranes independently of their side chain length. Both Q_6 and Q_{10} incorporated in HL-60 cells mitochondria were reduced at the same extent by either complex I or complex II, indicating that these exogenous Qs are functionally active in the inner membrane. However, only the uptake of Q_{10} induced a proportional increase of Q-dependent activities, complex I+III and complex II+III. This can be explained because Q is at non-saturating conditions in respiratory chain [44,45], and also because the amount of Q in the inner membrane can modify the maximum rates of mitochondrial electron transport probably by diffusion-based collisions of Q with its redox partners or by a kinetic saturating effect [44–46]. Q_6 was incorporated at the same levels as Q_{10} in mitochondria and, although probably showing a similar or higher diffusion degree than Q_{10} [47], did not modify the electron flow among complexes. As Q_6 was reduced by both complex I and complex II but the transfer of electrons to complex III was not increased and the $\Delta\psi_m$ in mitochondria of Q_6-supplemented cells decreased, we hypothesise that Q_6 shows a lower capability of interaction with human complex III than Q_{10}. In this sense, complex I can be inhibited by short side chains Q_2 and Q_3, indicating the importance of the length of the isoprenoid side chain in electron transport and interaction with respiratory complexes [48].

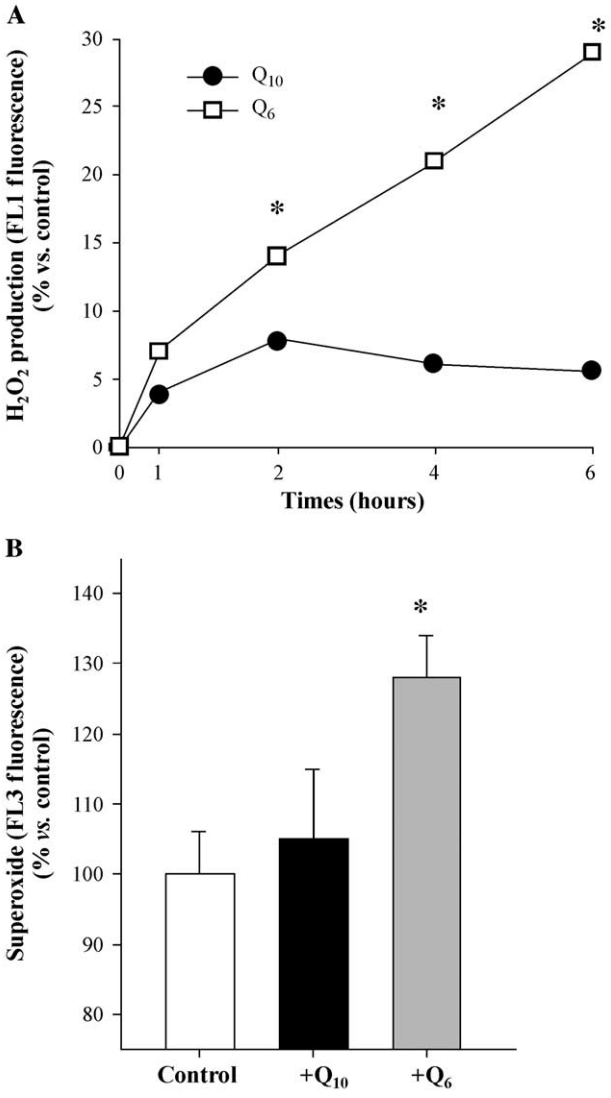

Fig. 6. ROS production in HL-60 cells cultured with exogenous Q. (A) Time course of H_2O_2 levels in HL-60 cells incubated with 2 μM Q_{10} or Q_6 measured by using c-DHCF-DA. Data represent the mean of the percentage of fluorescence vs. control levels of H_2O_2 from three different experiments performed in duplicate. (B) Superoxide anion levels after 6 h of incubation with 2 μM Q_{10} or Q_6. Data represent the mean±S.D. of the percentage of Eth fluorescence vs. control levels from three different experiments performed in duplicate. *Significant differences vs. control, $P \leq 0.05$.

