A Water Soluble CoQ10 Formulation Improves Intracellular Distribution and Promotes Mitochondrial Respiration in Cultured Cells

Christian Bergamini¹, Noah Moruzzi¹, Antonella Sblendido², Giorgio Lenaz¹, Romana Fato¹*¹

¹ Department of Biochemistry "G. Moruzzi", University of Bologna, Bologna, Italy, ² Scharper Therapeutics, Medical Department, Sesto S. Giovanni, Milano, Italy

Abstract

Background: Mitochondria are both the cellular powerhouse and the major source of reactive oxygen species. Coenzyme Q₁₀ plays a key role in mitochondrial energy production and is recognized as a powerful antioxidant. For these reasons it can be argued that higher mitochondrial ubiquinone levels may enhance the energy state and protect from oxidative stress. Despite the large number of clinical studies on the effect of CoQ₁₀ supplementation, there are very few experimental data about the mitochondrial ubiquinone content and the cellular bioenergetic state after supplementation. Controversial clinical and in vitro results are mainly due to the high hydrophobicity of this compound, which reduces its bioavailability.

Principal Findings: We measured the cellular and mitochondrial ubiquinone content in two cell lines (T67 and H9c2) after supplementation with a hydrophilic CoQ₁₀ formulation (Qter®) and native CoQ₁₀. Our results show that the water soluble formulation is more efficient in increasing ubiquinone levels. We have evaluated the bioenergetics effect of ubiquinone treatment, demonstrating that intracellular CoQ₁₀ content after Qter supplementation positively correlates with an improved mitochondrial functionality (increased oxygen consumption rate, transmembrane potential, ATP synthesis) and resistance to oxidative stress.

Conclusions: The improved cellular energy metabolism related to increased CoQ₁₀ content represents a strong rationale for the clinical use of coenzyme Q₁₀ and highlights the biological effects of Qter®, that make it the eligible CoQ₁₀ formulation for the ubiquinone supplementation.

Introduction

Coenzyme Q₁₀ (CoQ₁₀), also known as ubiquinone, is the predominant form of coenzyme Q in humans. It is a lipid-soluble molecule composed of a redox active quinone ring and a hydrophobic tail. In the mitochondrial respiratory chain it acts as a mobile electron transporter and is a cofactor of uncoupling proteins [1]. When reduced, it is a powerful antioxidant that prevents oxidative damage by free radicals, including oxidation of proteins [1]. When reduced, it is a powerful antioxidant that prevents oxidative damage by free radicals, including oxidation of proteins [1]. Heart, kidney, brain and liver tissues show the highest concentration of CoQ₁₀, which is endogenously synthesized and in small part assimilated from the diet [5].

The fundamental role of ubiquinone in mitochondrial function and cellular bioenergetics should make it the main dietary supplement in situations where its production is inadequate [6] or in pathological conditions where alterations of mitochondrial enzymes involved in CoQ₁₀ redox mechanisms occur [7] such as cardiovascular disease [8], metabolic diseases [9], oxidative stress and aging [10].

The rationale for CoQ₁₀ therapy is supported by the evidence of decreasing CoQ₁₀ levels with age in human and animal tissues, further suggesting a potential therapeutic role in age-related neurodegenerative disorders [11,12,13].

Despite these potential beneficial effects on disorders related to mitochondrial dysfunction, clinical studies showed controversial results. The use of CoQ₁₀ in neurodegenerative disorders failed to demonstrate any positive result in patients with Huntington’s [14] and Parkinson’s diseases [15] or amyotrophic lateral sclerosis [16]. Controversial results were observed in primary hypertension and statin induced myalgia [17] as well.

Therapeutic applications of CoQ₁₀ are greatly limited by its poor bio-availability, due to its lack of solubility in aqueous media. A recent study demonstrated that, in rats, only 3% of orally administered CoQ₁₀ can be absorbed [18]. Several advancements have been made to enhance the bioavailability of CoQ₁₀ using various approaches like size reduction, solubility enhancement (by solid dispersion, prodrug, complexation, ionization) and use of novel drug carriers such as liposomes, microspheres, nanoparticles,
nаноэмульсий и само-эмульгирующихся систем [19,20]. Для обновленной ревизии смотри: Villalba et al. [21].

