Protection by Exogenously Added Coenzyme Q9 against Free Radical-Induced Injuries in Human Liver Cells

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Summary Reduced coenzyme Q10 (CoQ10H2) is known as a potent antioxidant in biological systems. However, it is not yet known whether CoQ9H2 could act as an antioxidant in human cells. The aim of this study is to assess whether exogenously added CoQ9 can protect human liver cells against injuries induced by a water-soluble radical initiator, 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH) and a lipid-soluble radical initiator, 2,2’-azobis(2,4-dimethylvaleronitrile) (AMVN). CoQ9-enriched cells were obtained by treatment of HepG2 cells with 10 µM CoQ9 liposomes for 24 h. CoQ9-enriched cells were exposed to 10 mM AAPH and 500 µM AMVN over 4 h and 24 h, respectively. The loss of viability after treatment with AAPH or AMVN was much less in CoQ9-enriched cells than in naive HepG2 cells. The decrease in glutathione and the increase in thiobarbituric acid-reactive substance after treatment with AAPH or AMVN were also suppressed in CoQ9-enriched cells. The incubation of CoQ9-enriched cells with AAPH or AMVN led to a decrease in cellular CoQ9H2 and reciprocal increase in cellular CoQ9 resulting from its antioxidant function. Taken together, it was demonstrated for the first time that exogenously added CoQ9 could prevent oxidative stress-mediated damage to human cells by virtue of its antioxidant activity.

Key Words: coenzyme Q9, free radical, human liver cells, antioxidant

Introduction

Coenzyme Q (CoQ) is a quinone derivative with an isoprenoid tail. CoQ homologs (CoQn) containing 1–13 isoprene units occur in nature, and in mammals the most common forms contain 9 (CoQ9) and 10 (CoQ10) isoprene units [1, 2]. CoQ9 is the predominant form in mouse and rat, and CoQ10 is predominant in rabbit, guinea pig, dog, pig and human [3]. As for the physiological significance of CoQn, its role as an electron-carrying component of mitochondrial respiratory chain is well established [4, 5]. A number of in vitro and in vivo studies have revealed antioxidant function of CoQ10H2, reduced form of CoQ10 [6–16]. However, it is not yet known whether CoQ9H2, a minor homolog in human, could play a role as an antioxidant in human cells.

2,2’-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2’-azobis(2,4-dimethylvaleronitrile) (AMVN) are a water-soluble and a lipid-soluble radical initiators, respectively which undergo spontaneous thermal decomposition to form carbon-centered radicals [17]. These radicals can initiate a chain reaction of lipid peroxidation to generate lipid peroxides in the presence of oxygen and polyunsaturated fatty acids. AAPH and AMVN have therefore been used to produce free-radical stresses.

We have reported that exogenously added CoQ10 can
protected rat hepatocytes against cell death by AAPH [18]. However, it remains to be elucidated whether exogenously added CoQ9 can prevent oxidative damage to human cells, which have a considerable amount of CoQ10 but trace of CoQ9. In the present study, we determined the sensitivities of CoQ9-enriched human hepatic cells to oxidative stress induced by AAPH and AMVN.

Materials and Methods

Chemicals
AAPH and dimyristoyl-phosphatidylcholine (DMPC) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). AMVN was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Chromatographically pure CoQ9 and CoQ10 were generous gifts from Eisai Co. (Tokyo, Japan). Fetal calf serum (FCS) was purchased from PAA Laboratories GmbH (Linz, Austria). Dulbecco's modified eagle medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals used were of analytical grade.

Cell culture and establishment of CoQ9-enriched human liver cells
HepG2 human hepatoma cell line was obtained from Japan Health Science Foundation (Osaka, Japan), and grown in DMEM supplemented with 10% heat-inactivated FCS, 2 mM glutamine and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified incubator with 5% CO2. Cells from passages 3–7 were used for the experiments. The cell density of 5 × 104 cells/dish was plated in a 24-well plate at a cell density of 5 × 104 cells/dish and incubated for 24 h at 37°C in an atmosphere of 5% CO2/95% air. After non-adherent cells were removed by washing with culture medium, attached cells were enriched with CoQ9 (see below) before induction of free radical injuries.