In vitro experiments have shown that sub-mitochondrial particles supplemented with Q_9 produce higher amounts of superoxide anion than others supplemented with Q_{10} [49]. Our results suggest that part of the Q_6 incorporated into mitochondria is able to be reduced by mitochondrial complexes I and II but does not properly transfer electrons to complex III. The accumulation of reduced Q_6 could increase the levels of the semiquinone form, an important source of ROS [42,43]. Furthermore, Q_6 could also interact with the N2 Fe–S cluster of complex I and be reduced by one electron increasing the levels of the semiquinone form of Q_6 [48]. However, we cannot exclude other sources of ROS affected by Q_6 incorporation in cell membranes. On the other hand, accumulation of Q_{10} increased the electron flow but did not change the rate of ROS production in mitochondria. Thus, the specificity of the side chain of Q isoforms is an essential factor to prevent Q-dependent ROS production in mitochondria. In fact, it has been shown that supplementing the diet of rats with Q_{10} does not increase ROS production in mitochondria [9,50], but increases plasma membrane protection against oxidative stress [51], and extends life span [52].

Our results may explain the shortening of life span induced by exogenous Q_8 in *C. elegans*, which synthesises only Q_9 [10], although when nematodes were supplemented with Q_{10} a lengthening of life span was found [25]. Thus, a higher production of ROS in mitochondria mainly depends on the specificity of Q isoform inserted in the inner mitochondrial membrane but not on the amount of Q.

Q biosynthesis and its catabolism are very complex processes that are not well understood [11,53,54]. Q_{10} and Q_9 are the only Q isoforms that are present together in animals such as rodents, and their biosynthesis pathways seem to be affected by the dietary intake of Q and other antioxidants [50–52]. The final content of Q_{10} in human cells is a consequence of both synthesis rate and catabolism. In HL-60 cells, the incorporation of Q_6 but not Q_{10} induced a transient increase of catabolism of endogenous Q_{10} without affecting its biosynthesis rate. Total levels of Q_{10} returned to normal levels after 12 h of incubation, indicating a quick recovery of balanced metabolism of endogenous Q_{10}, in part to compensate the influence of Q_6. In agreement, supplementation with Q_{10} in the diet of rats also induces the biosynthesis of Q_9 [51]. The effect of Q_6

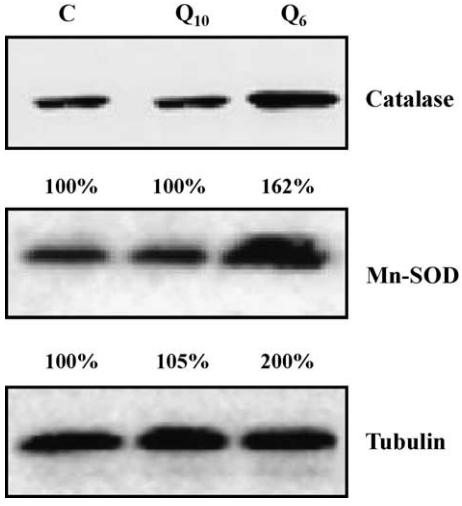

Fig. 7. Antioxidant enzymes expression in HL-60 cells cultured with exogenous Q. Catalase and Mn-SOD present in the whole extracts from HL-60 cells incubated in control conditions or supplemented with 2 μM Q_{10} and Q_6 or 6 h were analysed by Western blotting. Tubulin was determined as loading control and the density of tubulin blots used to homogenize data. Densitometric analysis results of blots are indicated as percentage vs. control. Data are the representative Western blotting of three.