Целью настоящего исследования было увеличение митохондриального содержания CoQ10 в культурированных клетках (H67 и H9c2 клеточные линии), с целью улучшения их биоэнергетических параметров. В целях достижения этого мы пополнили культурированные клетки водорастворимым CoQ10 в терклазированной форме Qter®, чтобы улучшить активность CoQ10. Митохондриальный респираторный коэффициент поддерживался различными углеводами (глюкоза, глутамат/малат и сукцинат/глицерол 3-фосфат), митохондриальная ATP и белковая концентрация подразумевали себе описать состояние энергии клетки. Антиоксидантные свойства CoQ10 и Qter® были оценены с помощью флуоресцентных проб (DCFDA и MitoSOX red). Мы также хотели подчеркнуть важность правильного внедрения ubiquinone в митохондриальную мембрану, которая основывается на его биодоступности, а не на вводимом количестве ubiquinone.

Результаты

Титрация CoQ10 uptake

Предварительные эксперименты были проведены, чтобы установить необходимую концентрацию ubiquinone, и описать влияние на биоэнергетические параметры. Мы пополнили культурированные клетки водорастворимым CoQ10 в концентрациях от 10 нМ до 10 мМ. В титрации CoQ10 uptake, проведённой с помощью trypan blue exclusion method (данные не показаны), было показано, что в клетках H9c2, подкормленных CoQ10, была более высокая концентрация CoQ10, чем в контрольной группе. В частности, на концентрацию ubiquinone, например, 100 нМ Qter®, потребовалось более высокое количество ubiquinone, чтобы достичь схожего уровня CoQ10. 

Респираторный и митохондриальный ATP

Митохондриальный респираторный коэффициент поддерживался различными углеводами (глюкоза, глутамат/малат и сукцинат/глицерол 3-фосфат), митохондриальная ATP и белковая концентрация подразумевали себе описать состояние энергии клетки. Антиоксидантные свойства CoQ10 и Qter® были оценены с помощью флуоресцентных проб (DCFDA и MitoSOX red). Мы также хотели подчеркнуть важность правильного внедрения ubiquinone в митохондриальную мембрану, которая основывается на его биодоступности, а не на вводимом количестве ubiquinone.

Оксидативный стресс

Известно, что митохондриальное депрессирование является основной причиной ROS в клетке, а также ROS может быть стимулирован, если используются радикалы, такие как tert-butyl hydroperoxide (TBH). Для оценки уровней ROS в биологических образцах мы использовали два флуоресцентных маркера: DCFDA и MitoSOX Red. 

Рисунок 4 показывает защитное действие Qter® против оксидативного стресса, вызванного 100 нМ Rotenone (специфический комплекс I)}
inhibitor) in T67 cells (Fig. 4A) and H9c2 (Fig. 4B) or 100 μM TBH in T67 cells (Fig. 4C) and H9c2 (Fig. 4D).

Pre-treating cells for 24 hours with 100 nM Qter® reduces the total amounts of cellular ROS (Fig. 4A, 4B, 4C, 4D), whereas in the same conditions native CoQ10 is less efficient. Moreover, cellular pre-treatment with higher Qter® concentration (10 μM), not only failed to improve protection against ROS production, but increased the oxidative stress. (Fig. 4A)

Figure 5A and 5B report the MitoSOX Red staining of H9c2 cells without (Fig. 5A) and with (Fig. 5B) 100 nM Qter® pre-treatment for 24 hours: the lower staining observed in Figure 5B suggests that cellular CoQ10 supplementation reduces the ROS level also in absence of an oxidative insult. In Figure 5C is reported the MitoSOX Red fluorescence intensity obtained by Image J software analysis. ROS damage can be evaluated by measuring the presence of oxidative products such as malondialdehyde (MDA) and conjugated dienes. We observed that Qter treatment caused a statistically significant reduction of all lipid oxidation markers. Figure 6A and 6B show the MDA levels in T67 cells, both in absence (Fig. 6A) and in presence (Fig. 6B) of an oxidative insult, induced by treatment with 100 μM TBH. Even in this case it is possible to appreciate the higher efficiency of Qter® supplementation with respect to native CoQ10.

