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Characterization of cellular uptake and distribution of coenzyme Q_{10} and vitamin E in PC12 cells☆

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Abstract

Coenzyme Q (CoQ) is a well-known electron transporter in the mitochondrial respiratory chain. Furthermore, ubiquinol (UQH_2) — a reduced form of ubiquinone (UQ) — has been shown to act as a radical-scavenging antioxidant. Some studies have reported the beneficial effect of CoQ addition to cultured cells; however, the cellular uptake and distribution of CoQ have not been elucidated. In the present study, we used rat pheochromocytoma PC12 cells to investigate and compare the cellular uptake and distribution of CoQ_{10} and α-tocopherol (αT). UQ_{10} or $UQ_{10}H_2$ treatment resulted in an increase in the cellular content of both CoQ_{10} in a time- and concentration-dependent manner. A subcellular fractionation study revealed that the added UQ_{10} as well as $UQ_{10}H_2$ mainly localized in the mitochondrial fraction, which is similar to the localization of endogenous CoQ but different from that of αT. The cellular distribution of αT directly corresponded to the lipid distribution, while the CoQ distribution did not show any relationship with the lipid distribution, particularly in the mitochondrial and microsomal fractions. These results indicate that the cellular distribution of CoQ is completely different from that of αT; moreover, a certain system which accumulates CoQ preferentially in mitochondria may be suggested.

Keywords: Coenzyme Q; Vitamin E; Cellular uptake; Cellular distribution; Subcellular fractionation study

1. Introduction

The involvement of lipid peroxidation in *in vivo* oxidative damage and in the pathogenesis of several disorders and diseases induced by reactive oxygen and nitrogen species is widely accepted. Lipid peroxidation may directly damage biological molecules and membranes and may also induce the generation of toxic and signaling molecules [1–3]. Based on this, the potential role of antioxidant nutrients has been investigated in relation to the prevention of cancer, cardiovascular disease, cataract, age-related macular degeneration and aging.

Coenzyme Q (CoQ) is a well-known electron transporter in complexes of the mitochondrial respiratory chain [4]. It has been known that in CoQ, there are two forms, namely, oxidized form (ubiquinone, UQ) and reduced form (ubiquinol, UQH_2). Redox functions of CoQ are due to its ability to exchange two electrons in a redox cycle between UQ and UQH_2. CoQ is synthesized *in vivo* and performs several functions that are of great importance with regard to cellular metabolism, including ATP synthesis. Furthermore, it has also been shown that UQH_2, a reduced form of UQ, acts as a radical-scavenging antioxidant [5,6]. For example, UQH_2 can prevent lipid peroxidation in most subcellular membranes [5]; it functions as a reducing agent against α-tocopheroxyl radicals in liposomal suspensions [6] and in low-density lipoprotein [7]. Based on the total hydroxyoctadecadienoic acid (tHODE) levels and stereoisomeric ratio,

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which are prominent biomarkers for the evaluation of *in vivo* oxidative status and antioxidant capacity, it has been demonstrated that CoQ plays a major role in the *in vivo* antioxidant network [8]. Its benzoquinone ring suggests a redox function, while the isoprenic side chain mediates the arrangement of CoQ in the lipid core of biomembranes. It has been known that CoQ is inhomogeneously distributed in various biomembranes [9]. The presence of CoQ has been demonstrated in all cellular membranes, including the plasma membrane [10]. Since the final reactions of the CoQ biosynthesis pathway occur exclusively in the mitochondria in yeast and mammalian cells [10], the existence of specific mechanisms for CoQ distribution has been suggested. It has been reported that CoQ is transferred to the plasma membrane by the brefeldin A-sensitive endomembrane pathway [10]; however, the detailed molecular mechanisms of CoQ distribution remain unclear.

