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Protection by Exogenously Added Coenzyme Q_9 against Free Radical-Induced Injuries in Human Liver Cells

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Summary **Reduced coenzyme Q_{10} ($CoQ_{10}H_2$) is known as a potent antioxidant in biological systems. However, it is not yet known whether CoQ_9H_2 could act as an antioxidant in human cells. The aim of this study is to assess whether exogenously added CoQ_9 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). CoQ_9-enriched cells were obtained by treatment of HepG2 cells with 10 μM CoQ_9 liposomes for 24 h. CoQ_9-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 CoQ_9-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 CoQ_9-enriched cells. The incubation of CoQ_9-enriched cells with AAPH or AMVN led to a decrease in cellular CoQ_9H_2 and reciprocal increase in cellular CoQ_9 resulting from its antioxidant function. Taken together, it was demonstrated for the first time that exogenously added CoQ_9 could prevent oxidative stress-mediated damage to human cells by virtue of its antioxidant activity.**

Key Words**: coenzyme Q_9, free radical, human liver cells, antioxidant**

Introduction

Coenzyme Q (CoQ) is a quinone derivative with an isoprenoid tail. CoQ homologs (CoQ_n) containing 1–13 isoprene units occur in nature, and in mammals the most common forms contain 9 (CoQ_9) and 10 (CoQ_{10}) isoprene units [*1*, *2*]. CoQ_9 is the predominant form in mouse and rat, and CoQ_{10} is predominant in rabbit, guinea pig, dog, pig and human [*3*]. As for the physiological significance of CoQ_n, 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 $CoQ_{10}H_2$, reduced form of CoQ_{10} [*6–16*]. However, it is not yet known whether CoQ_9H_2, 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 CoQ_{10} can

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protected rat hepatocytes against cell death by AAPH [*18*]. However, it remains to be elucidated whether exogenously added CoQ_9 can prevent oxidative damage to human cells, which have a considerable amount of CoQ_{10} but trace of CoQ_9. In the present study, we determined the sensitivities of CoQ_9-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 CoQ_9 and CoQ_{10} 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 CoQ_9-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% CO_2. Cells from passages 3–7 were used for the experiments. The cells were seeded in 94-mm dishes at a cell density of 5×10^5 cells/dish and incubated for 24 h at 37°C in an atmosphere of 5% CO_2/95% air. After non-adherent cells were removed by washing with culture medium, attached cells were enriched with CoQ_9 (see below) before induction of free radical injuries.

Small unilamellar liposomes containing CoQ_9 were prepared by dissolving 17 mg of DMPC in 1 ml of ethanol containing CoQ_9 (1 mg/ml) at a CoQ_9/DMPC molar ratio of 1:20. The solution was evaporated under N_2 stream. The resulting film was redissolved in 1.26 ml of phosphate-buffered saline (PBS) to obtain 1 mM CoQ_9, vortexed vigorously, and sonicated for 3 min. HepG2 cells were incubated at 37°C with varying concentrations of CoQ_9 liposomes for different time periods to make CoQ_9-enriched human liver cells.

Experimental protocol

AAPH and AMVN were dissolved in PBS and dimethyl sulfoxide (DMSO), respectively. CoQ_9-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 CoQ_9 and CoQ_{10}

The cell suspension was transferred to a centrifuge tube and centrifuged at $1,500 \times g$ 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 (CoQ_9, CoQ_{10}, CoQ_9H_2 and $CoQ_{10}H_2$) 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 CoQ_9 and CoQ_{10} 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 CoQ_9 and CoQ_{10}. The reduced form of CoQ_9 and CoQ_{10} 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 5×10^4 cells/well and incubated for 24 h after treatment with CoQ_9 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 a 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 NaH_2PO_4, 0.2 M Na_2HPO_4, 0.2 M Na-EDTA, pH 7.4) under N_2 stream. Cellular lipid peroxidation was measured by a fuluorometric reaction with thiobarbituric acid as previously described [*15*, *21*, *22*]. Lipid peroxide content was expressed as the amount of malondialdehyde (MDA) equivalents using tetraethoxypropane 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-

tion 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_9 in HepG2 cells with CoQ_9 liposomes

To establish CoQ_9-enriched HepG2 cells (referred to as CoQ_9-enriched cells below), intracellular concentration of CoQ_9, CoQ_{10}, CoQ_9H_2 and $CoQ_{10}H_2$ 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_9 liposomes (Fig. 1). Cellular CoQ_9 and CoQ_9H_2 levels increased in a time-dependent manner and the levels reached a maximum 24 h after addition of CoQ_9 liposomes. In contrast, there were few changes in cellular contents of CoQ_{10} and $CoQ_{10}H_2$ except for the CoQ_{10} content at 12 h.