Figure 7 reports the differential absorption spectra of conjugated dienes extracted from T67 cells pre-treated with CoQ10 after 100 μM TBH exposure. The spectrum of cells treated with 100 nM Qter showed the lowest absorbance and the peak was shifted towards shorter wavelengths according to the presence of a lower conjugation status. On the other hand, the spectrum obtained by cells treated with 10 μM of native CoQ10 showed a higher absorbance and the peak was red shifted, indicating a high amount of conjugated dienes.
CoQ10 extracted from T67 cells treated with 10 μM supplemented quinone. Figure 8 shows the absorption spectra of CoQ10 7.40 w quinone forms. When cells were treated with 10 μM ubiquinone is mainly present in the reduced form. When cells were treated cells showed a maximum close to 290 nm, indicating that the cytoplasm with a green fluorescence emission (Figure 9A).

Mitochondrial membrane potential

To assess whether Qter administration could alter mitochondrial membrane potential, JC-1 fluorescence assay was performed in H9c2 cells. In control cells with normal mitochondrial membrane potential, JC-1 accumulates in mitochondria as aggregates with a red fluorescence emission while the monomeric form is prevalent in the cytosol. The high concentration of CoQ10 in the mitochondria reflects its important role in electron transport chain: age-related decrease in mitochondrial CoQ10 content is responsible for oxygen consumption decline.

The red/green fluorescence ratios are summarized in Figure 9D. Respiriometric analyses were performed under endogenous and uncoupled conditions (500 nM FCCP). Respiratory rates are expressed as percentage of oxygen consumption respect to the endogenous respiration ± S.D.

doi:10.1371/journal.pone.0033712.t001

Respirometric analyses were performed under endogenous and uncoupled conditions. The maximal uncoupled respiration was measured in the presence of 500 nM FCCP. Respiratory rates are expressed as nmoles O2 min⁻¹/10⁶ cells ± S.D. from at least three independent experiments. *p<0.05 vs. control.

doi:10.1371/journal.pone.0033712.t002

The oxidative stress observed in cells treated with high amounts of native CoQ10 can be due to an incomplete reduction of the supplemented quinone. Figure 8 shows the absorption spectra of CoQ10 extracted from T67 cells treated with 10 μM native CoQ10 or 100 nM Qter. The spectrum obtained from 100 nM Qter treated cells showed a maximum close to 290 nm, indicating that ubiquinone is mainly present in the reduced form. When cells were treated with 10 μM of native CoQ10, the spectrum was broad and the maximum was shifted towards 275 nm, indicating the contemporary presence of oxidized and reduced quinone forms.

Table 1. Respiratory rates of intact H9c2 and T67 cells treated for 24 hours with 100 nM native CoQ10 or Qter.

| Cell treatment | % of endogenous respiration rate | - FCCP | +500 nM FCCP |
|----------------|----------------------------------|--------|--------------|
| No treatment   | 100±7.2                          | 131±13 |
| 10 μM CoQ10    | 100±8.6                          | 84±8.6 |
| 10 μM Qter     | 100±1.1                          | 102±14 |

The results obtained in vivo about CoQ10 tissue distribution are uncertain. Ibrahim et al. (Ibrahim et al., 2000) observed that CoQ10 oral administration did not alter the levels of this compound in the heart. Furthermore there is no evidence so far showing that dietary CoQ10, which is found to increase the CoQ10 content in lipoproteins in the liver, is taken up by other tissues under normal conditions. These uncertain results could be partially attributed to the poor water solubility of CoQ10 that impairs its intestinal absorption, tissue distribution and mitochondrial incorporation.