In order to bridge the gap between *in vitro* and *in vivo* systems, cultured cells have often been used to study oxidative stress and its inhibition. The protective effects of various antioxidants against cytotoxicity induced by different types of oxidative insults have been studied extensively [11–13]. The efficacy of an antioxidant depends on its cellular uptake and localization as well as its inherent activity [14]. Therefore, the uptake of antioxidants and their distribution in cells are important factors that determine their effects. With regard to vitamin E, which is well known as the most abundant and potent *in vivo* lipophilic radical-scavenging antioxidants, we have previously reported that the apparently higher cytoprotective effects of α-tocotrienol (αT3, an isoform of vitamin E) than those of α-tocopherol (αT, a major form of vitamin E) are primarily ascribed to the higher cellular uptake of the former [11,14]. Subcellular fractionation analysis of αT- and αT3-treated Jurkat cells revealed similar cellular distribution of these antioxidants, which are mainly enriched in the microsomal fraction of the cells [14]. It is also notable that the cellular distribution of these vitamin E isoforms is directly proportional to the lipid distribution [14]. The antioxidant action of CoQ has received substantial attention because of several biological activities including the potent radical-scavenging activity of UQH_2. The beneficial effect of its supplementation has been demonstrated not only in *in vivo* studies [8,15,16] but also in *in vitro* studies, including those involving cell culture systems [17–20]. It has been reported that CoQ exhibited protective effects against insults induced by excimer laser irradiation [17], hydrogen peroxide [18], paraquat [18], serum starvation [19], antimycin A [19] and ceramide [19,20] in several cultured cells; however, the cellular uptake and distribution of CoQ have not been elucidated.

In the present study using rat pheochromocytoma PC12 cells that have been widely used as a model to study oxidative stress in neuronal cells, we investigated the cellular uptake and distribution of CoQ_{10} and compared with those of αT. We found that exogenous UQ_{10} as well as $UQ_{10}H_2$ mainly localized in the mitochondrial fraction, which is similar to the localization of endogenous CoQ, but different from that of αT.

2. Methods and materials

2.1. Chemicals

Dulbecco's Modified Eagle's Medium/nutrient mixture F-12 Ham=1:1 (D-MEM/F-12) and horse serum (HS) were obtained from Gibco BRL (Rockville, MD, USA), and fetal bovine serum (FBS) was obtained from JRH Biosciences (Lenexa, KS, USA). αT was kindly supplied by Eisai Co. Ltd. (Tokyo, Japan). Water-soluble CoQ_{10} consisting of 10% CoQ_{10}, 10% polyglycerol ester of fatty acid, 5% sucrose ester of fatty acid and 75% glycerol was prepared as described previously [21]. Other chemicals were of the highest quality commercially available.

2.2. Cell culture and treatment with agents

Undifferentiated PC12 cells, rat pheochromocytoma cell line, were routinely maintained in D-MEM/F-12 medium containing 10% heat-inactivated FBS and 5% heat-inactivated HS at 37°C under an atmosphere of 95% air and 5% CO_2 as described previously [22]. Concentrations of CoQ and αT in serum used are as follows: UQ_{10}, 0.053±0.022 μM and αT, 0.15±0.01 μM in heat-inactivated FBS; UQ_{10}, 0.045±0.006 μM and αT, 2.61±0.23 μM in heat-inactivated HS. $UQ_{10}H_2$ and CoQ_9 were not detected in these serums. Cell viability was assessed using the trypan blue dye exclusion method prior to all treatments. PC12 cells were grown on plate at a density of 1.5×10^5 cells/ml. After the cells were attached (16–18 h), they were treated with CoQ_{10} or αT at different concentrations for the indicated times. Water-soluble CoQ_{10} was diluted with phosphate buffered saline (PBS) and used for cell experiments. Stock solution of αT was prepared in DMSO.

2.3. Determination of intracellular coenzyme Q and vitamin E

Intracellular CoQ and αT were detected using HPLC systems with electrochemical detection as described previously [8,14]. In the case of CoQ, cell samples in PBS were mixed with methanol/hexane (1:5:10, v/v) followed by centrifugation (15,000 rpm, 10 min); an aliquot of the upper layer was immediately injected into the HPLC with an amperometric electrochemical detector (NANOSPACE SI-1, Shiseido Co. Ltd., Tokyo, Japan) set at 700 mV. The samples were passed through a reverse-phase column (LC8, 5 μm, 250×4.6 mm, Sigma-Aldrich Japan Co., Tokyo, Japan) followed by a reducing column (RC-10, 30×4 mm, Shiseido Co. Ltd.); methanol-*tert*-butyl alcohol (85:15, v/v) containing 50 mM sodium perchlorate was used as the eluent at a rate of 1 ml/min. In the case of αT analysis, cell samples in PBS were mixed with chloroform/methanol (1:2:1, v/v), and then cellular vitamin E in the lower chloroform layer was analyzed with an HPLC using a post-column amperometric electrochemical detector (NANOSPACE SI-1) set at 800 mV

combined with an octadecyl-bonded silica column (LC18, 5 μm, 250×4.6 mm, Sigma-Aldrich Japan Co.); methanol-*tert*-butyl alcohol (95:5, v/v) containing 50 mM sodium perchlorate was used as the eluent at a rate of 1 ml/min.