We next examined whether CoQ_9 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_9 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_9 liposomes (data not shown).

Given these results, we used the cells incubated with CoQ_9 liposomes for 24 h as CoQ_9-enriched cells in the following experiments.

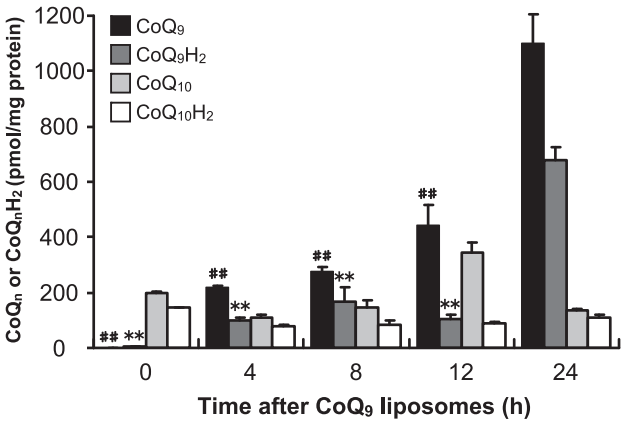

Fig. 1. Changes in contents of CoQ_n and CoQ_nH_2 in HepG2 cells after exposure to CoQ_9 liposomes. HepG2 cells were exposed to CoQ_9 liposomes (10 μM) and harvested after 4, 8, 12, or 24 h. Intracellular CoQ_9, CoQ_9H_2, CoQ_{10}, and $CoQ_{10}H_2$ were measured by HPLC as described in Materials and Methods. Data points represent the means ± SE ($n = 3$). $^{**}p<0.01$ vs CoQ_9H_2 at 24 h, $^{\#\#}p<0.01$ vs CoQ_9 at 24 h.

Resistance of CoQ_9-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_9-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_9-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_9-enriched cells with AMVN yielded similar results (Fig. 2B). These results suggested that CoQ_9-enriched cells are strongly resistant to oxidative stress.

Lipid peroxidation in CoQ_9-enriched cells exposed to oxidative stress

We examined the effect of CoQ_9 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_9-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_9-enriched cells kept almost normal TBARS levels during the incubation time period.

Changes in GSH level in CoQ_9-enriched cells exposed to oxidative stress

Since GSH is the major intracellular reductant, we measured its concentration in control cells and CoQ_9-enriched

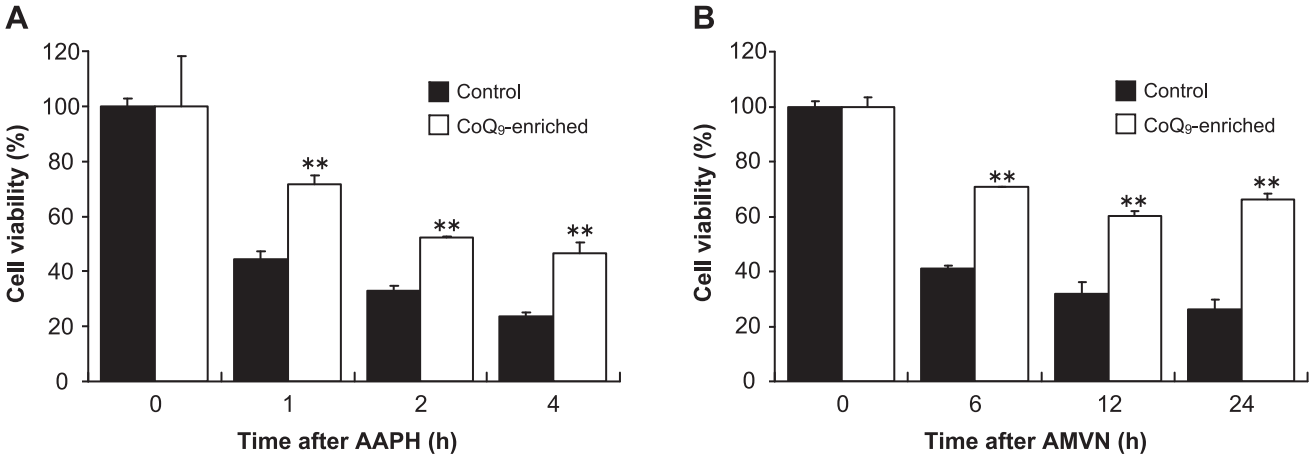

Fig. 2. Changes in cell viability in control cells and CoQ_9-enriched cells following treatment with AAPH (A) or AMVN (B). HepG2 cells were incubated for 24 h in the absence (for control cells) or presence (for CoQ_9-enriched cells) of CoQ_9 liposomes (10 μM), and then exposed to 10 mM AAPH and 500 μM AMVN over 4 h and 24 h, respectively. The cell viability was analyzed using WST-8 assay kit. Data points represent the means ± SE ($n = 3$). $^{**}p<0.01$ vs control cells at the same incubation time.