Table 2. Respiratory rates of permeabilized H9c2 and T67 cells treated for 24 hours with 100 nM native CoQ10 or Qter.

| Glutamate/Malate | Succinate/Glycerol 3-phosphate |
|------------------|-------------------------------|
|                  | nmoles O2 min⁻¹/10⁶ cells     | nmoles O2 min⁻¹/10⁶ cells |
| T67              |                               |                           |
| Control          | 3.92±1.02                     | 5.26±1.31                 |
| CoQ10            | 3.26±1.05                     | 6.25±0.92                 |
| Qter             | 4.3±1.14                      | 7.85±0.07 *               |
| H9c2             |                               |                           |
| Control          | 8.81±0.16                     | 12.60±0.70                |
| CoQ10            | 9.20±0.14                     | 13.76±0.28                |
| Qter             | 10.32±0.66*                   | 16.95±0.21**              |

Respirometric analyses were performed in the presence of 5 mM Glutamate/ Malate or 12.5 mM Succinate/Glycerol 3-phosphate. Respiratory rates are expressed as nmoles O2 min⁻¹/10⁶ cells ± S.D. from at least three independent experiments. *p<0.05 vs. control. **p<0.001 vs. control.

doi:10.1371/journal.pone.0033712.t003

Discussion

Coenzyme Q10 is a lipid-soluble compound mainly found in mitochondria. It is mostly endogenously produced within cells though small amounts can be provided by food intake. Analysis of CoQ10 subcellular distribution shows that a large portion of CoQ10 (40–50%) is localized in the mitochondrial inner membrane, with smaller amounts in the other organelles and in the cytosol. The high concentration of CoQ10 in the mitochondria reflects its important role in electron transport chain: age-related decrease in mitochondrial CoQ10 content is responsible for oxygen consumption decline.

From a physiological point of view, tissue CoQ10 content is subject to regulation by several factors including oxidative stress and aging [1,22].

Supplementation with CoQ10 has been thought to be beneficial, especially for situations in which adequate CoQ10 production is adversely affected [6]. A large number of clinical studies have evaluated the effects of CoQ10 supplementation on oxidative stress, both in physiological or pathological conditions.

The results obtained in vivo about CoQ10 tissue distribution are quite controversial. Ibrahim et al. (Ibrahim et al., 2000) observed that CoQ10 oral administration did not alter the levels of this compound in the heart. Furthermore there is no evidence so far showing that dietary CoQ10, which is found to increase the CoQ10 content in lipoproteins in the liver, is taken up by other tissues under normal conditions. These uncertain results could be partially attributed to the poor water solubility of CoQ10 that impairs its intestinal absorption, tissue distribution and mitochondrial incorporation.

The aim of the present study is to evaluate the in vitro efficacy of CoQ10 supplementation in improving mitochondrial function and protection against oxidative stress. Cells supplemented with CoQ10 do not often show any improvement in their bioenergetics status.

These negligible effects can be explained by several factors, first of all its strong lipophilic nature that results in its accumulation in extra-mitochondrial membranes [24,25] while only a small portion (~11%) can reach the mitochondria [26,27]. The exogenous
CoQ₁₀ found in mitochondria is likely to be localized in the outer membrane, thus it is not available to the respiratory chain [28,29].

Our data showed that Qter™ has a better cellular uptake and mitochondrial incorporation compared to native CoQ₁₀: from 10 to 100 fold lower concentrations are required to achieve similar cellular and mitochondrial CoQ₁₀ amount.

A better uptake is the first step to proper CoQ₁₀ insertion into biological membranes and in particular for a significant incorporation in the inner mitochondrial membrane (IMM). We can assume that Qter™ promotes a correct CoQ₁₀ insertion into the IMM since an increase in mitochondrial respiration, ATP production, mitochondrial membrane potential and protein synthesis are observed in the cell lines tested. In an interesting paper by Somayajulu et al. similar results were reported using a different water soluble CoQ₁₀ formulation in human neuroblastoma cells (SH-SY5Y) and teratocarcinoma cells (NT2); in particular the authors described a protective effect of CoQ₁₀ treatment on mitochondrial potential, ATP levels and oxidative stress after hydrogen peroxide exposure [30].