2.4. Protein assay

The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA) with bovine serum albumin as a standard.

2.5. Subcellular fractionation of cells

CoQ- and αT-treated cells were fractionated by the previously described method [23] with slight modification. The cells were homogenized by nitrogen decompression using Parr Cell Disruption Bombs (Parr Instrument Co., Moline, IL, USA). The nuclear fractions were prepared by centrifugation at 500×*g* for 10 min and suspended in a homogenizing buffer. Mitochondrial fraction was obtained from post nuclear fractions by centrifugation at 5000×*g* for 10 min, and the microsomal and cytosolic fractions were obtained by centrifugation at 105,000×*g* for 60 min. The distribution of each subcellular fraction was judged by standard enzymatic measurements, cytochrome *c* oxidase (mitochondrial marker), NADPH-cytochrome *c* reductase (microsomal marker) and lactate dehydrogenase (cytosolic marker) as reported previously [14,23].

2.6. Determination of intracellular lipids

The cell constituents were extracted with chloroform and methanol (1:5:10, v/v), and lipids in the chloroform layer were analyzed by using a thin-layer chromatography equipped with a flame ionization detection system [24]. The total molar lipid content of each cell fraction was determined using these authentic samples and average molecular weight as follows: for phosphatidylcholine, 1,2-dimyristoyl-rac-glycero-3-phosphocholine (Sigma) and 677.9; for phosphatidylethanolamine, L-α-phosphatidylethanolamine from egg yolk (Sigma) and 746.1; for triacylglycerol, triolein (Nacalai) and 885.4; for cholesterol, cholesterol (Sigma) and 386.7.

3. Results

3.1. Characterization of cellular contents and distribution of endogenous CoQ and αT

In order to investigate the cellular uptake and distribution of the added CoQ, we first determined the endogenous CoQ and αT levels in undifferentiated PC12 cells maintained in a serum medium containing 10% FBS and 5% HS. UQ_9 content was detected as a major CoQ in PC12 cells and the cellular content of its reduced form, i.e., UQ_9H_2, was lower than that of UQ_9 (Fig. 1A). The total cellular CoQ_9 content was higher than the CoQ_{10} content, while the αT content was lower than both the CoQ_9 and CoQ_{10} contents. PC12 cells have been widely used in recent years as model to study oxidative stress in neuronal cells. We next compared the contents of these lipid-soluble antioxidants between PC12 cells and the rat brain. As a result, the total CoQ content in the PC12 cells was largely similar to that in the rat brain (Fig. 1B). On the other hand, it was evident that the αT content of the PC12 cells was lower than the other antioxidant contents.

To investigate the distribution of endogenous CoQ and αT in cellular organelles, we fractionated cells into their organelles under normal culture conditions by centrifugation and determined the CoQ and αT contents. Both CoQ_9 and CoQ_{10} were mainly enriched in the mitochondrial fraction of the cells and showed similar distribution to the mitochondrial marker enzyme cytochrome *c* oxidase (Fig. 1C). On the other hand, endogenous αT was mainly enriched in the microsomal fraction and showed similar distribution to the microsomal marker enzyme cytochrome *c* reductase (Fig. 1C). These results indicated the difference in the cellular distribution of CoQ and αT.

3.2. Difference between CoQ_{10} and αT with regard to their cellular uptake and distribution

Next, we determined the cellular uptake of CoQ_{10}. On culturing PC12 cells in a serum medium containing 10 μM $UQ_{10}H_2$ or UQ_{10}, the cellular content of CoQ_{10} under both conditions increased with the incubation time and reached a plateau after 24 h of incubation (Fig. 2A and B). Strikingly, increased $UQ_{10}H_2$ or UQ_{10} existed predominantly in its form inside the cells up to 72 h. A concentration-dependent study of the cellular uptake of CoQ_{10} under both conditions (addition of $UQ_{10}H_2$ or UQ_{10}) revealed that the cellular content of the added $UQ_{10}H_2$ or UQ_{10} increased in a concentration-dependent manner (Fig. 2C and D). Treatment with 10 μM $UQ_{10}H_2$ or UQ_{10} resulted in an increase in the total cellular CoQ content (11.7 and 9.1 nmol/mg protein, respectively), which in the latter case was approximately 50-fold higher than the total endogenous CoQ content (0.18 nmol/mg protein). The sole effect of surfactants used for CoQ solubilization on cellular CoQ content was not observed (data not shown). On the other hand, treatment with 10 μM αT resulted in an increase in the cellular αT content from 0.02 to 0.25 nmol/mg protein.