Small unilamellar liposomes containing CoQ9 were prepared by dissolving 17 mg of DMPC in 1 ml of ethanol containing CoQ9 (1 mg/ml) at a CoQ9/DMPC molar ratio of 1:20. The solution was evaporated under N2 stream. The resulting film was redissolved in 1.26 ml of phosphate-buffered saline (PBS) to obtain 1 mM CoQ9, vortexed vigorously, and sonicated for 3 min. HepG2 cells were incubated at 37°C with varying concentrations of CoQ9 liposomes for different time periods to make CoQ9-enriched human liver cells.

Experimental protocol
AAPH and AMVN were dissolved in PBS and dimethyl sulfoxide (DMSO), respectively. CoQ9-enriched HepG2 cells and naive HepG2 cells were exposed to 10 mM AAPH and 500 μM AMVN over 4 h and 24 h, respectively to induce oxidative stress. The cells were harvested with rubber policeman, washed with PBS twice, and resuspended in PBS.

Measurement of CoQ9 and CoQ10
The cell suspension was transferred to a centrifuge tube and centrifuged at 1,500 × g for 5 min. The resulting pellet was washed with ice-cold PBS and stored at −80°C until assayed. The determination of cellular oxidized and reduced CoQ homologs (CoQ9, CoQ10, CoQ9H2: and CoQ10H2) was carried out as described previously [3, 19, 20]. Briefly, the frozen cells were homogenized with ice-cold water (1.3–1.5 ml/ sample) under an atmosphere of nitrogen gas, and then oxidized and reduced forms of CoQ9 and CoQ10 were extracted with a mixture of 2 volumes of ethanol and 5 volumes of n-hexane to 1 volume of homogenate, and further extracted two times. The n-hexane layers were collected and evaporated under nitrogen gas. The residue was redissolved in ethanol, and subjected to high-performance liquid chromatography (HPLC).

All standards used for HPLC were pure samples of CoQ9 and CoQ10. The reduced form of CoQ9 and CoQ10 were prepared by reduction with sodium borohydride.

Cell Viability
The viability of cells was determined using a commercially available WST-8 assay kit (Seikagaku Biobusiness Co., Tokyo, Japan) according to the manufacture’s instruction as described previously [21]. The cells were seeded in a 24-well plate at a cell density of 5 × 104 cells/well and incubated for 24 h after treatment with CoQ9 liposomes, and then exposed to 10 mM AAPH and 500 μM AMVN over 4 h and 24 h, respectively. Cell viability was assessed by measurement of the absorbance at 492 nm in microplate reader (Biotrak II, Amersham) after incubation of cells in WST-8 solution for 1 h at 37°C.

Lipid peroxidation and glutathione assay
Cells were collected and washed with PBS twice. The cells were resuspended in PBS and lysed by freezing and thawing. Then, the cell lysates were homogenized in 0.05 M phosphate buffer (0.2 M Na2HPO4, 0.2 M NaH2PO4, 0.2 M Na-EDTA, pH 7.4) under N2 stream. Cellular lipid peroxidation was measured by a fluorometric reaction with thiobarbituric acid as previously described [15, 21, 22]. Lipid peroxide content was expressed as the amount of malondialdehyde (MDA) equivalents using tetraethoxy-propane as a standard.

Cellular reduced glutathione (GSH) contents were determined fluorometrically using Thio-Glo1® as previously described [23]. Briefly, cells treated with AAPH or AMVN were harvested with rubber policeman and washed twice with PBS. Thereafter cells were lysed by the same procedure as that in lipid peroxidation assay. Immediately after addi-
of 10 μM Thio-Glo1® to the cell lysates, fluorescence was measured in a CytoFluor II (Applied Biosystems, Foster city, CA) fluorescence microplate reader using excitation at 360 ± 40 nm and emission at 530 ± 25 nm.

**Protein assay**
Protein contents were determined by the method of Bradford [24] with bovine serum albumin as a standard.

**Statistical analysis**
Data are expressed as means ± standard error (SE). Changes in variables for different assays were analyzed by Student’s *t* test or one-way ANOVA. Differences were considered to be significant at *p*<0.05.