Figure 3. Effect of Qter™ treatment on ATP, protein content and cell growth in H9c2 cells. H9c2 cells were treated up to 72 hours with 100 nM Qter™ and the ATP content was measured at 24, 48 and 72 hours by HPLC analysis (A). Panel B shows the intracellular ATP content after 24 hours treatment with 100 nM Qter™ or native CoQ₁₀ measured using luminescence ATP detection assay. Data are reported as arbitrary luminometric units and normalized on total protein content. (Values are means ± S.D., n = 5, * p<0.01 vs control). H9c2 cells treated with 100 nM Qter up to 72 hours were assayed for protein content at 24, 48 and 72 hours. Protein content was evaluated by Lowry method (C). (Values are means ± S.D., n = 5, * p<0.05 vs. control). Cell growth was assessed by trypan blue exclusion method (D). doi:10.1371/journal.pone.0033712.g003

The great importance of a correct insertion is well explained by our data: cells treated with 10 μM of native CoQ₁₀ present a mitochondrial ubiquinone concentration close to the one observed in cells treated with 100 nM Qter™, but the bioenergetic effects are quite different.

Moreover lipid peroxidation induced by an oxidative insult is reduced in 100 nM Qter™ treated cells as shown in Figure 6B and Figure 7.

For this reason the chemical formulation of CoQ₁₀ may play a crucial role in determining the correct integration of the molecule in the mitochondrial membrane. Nevertheless an increased content of CoQ₁₀ in the mitochondrial membrane does not necessarily imply an automatic increase in mitochondrial function (Table 3). It is well known that respiration rate and ATP synthesis are highly regulated processes that are affected by many factors, primarily cell energy requirements.

Respiration data reported in Table 2 suggest that the mitochondrial ubiquinone content can affect the oxygen consumption under high energy requirement conditions (e.g. high
ADP content). In this condition the oxygen consumption rate increases and the CoQ10 content could become the rate limiting factor. A similar behavior is observed in intact cells under FCCP uncoupled condition; in fact, the reported values of endogenous respiration rate were most likely due to an intermediate respiration state (state 4/state 3 mixed state) in which the rate-limiting step was not affected by CoQ10 addition (Table 1).

Our data show that a high respiration rate is positively correlated with the increased amount of mitochondrial CoQ10, suggesting that its supplementation can play an important role in diseases related to CoQ10 deficiency (aging, Parkinson Disease, Alzheimer and mitochondrial myopathies). These data correlate with increased NADH-Cyt.c and Succinate-Cyt.c reductase activity observed in HL-60 cells treated with CoQ10 reported by Navas and co-workers [31].

The mitochondrial respiratory chain organization could play an important role in the increase of respiratory activity. Currently, two models have been proposed: the random collision model [32] and a supercomplex organization called Respirasome [33]. In the first model the electron transfer through the respiratory chain is assured by free diffusion of each component within the IMM. In this scenario, CoQ10 forms a pool used by all the CoQ-dependent respiratory Complexes (mainly Complex I, II and III). On the other hand, the Respirasome requires a solid state organization in which only bound CoQ10 is involved in electron transfer. This last model was evaluated by Lowry method. Asterisks refer to the statistically significant decrease of ROS production in Rotenone/TBH treated samples supplemented with quinones (n = 5, *p ≤ 0.05; **p ≤ 0.001).

Figure 4. Effects of CoQ10 supplementation on oxidative stress induced by Rotenone and t-Butyl hydroperoxide (TBH). ROS were detected following DCFDA fluorescence in cells treated for 24 hours with 100 nM or 10 uM native CoQ10 or Qter®. ROS were induced by 48 hours treatment with 100 nM Rotenone in T67 (A) and H9c2 cells (B) or by 30 minutes exposure to 100 μM TBH in T67 cells (C) and H9c2 cells (D). Data are the mean ± S.D. of at least three different determinations and are expressed as arbitrary fluorescence units (A.F.U.) normalized on protein content. Protein content was evaluated by Lowry method. Asterisks refer to the statistically significant decrease of ROS production in Rotenone/TBH treated samples supplemented with quinones (n = 5, * p ≤ 0.05; ** p ≤ 0.001).

doi:10.1371/journal.pone.0033712.g004
hypothesis seems to be in contrast with a dose dependent effect of CoQ10 addition on the respiratory rate. However, it may be possible that the bound ubiquinone should be in equilibrium with the pool. This hypothesis could explain the beneficial effect of exogenous CoQ10 supplementation [34].