We further pretreated cells with 10 μM $UQ_{10}H_2$ or UQ_{10} for 24 h, fractionated them into their organelles and determined the CoQ contents. Both $UQ_{10}H_2$ and UQ_{10} were mainly enriched in the mitochondrial fraction, and the distribution of the added $UQ_{10}H_2$ or UQ_{10} was similar to that of endogenous CoQ_{10} (Fig. 3). In the case of αT, the distribution of the added αT was similar to that of endogenous αT despite the different concentrations in the organelles (data not shown).

We have previously observed that the subcellular contents of both αT and αT3 in Jurkat cells were directly proportional to the lipid distribution [14]. In the present study, we further investigated the correlation between lipid

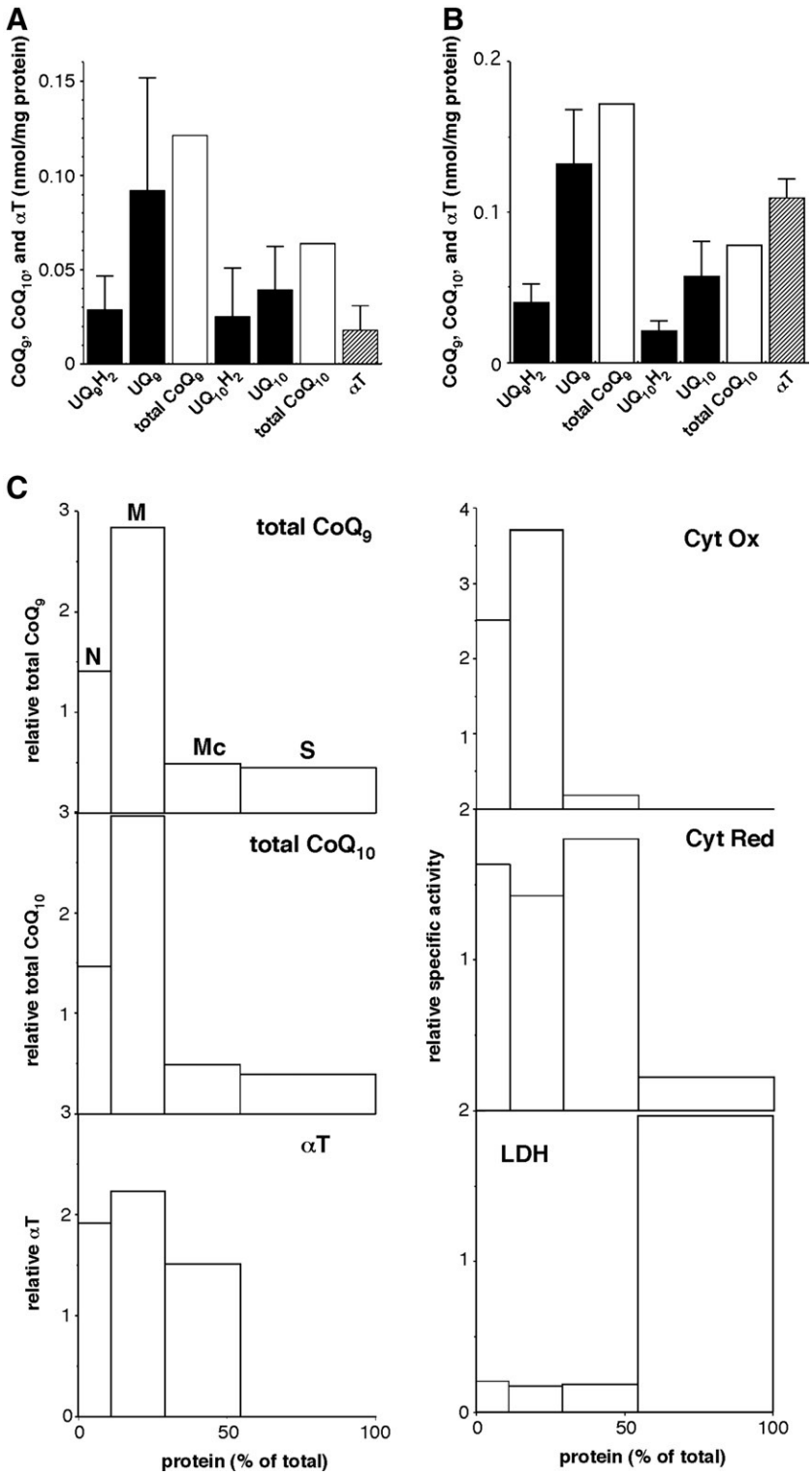

Fig. 1. Cellular content and distribution of CoQ and αT in PC12 cells. (A and B) CoQ and αT contents of PC12 cells cultured with serum medium (A) and rat brain (B) were measured using an HPLC system with an electrochemical detector as described in Methods and Materials. Data are represented as means±S.D. of at least three determinations. (C) Subcellular localization of CoQ and αT in PC12 cells. Cells cultured with serum medium were fractioned by differential centrifugation into nuclear fraction (N), mitochondrial fraction (M), microsomal fraction (Mc) and cytosolic fraction (S), and the contents of CoQ, αT and protein were measured as described in Methods and Materials. The distribution of marker proteins was also determined by enzymatic activity. Cytochrome *c* oxidase (Cyt Ox), NADPH-cytochrome *c* reductase (Cyt Red) and lactate dehydrogenase (LDH) were measured as marker enzymes of mitochondria, microsome and cytosol, respectively. Mean values of each content are shown (*n*=2).