**Results**

**Enrichment of CoQ<sub>n</sub> in HepG2 cells with CoQ<sub>n</sub>-enriched liposomes**
To establish CoQ<sub>n</sub>-enriched HepG2 cells (referred to as CoQ<sub>n</sub>-enriched cells below), intracellular concentration of CoQ<sub>n</sub>, CoQ<sub>10</sub>, CoQ<sub>9</sub>H<sub>2</sub> and CoQ<sub>9</sub>H<sub>2</sub> was measured at 0, 4, 8, 12 or 24 h after treatment of naive HepG2 cells (referred to as control cells below) with 10 μM CoQ<sub>n</sub> liposomes (Fig. 1). Cellular CoQ<sub>n</sub> and CoQ<sub>n</sub>H<sub>2</sub> levels increased in a time-dependent manner and the levels reached a maximum 24 h after addition of CoQ<sub>n</sub> liposomes. In contrast, there were few changes in cellular contents of CoQ<sub>10</sub> and CoQ<sub>9</sub>H<sub>2</sub> except for the CoQ<sub>10</sub> content at 12 h.

We next examined whether CoQ<sub>n</sub> liposomes were really taken up by control cells, and whether they led to morphological changes of the cells. The cells were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with Oil-red-O and hematoxylin. Thereafter, the specimen was observed under a light microscope. Although CoQ<sub>n</sub> liposomes were time-dependently taken up by the cells as shown by the increase in intracellular lipid droplets, there were no morphological changes in the cytosol as well as the nucleus until 24 h after treatment with CoQ<sub>n</sub> liposomes (data not shown).

Given these results, we used the cells incubated with CoQ<sub>n</sub> liposomes for 24 h as CoQ<sub>n</sub>-enriched cells in the following experiments.

**Resistance of CoQ<sub>n</sub>-enriched cells to oxidative stress**
To determine the concentration of AAPH and AMVN required to induce cell death in control cells, we treated the cells with AAPH (1 to 10 mM) and AMVN (200 to 500 μM) for 4 h and 24 h, respectively. Both AAPH and AMVN induced the decrease in cell viability in a dose-dependent manner and the decrease reached a maximum at 10 mM and 500 μM, respectively (data not shown).

We next examined the time course of changes in cell viability in control cells and CoQ<sub>n</sub>-enriched cells following treatment with AAPH (10 mM) or AMVN (500 μM). The viability of control cells after AAPH treatment decreased in a time-dependent manner and was 24% of normal level after 4 h. On the other hand, the viability of CoQ<sub>n</sub>-enriched cells after AAPH was significantly higher than that of control cells during the incubation time period (Fig. 2A). Treatment of control cells and CoQ<sub>n</sub>-enriched cells with AMVN yielded similar results (Fig. 2B). These results suggested that CoQ<sub>n</sub>-enriched cells are strongly resistant to oxidative stress.

**Lipid peroxidation in CoQ<sub>n</sub>-enriched cells exposed to oxidative stress**
We examined the effect of CoQ<sub>n</sub> enrichment on lipid peroxidation in control cells after AAPH (10 mM) exposure (Fig. 3A). Thiobarbituric acid-reactive substance (TBARS) level in control cells increased in a time-dependent manner up to 2 h after AAPH, and thereafter reached the plateau. The TBARS level was 2.5-fold of normal level at 4 h after AAPH. In contrast, CoQ<sub>n</sub>-enriched cells kept normal TBARS levels during the incubation time period. TBARS level in control cells increased to 2.8-fold of normal level at 6 h after AMVN (500 μM) exposure, and thereafter reached the plateau (Fig. 3B). However, CoQ<sub>n</sub>-enriched cells kept almost normal TBARS levels during the incubation time period.

**Changes in GSH level in CoQ<sub>n</sub>-enriched cells exposed to oxidative stress**
Since GSH is the major intracellular reductant, we measured its concentration in control cells and CoQ<sub>n</sub>-enriched

![Fig. 1. Changes in contents of CoQ<sub>n</sub> and CoQ<sub>n</sub>H<sub>2</sub> in HepG2 cells after exposure to CoQ<sub>n</sub> liposomes. HepG2 cells were exposed to CoQ<sub>n</sub> liposomes (10 μM) and harvested after 4, 8, 12, or 24 h. Intracellular CoQ<sub>n</sub>, CoQ<sub>n</sub>H<sub>2</sub>, CoQ<sub>10</sub>, and CoQ<sub>9</sub>H<sub>2</sub> were measured by HPLC as described in Materials and Methods. Data points represent the means ± SE (n = 3). **p<0.01 vs CoQ<sub>n</sub>H<sub>2</sub> at 24 h, *p<0.01 vs CoQ<sub>n</sub> at 24 h.](image-url)
Prevents Human Liver Cell Injuries

Intracellular GSH in control cells significantly decreased by 20% and 40% at 1 h and 2 h, respectively after treatment with AAPH. The GSH level in CoQ9-enriched cells was significantly higher than that in control cells at 1 h after AAPH. When control cells were incubated with 500 μM AMVN over 24 h, cellular GSH content significantly decreased by 20% at 12 h after AMVN (Fig. 4B). At that time, the GSH content in CoQ9-enriched cells was significantly higher than that in control cells.