Nevertheless, treatment with high doses of ubiquinone, despite of its formulation, induces a loss of sensitivity to uncoupling agents and increases oxidative stress. We can argue that an excessive incorporation of CoQ10 may perturb the lipid environment of cellular membranes while oxidative stress may be due to the excess of ubiquinone that remains in its oxidized form. For this reason it is not recommended to treat patients with high doses of coenzyme Q10. In particular, our results show that it is necessary to use from 10 to 100 fold concentrations of native CoQ10 to achieve comparable amounts of ubiquinone respectively in whole cells or isolated mitochondria.

The antioxidant role of the CoQ10 reduced form is well known. It localizes in cellular membranes where it acts as a ROS scavenger together with vitamin E. Cells treated with 100 nM CoQ10 showed a significant decrease in mitochondrial superoxide production compared to untreated cells. Furthermore, treatment with CoQ10 at 100 nM was able to reduce the levels of MDA in T67 cells treated with TBH.

**Figure 5. Analysis of physiological mitochondrial superoxide production using MitoSOX Red.** The representative fluorescence images showed the oxidized MitoSOX fluorescence signal in control H9c2 cells (A) and H9c2 cells following 24 treatment with 100 nM Qter® (B). The fluorescence intensity reported in panel C was quantified by Image J software. Values are presented as means ± SD; n = 20. * p<0.001. doi:10.1371/journal.pone.0033712.g005

**Figure 6. Malondialdehyde (MDA) levels in T67 cells treated with native CoQ10 or Qter.** Cells were pre-treated for 24 hours with native CoQ10 and QTer (100 nM and 10 μM). Panel A shows the MDA levels in the absence of external oxidative stress. Panel B shows the MDA levels after 30 minutes exposure to 100 μM TBH. Data are the mean of two different experimental determinations and are normalized on total protein content.

doi:10.1371/journal.pone.0033712.g006
Qter\(^{\text{H}}\) appear to be more resistant to oxidative stress; in fact, Figure 8 shows that in this condition the quinone is mainly in the reduced form. The higher Qter\(^{\text{H}}\) efficiency is mainly due to its greater water solubility. In fact, compared with native CoQ\(_{10}\), Qter\(^{\text{H}}\) is about 200 times more soluble in water, while retaining its antioxidant capacity [35,36].

Conclusion

Our in vitro study underlines important issues regarding CoQ\(_{10}\) treatment. Present results demonstrate that adequate channeling of CoQ\(_{10}\) is important to ensure proper cellular uptake. The vehicle used to terclatrate CoQ\(_{10}\) maintains it in a monomeric form, that results in a correct insertion into membranes, in particular in the inner mitochondrial membrane. The improved bioavailability allows treatments with low doses of ubiquinone that prevent unspecific accumulation with deleterious effects on cell viability.

Although CoQ\(_{10}\) supplementation has shown beneficial effect in many physiopathological alterations, there are few experimental evidences of a direct improvement of mitochondrial functions after CoQ\(_{10}\) treatment. Some interesting papers by Somayajulu and McCarthy describe the protective effect of a water soluble CoQ\(_{10}\) in in vitro and in vivo studies [30,37,38].

We demonstrated that increased mitochondrial ubiquinone content results in a general improvement of bioenergetic parameters, like oxygen consumption, ATP content, mitochondrial potential and protein synthesis.

Recently, the beneficial effect of terclatrated CoQ\(_{10}\) supplementation in vivo, both in animal models [39,40] and humans [41] has been reported. Thus, this work represents a strong rationale for the clinical use of Coenzym Q\(_{2}\) and highlights the enhanced biological effects of Qter\(^{\text{H}}\) that make it the eligible CoQ\(_{10}\) formulation for ubiquinone supplementation in patients.