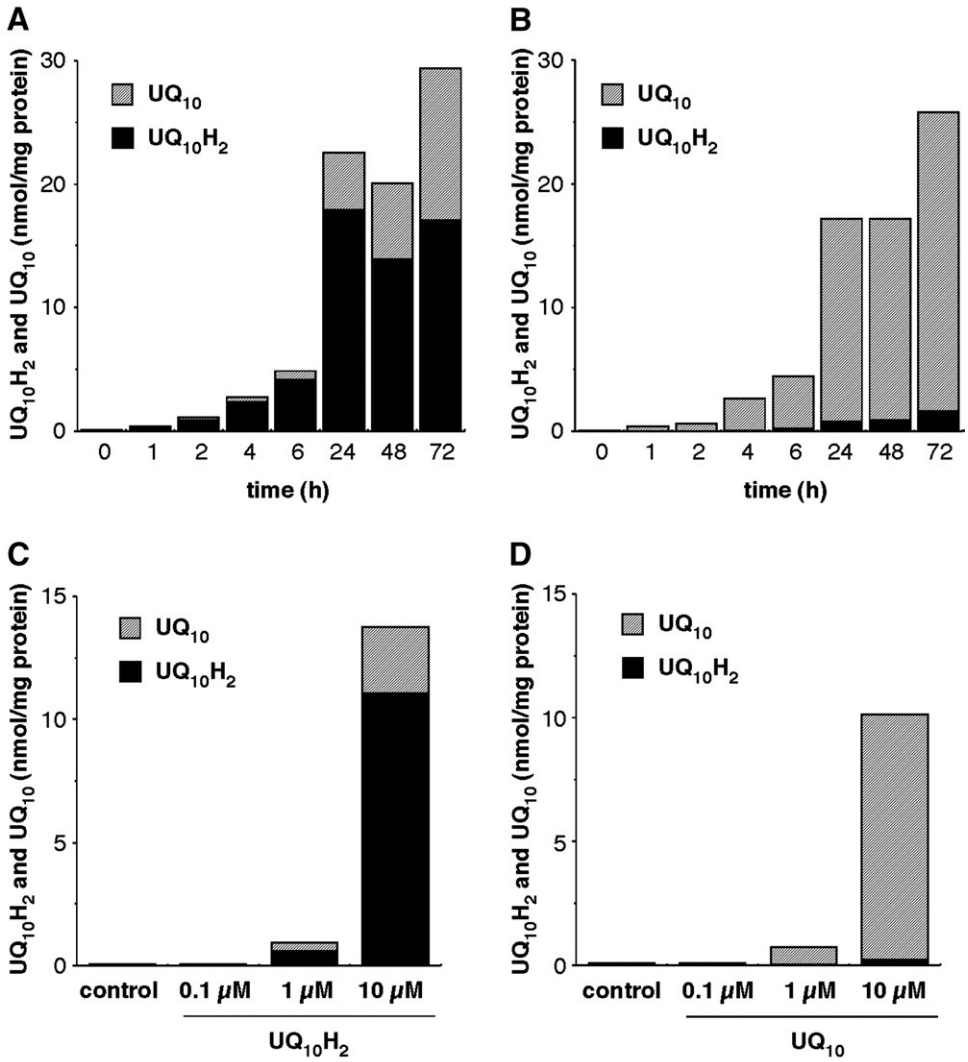

Fig. 2. Cellular uptake of $UQ_{10}H_2$ and UQ_{10} into PC12 cells. The cells were cultured in serum medium with $UQ_{10}H_2$ and UQ_{10} for the indicated times, and the cellular vitamin E content was measured using an HPLC system with an electrochemical detector. (A and B) 10 μM $UQ_{10}H_2$ (A) and UQ_{10} (B) for the indicated times. (C and D) Variable amounts of $UQ_{10}H_2$ (C) and UQ_{10} (D) for 24 h. Mean values of each content are shown (n=2).

distribution and the contents of lipid-soluble antioxidants in PC12 cells treated with 10 μM $UQ_{10}H_2$ or αT for 24 h. Although the cellular distribution of αT in the PC12 cells was directly proportional to the lipid distribution, CoQ_{10} distribution exhibited a nonlinear relationship with lipid distribution, particularly in the mitochondrial and microsomal fractions (Fig. 4).

4. Discussion

The CoQ_{10} content in human tissues is altered in a number of diseases; moreover, even under nonpathological conditions, the ability of tissues to synthesize CoQ_{10} has been reported to decrease with age [25]. Based on the level of tHODE, a biomarker for the evaluation of *in vivo* oxidative status, it has also been reported that CoQ plays a major role in the *in vivo* antioxidant network. Therefore, CoQ supplementation has received considerable attention. Information regarding cellular distribution is essential for clarifying how antioxidants function at certain concentrations and at specific sites. In the present study, we successfully demonstrated that exogenous CoQ was mainly localized in the mitochondrial fraction, which is similar to the localization of endogenous CoQ but completely different from that of αT.

It should be noted that the CoQ content of PC12 cells, a neuronal cell model, was largely similar to that of the rat brain (Fig. 1). Since it is known that rats synthesize CoQ_9 [26], it is believed that the detected CoQ_{10} is completely exogenous. In order to investigate the cellular uptake and distribution of exogenous CoQ, CoQ_{10} was added to PC12 cells in this study. A significant amount of UQ_{10} was detected in the bovine and horse sera that were used in the