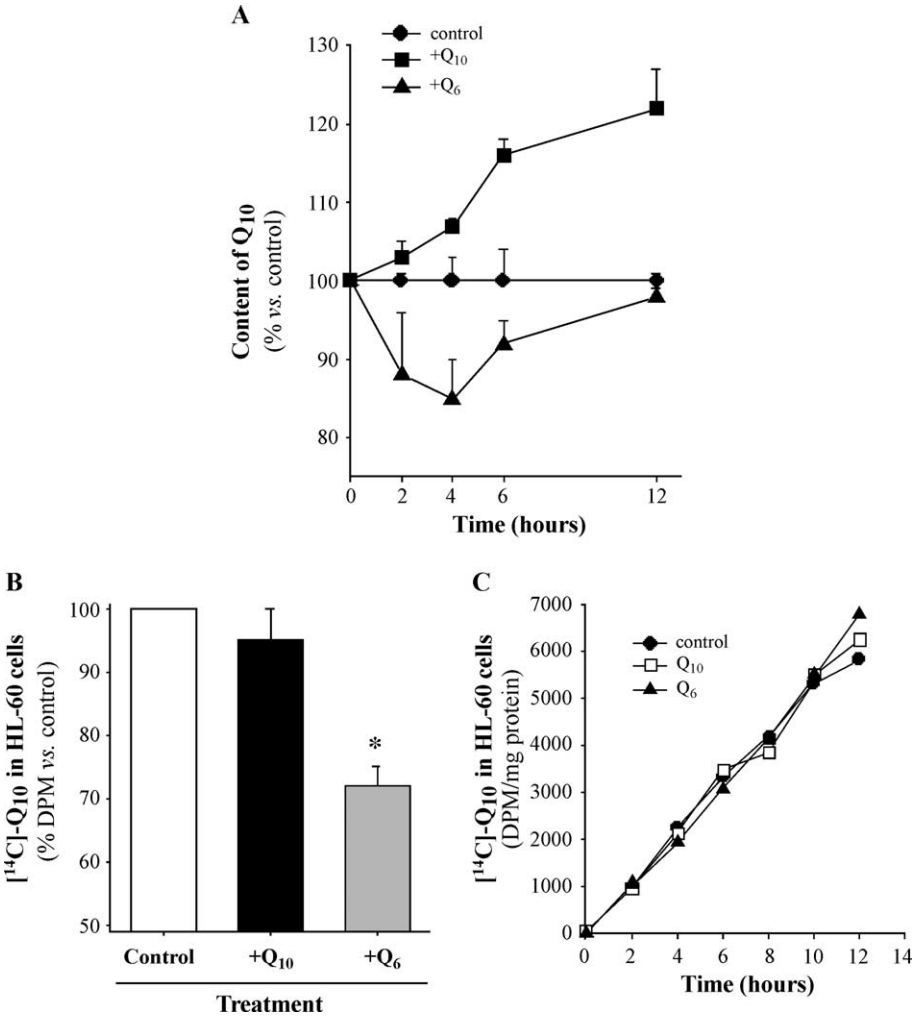

Fig. 8. Endogenous Q_{10} levels in mitochondria of HL-60 after exogenous Q incorporation. (A) Endogenous Q_{10} levels in pure mitochondria isolated from fraction 3. In the case of Q_{10} supplementation, the total level of Q_{10} found in mitochondria along incubation time is indicated due the impossibility of discriminating between endogenous and exogenous Q_{10}. Data represent the mean from three different experiments performed in duplicate and are normalised to control levels as the percentage of total Q_{10} vs. control Q_{10}. (B) Levels of [^{14}C]-Q_{10} in HL-60 cells after incubation with exogenous Q_{10} or Q_6 for 6 h. Cells were incubated with the radioactive precursor [^{14}C]-pHB for 12 h, washed twice with serum-free medium and further incubated with exogenous Q_{10} or Q_6 for 6 h. [^{14}C]-Q_{10} was determined as described in Materials and methods. Data are normalised to the DPMs found in control levels obtained from three different experiments performed in duplicate. *Significant differences vs. control, $P \leq 0.05$. (C) Levels of [^{14}C]-Q_{10} in HL-60 cells incubated with vehicle or exogenous Q_{10} or Q_6 for 12 h. The precursor, [^{14}C]-pHB, was added just at the beginning of the experiment and samples harvested at indicated times. The presence of [^{14}C]-Q_{10} in cells was determined as described in Materials and methods. Data represent an experiment of three performed in duplicate and are indicated as DPMs/mg protein.

on mitochondrial metabolism is very important to understand some aspects of dietary Q supplementation, particularly in the treatment of Q_{10}-deficiency pathologies. In fact, the supplementation of Q_{10} has already shown clear benefits [12,20,55]. The efficiency of dietary Q_{10} intake is rather low in the available formula [11,56] and alternative Q analogues such as idebenone have been used to treat mitochondrial pathologies [57–61] although its use has been questioned due to a possible increase in ROS production [62]. Thus, the supplementation with Q_{10} in human cells, in appropriate conditions to increase availability, appears more convenient in dietary supplementation, to prevent both oxidative damage and the modification of Q metabolism.

Acknowledgements

This work has been partially supported by Spanish MCyT grant no. BMC2002-01640. We acknowledge Dr. Jerker Olsson and Dr. Giorgio Lenaz for the critical reading of the manuscript. Daniel J.M. Fernández-Ayala was a recipient of a PDI fellowship of the Junta de Andalucía.

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