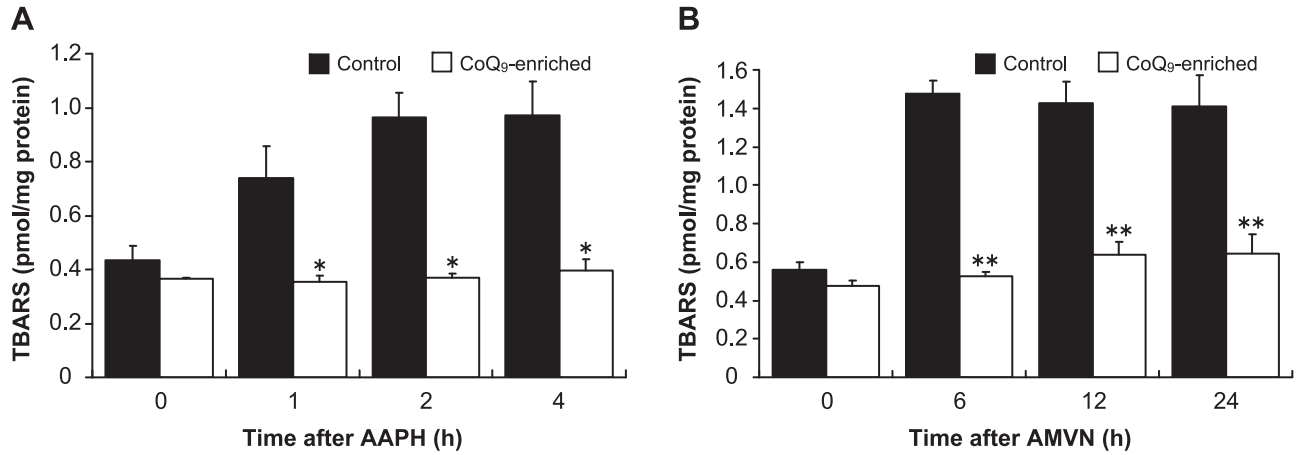

Fig. 3. Changes in lipid peroxidation in control cells and CoQ_9-enriched cells following treatment with AAPH (A) or AMVN (B). Control and CoQ_9-enriched cells were incubated as described in the legend to Fig. 2. Cellular TBARS content was measured as described in Materials and Methods. Data points represent the means ± SE ($n = 3$). $^{*}p<0.05$, $^{**}p<0.01$ vs control cells at the same incubation time.

cells over 4 h period of time after AAPH (10 mM) exposure (Fig. 4A). 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 CoQ_9-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 CoQ_9-enriched cells was significantly higher than that in control cells.

Changes in the concentration of CoQ_9, CoQ_{10}, CoQ_9H_2 and $CoQ_{10}H_2$ in CoQ_9-enriched cells exposed to oxidative stress

To determine whether CoQ_9H_2 derived from intracellular conversion of CoQ_9 taken up acts as an antioxidant, we measured the concentration of CoQ_9, CoQ_{10}, CoQ_9H_2 and $CoQ_{10}H_2$ in CoQ_9-enriched cells over 4 h period of time after AAPH (10 mM) exposure (Fig. 5A). The concentration of CoQ_9H_2 in CoQ_9-enriched cells decreased linearly from 1 to 4 h after addition of AAPH, whereas that of CoQ_9 tended to increase reciprocally (Fig. 5A). In contrast, the concentra-