Materials and Methods

Reagents

All chemicals used throughout the present study were of the highest analytical grade, purchased from Sigma-Aldrich, unless otherwise specified. Dulbecco’s modified Eagle’s medium, trypsin, penicillin, streptomycin and fetal bovine serum were purchased from Invitrogen. Qter\(^{\text{H}}\) was supplied by Scharper Therapeutics S.r.l. (Milan, Italy). Native CoQ\(_{10}\) was from Kaneka, Japan.

Drug preparation

Qter\(^{\text{H}}\) is described in the patent number WO/2003/097012 by Actimex S.r.l. CoQ\(_{10}\) is 10% (w/w) of Qter\(^{\text{H}}\) formulation. Qter\(^{\text{H}}\) concentration refers to the CoQ\(_{10}\) amount into the multicomposite material. Qter solution was freshly prepared dissolving Qter in DMEM at 100 nM or 10 \(\mu\)M CoQ\(_{10}\) final concentration.

Native CoQ\(_{10}\) stock solution was prepared in ethanol at 5 mM concentration and diluted with DMEM to final concentration of 100 nM or 10 \(\mu\)M CoQ\(_{10}\) final concentration.

Cell culture

The T67 human glioma cell line was derived by Lauro et al. [42] from a World Health Organization (WHO) Grade III gemistocytic astrocytoma. H9c2 embryonal rat heart-derived cells were obtained from European Collection of Cell Cultures, ECACC. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 40 \(\mu\)g/ml gentamycin, in a 5% CO\(_2\)
atmosphere at 37°C, with saturating humidity. Cell viability and number were measured by trypan blue exclusion method [43].

Preparation of Mitochondria Fractions
Mitochondria were isolated according to procedures previously described [44].

Extraction and quantification of Coenzyme Q
Treated cells were carefully washed with PBS before extraction procedures. Extraction of coenzyme Q from cells and isolated mitochondria was performed as described by Takada et al. [45]. Quantification of CoQ_{10} was performed by HPLC analysis. 50–100 ml of ethanolic extract was chromatographed on a C18 column (Kinetex, Phenomenex, 2.6 μm, 100 × 4.6 mm), using a mobile phase consisting of ethanol: water (97:3, v/v) at a flow rate of 0.6 ml/min. The concentrations of CoQ_{10} were obtained by comparison of the peak areas with those of standard solutions. Data are reported as the mean ± standard deviation of at least three independent experiments.

To evaluate the reduction state of ubiquinone, cells were treated for 24 h at 37°C in 5% CO2 with 100 nM CoQ_{10} or Qter® in DMEM plus FBS. Intact cells (1 × 10^6 cells) were assayed for glucose supported oxygen consumption at 30°C in DMEM using a thermostatically controlled oxygraph (Instech Mod.203).

Permeabilized cells. Cells were treated as above and assayed for oxygen consumption in respiration buffer (250 mM sucrose, 20 mM HEPES, 10 mM MgCl2, 1 mM ADP, 2 mM KH2PO4, pH 7.4) after permeabilization. Mitochondrial respiration (state 3 respiration) from complex I was started by adding 5 mM glutamate/malate (G/M) and then stopped with 2.5 μM Rotenone. Subsequently 12.5 mM succinate/glycerol-3-phosphate (S/G3P) was added to restart the respiration. In all experiments maximal respiration rate (uncoupled respiration) was achieved by adding 500 nM FCCP and oxygen consumption was completely inhibited by adding 4 μM Antimycin A at the end of the experiments.