Changes in the concentration of CoQ9, CoQ10, CoQ9H2, and CoQ10H2 in CoQ9-enriched cells exposed to oxidative stress

To determine whether CoQ9H2 derived from intracellular conversion of CoQ9 taken up acts as an antioxidant, we measured the concentration of CoQ9, CoQ10, CoQ9H2, and CoQ10H2 in CoQ9-enriched cells over 4 h period of time after AAPH (10 mM) exposure (Fig. 5A). The concentration of CoQ9H2 in CoQ9-enriched cells decreased linearly from 1 to 4 h after addition of AAPH, whereas that of CoQ9 tended to increase reciprocally (Fig. 5A). In contrast, the concentra-
tion of CoQ\(_{9}\)H\(_2\) in CoQ\(_{9}\)-enriched cells did not decrease significantly during the incubation time period. When CoQ\(_{9}\)-enriched cells were incubated with 500 \(\mu\)M AMVN over 24 h, the concentration of cellular CoQ\(_{9}\)H\(_2\) decreased linearly from 6 to 24 h after AMVN exposure with a reciprocal increase in cellular CoQ\(_{9}\) (Fig. 5B). The concentration of CoQ\(_{10}\)H\(_2\), however, did not decrease significantly over 24 h following AMVN addition.

Discussion

The present study demonstrated for the first time that exogenously added CoQ\(_{9}\) was converted to CoQ\(_{9}\)H\(_2\) in human liver cells and subsequently acted as an antioxidant to suppress lipid peroxidation in the cells, resulting in protection of the cells against free radical-induced injuries.

Previous in vivo and in vitro studies showed that exogenously administered CoQ\(_{10}\) prevented a variety of injuries associated with oxidative stress [9–16]. In an experiment using endotoxicemic mice, administered CoQ\(_{10}\) was converted to CoQ\(_{10}\)H\(_2\) in the liver, suppressed hepatic lipid peroxidation, spared endogenous CoQ\(_{9}\)H\(_2\), and increased the survival rate of mice via its antioxidant function [12]. In this context, recent in vitro study has revealed that exogenously added CoQ\(_{9}\)H\(_2\) suppressed the secretion of pro-inflammatory cytokine TNF-\(\alpha\) as well as different
chemokines in LPS-stimulated human monocytic THP-1 cells [25]. Furthermore, CoQ10 pretreatment was shown to protect rat liver and kidney against injuries caused by ischemia-reperfusion [10, 11], and orthotopic liver transplantation [13], and also to protect canine heart against reperfusion injury following cold preservation [14]. We have shown that pretreatment with CoQ10 resulted in an increase in hepatic CoQ9H2 and a marked reduction in hepatic lipid peroxide content and plasma alanine aminotransferase activity without affecting hepatic GSH after acetaminophen injection [15]. In addition to the in vivo study, we have demonstrated that exogenously added CoQ10 was actually taken up by rat hepatocytes and reduced to CoQ9H2 in the cells to protect against AAPH-induced cell death [18].

As described above, exogenously added CoQ9 has to be reduced to CoQ9H2 in the cells to exhibit its antioxidant activity. Very important therefore is the presence of enzymes, which can reduce CoQ9 and/or CoQ9 semiquinone radicals. It is well known that mitochondrial CoQ9H2 is efficiently regenerated by the respiratory chain [26]. On the other hand, to date, the following enzymes are proposed as reduction enzymes for non-mitochondrial CoQ9: NAD(P)H oxidoreductase (DT diaphorase) [27]; NADPH-dependent CoQ reductase [28]. The DT diaphorase is unique since it can directly reduce CoQ9 by two-electron transfer without intermediate formation of semiquinone, but it is less efficient for longer isoprenoid side chain length, i.e. those with 9 or 10 isoprene units [29]. NADH-dependent CoQ reductase is also a two-electron reducing enzyme, located in the cytosol, and can reduce CoQ9 with a long isoprenoid side chain such as CoQ10 and CoQ10, to CoQ9H2 [28]. Furthermore, lipoamide dehydrogenase in the matrix surface of the mitochondria, thioredoxin reductase 1 in the cytosol and nuclei, glutathione reductase in the cytosol also could reduce CoQ10 to CoQ9H2: [28]. However, it remains unknown which reductase plays a primary role in the reduction of CoQ9.