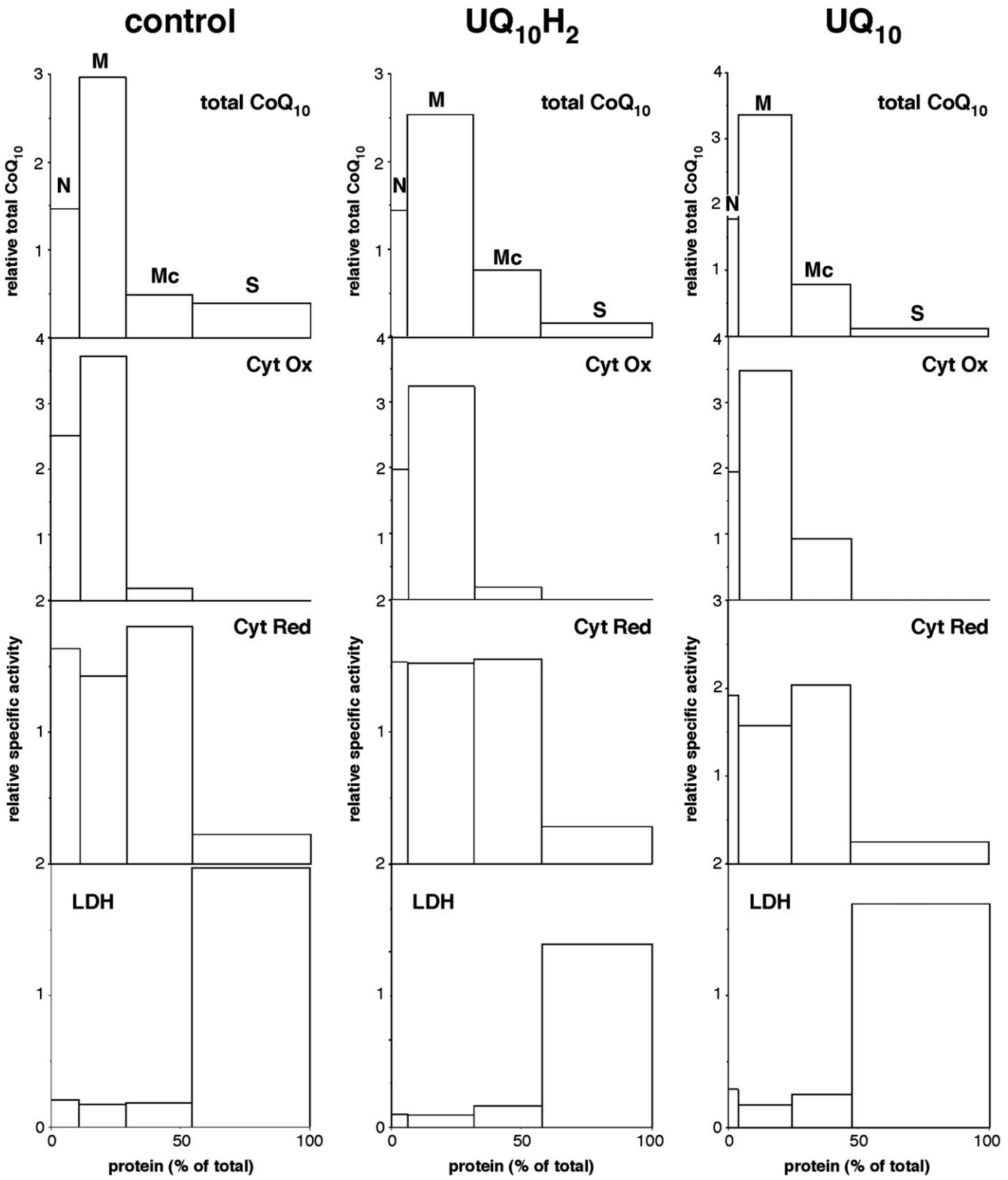

Fig. 3. Subcellular localization of $UQ_{10}H_2$ and UQ_{10} in PC12 cells. Cells were cultured in serum medium containing 10 μM $UQ_{10}H_2$ and UQ_{10} for 24 h. These cells were fractioned, and the contents of CoQ_{10}, marker enzymes and protein were measured. Mean values of each content are shown (n=2).

culture media, i.e., 53 and 45 nM, respectively. Therefore, the UQ_{10} content of the culture media was calculated to be 7.6 nM. It is considered that the CoQ_{10} in serum lipoproteins is taken up by PC12 cells and distributed similarly to that of endogenous CoQ_9. It is also notable that the cellular αT content in PC12 cells was 18% lower than that in the rat brain

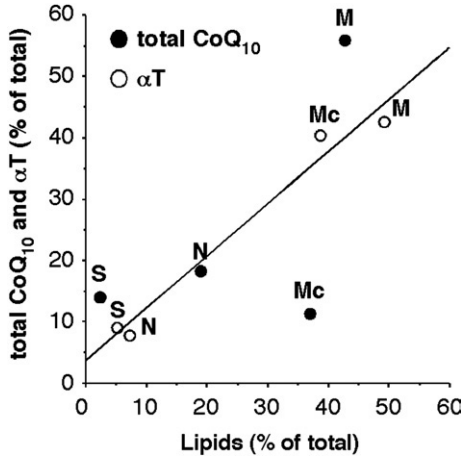

Fig. 4. Cellular contents of CoQ_{10}, αT and total lipid in subcellular fractions. Cells were cultured in serum medium containing 10 μM $UQ_{10}H_2$ and αT for 24 h. These cells were fractioned, and the contents of CoQ_{10}, αT and total lipids were measured as described in Methods and Materials. Mean values of each content are shown (n=2). The correlation of αT with total lipids is shown (y=3.75+0.85×x, R^2=0.974).

(Fig. 1). Leist et al. [27] previously reported that conventional cell culture media containing bovine serum do not adequately supply cells with vitamin E because bovine serum contains lower amounts of this antioxidant as compared to human serum. A standardized vitamin E supplement is important for obtaining reproducible results in investigations related to the function of antioxidants, oxidative stress and redox-modulated signaling pathways.