Reactive oxygen species (ROS) detection
H9c2 and T67 cells were seeded in 24-well plates at 4 × 10^4 cells/well. After 24 h incubation at 37°C in 5% CO2 in culture medium supplemented with 100 nM CoQ_{10} or Qter®, cells were washed with phosphate buffered saline (PBS) and treated for 48 h with 100 nM Rotenone. Alternatively cells were treated for 30 minutes with 100 μM tert-butyl hydroperoxide (TBH) in PBS. Subsequently, cells were washed with PBS and treated with 10 μM DCFDA (2',7'-dichlorofluorescein diacetate, DCFH-DA) in DMEM for 30 minutes, then washed again with PBS and the fluorescence increase in each well was measured (λex = 485 nm; λem = 535 nm) with a plate reader (Wallac Victor, Perkin-Elmer, USA). Data are reported as the mean ± standard deviation of at least three independent experiments. In a separate set of experiments, basal oxidative stress in H9c2 cells was measured using the mitochondrial superoxide indicator MitoSOX Red.
were treated with 100 nM Qtr® for 24 hours at 37°C in 5% CO2, then 5 μM MitoSOX was added. After 20 minutes of incubation, cells were washed twice with PBS and images were obtained using an IX50 inverted fluorescence microscope (Olympus, Tokyo) at 20× magnification. Fluorescence intensity was quantified by Image J software (NIH).

**ATP content**

Intracellular ATP level was measured using luminescence ATP detection assay (ATPlite, PerkinElmer, USA) according to manufacturer’s instructions. Data were reported as arbitrary luminescent units, measured with the microplate reader Wallac Victor multilabel counter and normalized to total protein content, determined by Lowry method [47]. Alternatively, intracellular ATP was measured by HPLC method. ATP was extracted essentially as described by Streher et al. [48]. Distilled water (180 μl) preheated to 100°C was added to cellular samples in Eppendorf tubes and boiled for 5 min with occasional vortexing. Tubes were transferred to ice until HPLC analysis. A mobile phase containing 100 mM K2HPO4 (pH 5.75), 0.1% TBAF (tetraethylammonium fluoride) and 2% acetonitrile was pumped through a Kinex C18 (Phenomenex) column at ambient temperature at a flow rate of 0.6 ml/min [49]. Absorbance at 254 nm was monitored by a photodiode array detector (Waters 996). ATP peak was identified by its retention time and by using co-chromatography with standard.

**Thiobarbituric acid assay of malondialdehyde and lipid-conjugated dienes assay**

T67 cells were treated for 24 h at 37°C in 5% CO2 with native CoQ10 (10 μM and 100 nM) or Qtr® (10 μM or 100 nM) in DMEM plus FBS and washed with PBS before treating for 30 minutes with 100 μM tert-butyl hydroperoxide (TBH) in PBS.

After TBH treatment, adherent cells were washed twice with PBS, then detached by trypsin-EDTA and centrifuged at 300 × g for 3 min; then the pellet was resuspended in PBS and centrifuged again.

Quantification of thiobarbituric acid reactive substances (TBARS) was carried out as described by Buege and Aust [50]. The formation of conjugated dienes, in T67 cells treated as above was assayed according to Buege and Aust [50].

**JC-1 stain for mitochondrial membrane potential (ΔΨm) measurement**

The fluorescent probe JC-1 (5, 5′, 6, 6′-tetrachloro-1′, 3′, 3′-tetraethylbenzimidazol carbonyl cyanide iodide) was used to measure the mitochondrial membrane potential (ΔΨm) of H9c2 cells. JC-1 is a cationic dye that is accumulated in mitochondria following membrane potential.

At low concentrations the probe is present in monomeric form, with green fluorescence emission (525±10 nm), but at higher concentrations it forms J-aggregates after accumulation in the mitochondrion, with red fluorescence emission (590±10 nm).

After incubation with 10 μM JC-1 at 37°C for 10 min, the culture medium containing JC-1 was removed. Cells were washed twice with PBS, and analyzed by IX50 fluorescence microscope (Olympus, Tokyo)) at 20× magnification. Fluorescence intensity was quantified by Image J software (NIH). Mitochondrial depolarization was achieved by treating cells with 500 nM FCCP, indicated by a decrease in the red/green fluorescence intensity ratio.

**Statistical analysis**

Statistical analysis of data was performed with Student’s t-test using GraphPad Prism software.

**Author Contributions**

Conceived and designed the experiments: RF CB GL. Performed the experiments: CB NM. Analyzed the data: CB NM RF AS GL. Contributed reagents/materials/analysis tools: RF. Wrote the paper: RF CB GL.

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