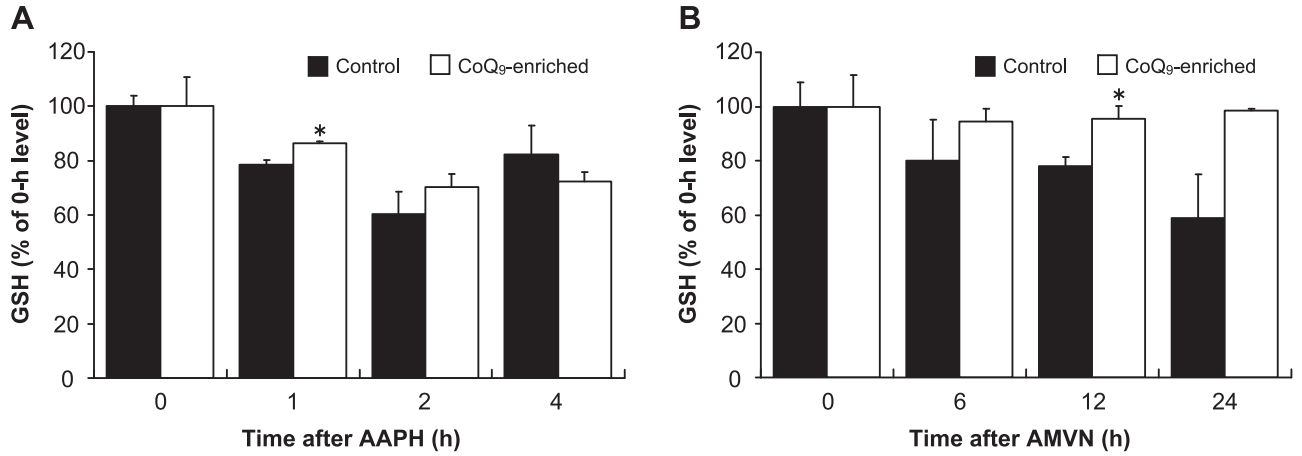

Fig. 4. Changes in GSH in control cells and CoQ_9-enriched cells following treatment with AAPH (A) or AMVN (B). Control and CoQ_9-enriched cells were incubated as described in the legend to Fig. 2. Cellular GSH content was measured as described in Materials and Methods. Data points represent the means ± SE (n = 3). *p<0.05 vs control cells at the same incubation time.

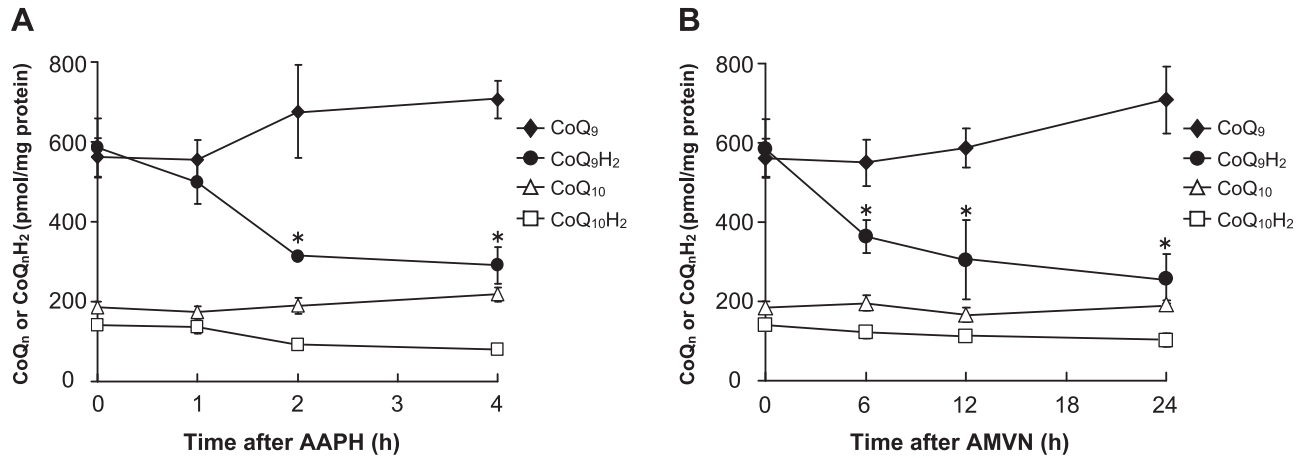

Fig. 5. Changes in contents of CoQ_n and CoQ_nH_2 in control cells and CoQ_9-enriched cells following treatment with AAPH (A) or AMVN (B). Control and CoQ_9-enriched cells were incubated as described in the legend to Fig. 2. The concentrations of CoQ_n and CoQ_nH_2 in the cells were determined by HPLC as described in Materials and Methods. Data points represent the means ± SE (n = 3). *p<0.05 vs 0-h incubation time.

tion of $CoQ_{10}H_2$ in CoQ_9-enriched cells did not decrease significantly during the incubation time period. When CoQ_9-enriched cells were incubated with 500 μM AMVN over 24 h, the concentration of cellular CoQ_9H_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_9H_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_9H_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 $CoQ_{10}H_2$ suppressed the secretion of pro-inflammatory cytokine TNF-α as well as different

chemokines in LPS-stimulated human monocytic THP-1 cells [*25*]. Furthermore, CoQ_{10} 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 CoQ_{10} resulted in an increase in hepatic $CoQ_{10}H_2$ 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 CoQ_{10} was actually taken up by rat hepatocytes and reduced to $CoQ_{10}H_2$ in the cells to protect against AAPH-induced cell death [*18*].