We previously examined the difference in antioxidant activity between endogenous CoQ9H2 and CoQ9H2: using rat and guinea pig hepatocytes, which have CoQ9 and CoQ10, respectively as a primary CoQ homolog [3]. We found that endogenous CoQ9H2 constantly acted as a potent antioxidant in rat as well as guinea pig hepatocytes exposed to AAPH, whereas endogenous CoQ10H2 did so mainly in cells such as guinea pig hepatocytes, in which CoQ10 was the predominant CoQ homolog. Since endogenous CoQ9H2 is a potent lipid-soluble antioxidant, supplementation with CoQ9 is strongly suggested to be a promising antidote for oxidative stresses. Recent study based on the measurement of CoQ9 and CoQ9H2 has reported that exogenously added CoQ9 exhibits antioxidant activity in mice, which have CoQ9 as a major CoQ homolog [30].

In this study, we administered CoQ9 to the human liver cells in which CoQ10 is predominant, and determined the antioxidant activity of extrinsic CoQ9H2 in the cells exposed to a water-soluble or a lipid-soluble radical initiator. We used two kinds of azo compounds well known as radical initiators, i.e. AAPH and AMVN [17]. AAPH generates carbon radicals primarily and constantly through thermal decomposition in the aqueous phase, and the radicals thus formed react with oxygen rapidly to give peroxyl radicals. This azo compound is therefore a useful tool for studying the cell damage by extracellular free radicals. On the other hand, AMVN is a lipid-soluble and water-insoluble azo compound, and generates radicals initially within the lipid region of the membranes. Therefore, we can investigate the effect of extrinsic CoQ9H2 on two different kinds of free radical-mediated damages. CoQ9-enriched cells were much more resistant to extracellular free radical-mediated as well as intramembranous free radical-mediated oxidative stress compared with control cells. In this context, suppression of lipid peroxidation was well correlated with the linear decrease in abundant CoQ9H2 with reciprocal increase in CoQ9 in CoQ10-enriched cells, indicating that a loss of CoQ9H2 in the cells exposed to two different kinds of radical initiators was caused by its acting as an antioxidant. Moreover, CoQ9H2 content in CoQ9-enriched cells remained unchanged during the exposure to the radical initiators, suggesting that extrinsic CoQ9H2 could spare endogenous CoQ9H2: by its serving as a primary antioxidant.

While CoQ10 administration was reported to enhance endogenous CoQ9 by a mechanism that remains to be elucidated [31], it has been shown that dietary CoQ10 did not influence the endogenous biosynthesis of CoQ9 [32]. There was no difference in the CoQ9H2 levels between control cells and CoQ9-enriched cells. These discrepancies may be ascribed to the differences in the duration of CoQ administration and/or in CoQ homologs administered. However, the further studies should be required.

Extrinsic CoQ9H2 was the same potent antioxidant as endogenous CoQ9H2 even when it was administered to the cells that have CoQ10 as a predominant homolog. It is evident from the studies using rodent models that exogenously added CoQ10 is converted to CoQ9H2: and subsequently plays an antioxidant role in various tissues, which have CoQ9 as a predominant homolog. Therefore, CoQ9 supplementation for mammals that have mainly CoQ10 is potentially one of promising antioxidative procedures like CoQ10 supplementation. The previous study has revealed that CoQ10 is taken up from the intestine into the circulation with a low rate in rats, and only about 2–4% can be recovered [32]. Accordingly, in the case of CoQ9 supplementation in vivo, absorption efficiency of CoQ9 from the intestine in CoQ10-predominant mammals has to be elucidated. Furthermore, CoQ9 taken up by the cells has to be delivered to the cellular compartments appropriately. In this connection, saposin B, a CoQ10-binding and transfer protein,
has been shown to also bind to CoQ\(_9\) [33]. In conclusion, the present study has shown that exogenously added CoQ\(_9\) protects human cells against oxidative stress via its potent antioxidant activity.

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