Interestingly, the incorporated UQ_{10} as well as $UQ_{10}H_2$ stably existed in its respective form in PC12 cells; however, a significant increase in $UQ_{10}H_2$ (from 0.02 to 0.78 nmol/mg protein) was observed in the PC12 cells treated for 24 h with 10 μM UQ_{10} (Fig. 2). It has also been observed that the ratio of UQH_2 to the total CoQ in the brain (CoQ_9=0.23 and CoQ_{10}=0.27 in this study) is lower than that of the liver (CoQ_9=0.55) [8] and plasma (CoQ_9=0.77) [8]. It has been previously reported that NADPH-UQ reductase activity in rat tissues is approximately proportional to the UQH_2/total CoQ ratio in the respective tissues and that the highest activity is found in the liver [28]. Therefore, the lower ratio observed in the rat brain as well as in PC12 cells might be ascribed to the lower reducing capacity of UQ reductase. Several enzymes have been reported to function as UQ reductases [29–31]. These include NADH-cytochrome *b5* reductase [29] and NADPH-cytochrome P450 reductase [30], which are one-electron UQ reductases, and NAD(P)H:quinone reductase 1 (NQO1, formerly known as DT-diaphorase) [31] and a distinct NADPH-UQ reductase [28], which are cytosolic two-electron UQ reductases. On the other hand, we previously detected not only α-tocopheryl quinone (αTQ, 1.2 nmol/mg protein), an oxidized metabolite of αT, but also α-tocopheryl hydroquinone (αTQH_2, 0.7 nmol/mg protein), which is the reduced form of αTQ, in PC12 cells cultured for 24 h in a serum medium containing 8 μM αTQ; however, endogenous αTQ was undetectable in the PC12 cells [32]. The increased CoQ_{10} content in PC12 cells treated with 1 and 10 μM UQ_{10} for 24 h was 0.69 and 10.06 nmol/mg protein, respectively (Fig. 2). The ratios of $UQ_{10}H_2$ to the total CoQ_{10} under these conditions were 0.03 and 0.02, respectively. Considering the increased CoQ_{10} content and the abovementioned reduced ratios in UQ_{10}- and αTQ-treated cells, the ratio of αTQH_2 to total αTQ (0.37) is considered higher than that of UQ_{10} to total CoQ_{10}. This observation suggests that αTQ is a better substrate than UQ_{10} for quinone reductase in PC12 cells. It has been reported that the reduction rate of αTQ by NQO1 is higher than that of UQ [33]. We did not obtain data indicating that the higher reduction of αTQ reflects the substrate specificity of NQO1; however, these results suggest that the reduction of quinone depends not only on the type of tissue but also on the structure of the quinone.

The most interesting finding of this study was that the distribution of exogenous UQ_{10} as well as $UQ_{10}H_2$ was similar to that of endogenous CoQ, which was mainly enriched in the mitochondrial fraction, even though the content of the latter was approximately 50-fold higher than that of endogenous CoQ (Fig. 3). It has been reported that CoQ distribution in HL-60 cells depends on the endomembrane system, which is sensitive to brefeldin A [10]. On the other hand, it is well known that lipophilic cations accumulate within the mitochondria due to their large membrane potential (150–170 mV, negative inside) [34]; however, the CoQ accumulating system in the mitochondria has not been completely elucidated. Many CoQ-related proteins are present in the mitochondria, namely, enzymes for the biosynthesis of CoQ as well as electron transporters in the mitochondrial respiratory chain [4,35]. It has also been reported that mitochondrial CoQ exists in at least two distinct pools, one of which is associated with membrane proteins [26]. It is postulated that mitochondrial proteins possessing CoQ-binding properties are some of the mediators in this accumulating system. The role of mitochondrial dysfunction in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease is identical for providing a rationale based on which mitochondrial treatment should be implemented to improve the progress and ultimately prevent the occurrence of these diseases. Increasing evidence indicates the effectiveness of mitochondria-targeted antioxidants that possess lipophilic cations [34,36]. Furthermore, other strategies for delivering compounds specifically to the mitochondria have also been developed [37]. In order to develop therapies for the protection of the mitochondria, further studies are necessary for the elucidation of the CoQ cellular transport system, particularly the CoQ accumulation mechanisms in the mitochondria.

In conclusion, the present study clearly indicated that the distribution of exogenous UQ_{10} as well as $UQ_{10}H_2$ was similar to that of endogenous CoQ, which was mainly localized in the mitochondrial fraction. Our results might contribute to the development of a novel strategy for the prevention of mitochondria-related neurodegenerative diseases.

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