As described above, exogenously added CoQ_n has to be reduced to CoQ_nH_2 in the cells to exhibit its antioxidant activity. Very important therefore is the presence of enzymes, which can reduce CoQ_n and/or CoQ_n semiquinone radicals. It is well known that mitochondrial CoQ_nH_2 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 CoQ_n: NAD(P)H oxidoreductase (DT diaphorase) [*27*]; NADPH-dependent CoQ reductase [*28*]. The DT diaphorase is unique since it can directly reduce CoQ_n 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 CoQ_n with a long isoprenoid side chain such as CoQ_9 and CoQ_{10}, to CoQ_nH_2 [*28*]. Furthermore, lipoamide dehydrogenase in the matrix surface of the mitochondria, thioredoxine reductase 1 in the cytosol and nuclei, glutathione reductase in the cytosol also could reduce CoQ_{10} to $CoQ_{10}H_2$ [*28*]. However, it remains unknown which reductase plays a primary role in the reduction of CoQ_n.

We previously examined the difference in antioxidant activity between endogenous CoQ_9H_2 and $CoQ_{10}H_2$ using rat and guinea pig hepatocytes, which have CoQ_9 and CoQ_{10}, respectively as a primary CoQ homolog [*3*]. We found that endogenous CoQ_9H_2 constantly acted as a potent antioxidant in rat as well as guinea pig hepatocytes exposed to AAPH, whereas endogenous $CoQ_{10}H_2$ did so mainly in cells such as guinea pig hepatocytes, in which CoQ_{10} was the predominant CoQ homolog. Since endogenous CoQ_9H_2 is a potent lipid-soluble antioxidant, supplementation with CoQ_9 is strongly suggested to be a promising antidote for oxidative stresses. Recent study based on the measurement of CoQ_9 and CoQ_9H_2 has reported that exogenously added CoQ_9 exhibits antioxidant activity in mice, which have CoQ_9 as a major CoQ homolog [*30*].

In this study, we administered CoQ_9 to the human liver cells in which CoQ_{10} is predominant, and determined the antioxidant activity of extrinsic CoQ_9H_2 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 CoQ_9H_2 on two different kinds of free radical-mediated damages. CoQ_9-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 CoQ_9H_2 with reciprocal increase in CoQ_9 in CoQ_9-enriched cells, indicating that a loss of CoQ_9H_2 in the cells exposed to two different kinds of radical initiators was caused by its acting as an antioxidant. Moreover, $CoQ_{10}H_2$ content in CoQ_9-enriched cells remained unchanged during the exposure to the radical initiators, suggesting that extrinsic CoQ_9H_2 could spare endogenous $CoQ_{10}H_2$ by its serving as a primary antioxidant.

While CoQ_{10} administration was reported to enhance endogenous CoQ_9 by a mechanism that remains to be elucidated [*31*], it has been shown that dietary CoQ_{10} did not influence the endogenous biosynthesis of CoQ_9 [*32*]. There was no difference in the CoQ_{10} levels between control cells and CoQ_9-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 CoQ_9H_2 was the same potent antioxidant as endogenous CoQ_9H_2 even when it was administered to the cells that have CoQ_{10} as a predominant homolog. It is evident from the studies using rodent models that exogenously added CoQ_{10} is converted to $CoQ_{10}H_2$ and subsequently plays an antioxidant role in various tissues, which have CoQ_9 as a predominant homolog. Therefore, CoQ_9 supplementation for mammals that have mainly CoQ_{10} is potentially one of promising antioxidative procedures like CoQ_{10} supplementation. The previous study has revealed that CoQ_{10} 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 CoQ_9 supplementation *in vivo*, absorption efficiency of CoQ_9 from the intestine in CoQ_{10}-predominant mammals has to be elucidated. Furthermore, CoQ_9 taken up by the cells has to be delivered to the cellular compartments appropriately. In this connection, saposin B, a CoQ_{10}-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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