Original Article

Resveratrol is converted to the ring portion of coenzyme Q10 and raises intracellular coenzyme Q10 levels in HepG2 cell

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(Received 25 April, 2024; Accepted 18 May, 2024; Released online in J-STAGE as advance publication 28 May, 2024)

Coenzyme Q10 is an essential lipid in the mitochondrial electron transport system and an important antioxidant. It declines with age and in various diseases, there is a need for a method to compensate for the decrease in coenzyme Q10. Resveratrol, a well-known anti-aging compound, has been shown to undergo metabolism to coenzyme Q10's benzene ring moiety in cells. However, administration of resveratrol did not alter or only slightly increased total intracellular coenzyme Q10 levels in many cell types. Synthesis of coenzyme Q10 requires not only the benzene ring moiety but also the side chain moiety. Biosynthesis of the side chain portion of coenzyme Q10 is mediated by the mevalonic acid pathway. Here, we explore the impact of resveratrol on coenzyme Q10 levels in HepG2 cells, which possess a robust mevalonic acid pathway. As a results, intracellular coenzyme Q10 levels were increased by resveratrol administration. Analysis using $^{13}C_6$-resveratrol revealed that the benzene ring portion of resveratrol was converted to coenzyme Q10. Inhibition of the mevalonic acid pathway prevented the increase in coenzyme Q10 levels induced by resveratrol administration. These results indicate that resveratrol may be beneficial as a coenzyme Q10-enhancing reagent in cells with a well-developed mevalonic acid pathway.

***Key Words*: coenzyme Q10, resveratrol, mevalonic acid pathway, HepG2**

Coenzyme Q10 (CoQ10) plays a vital role as a lipid component in the mitochondrial electron transport chain, while also serving as an important lipid-soluble antioxidant.[1,2] Its levels within cells and organs have been reported to decline with aging and in various diseases, such as post-myocardial infarction and fibromyalgia.[3–6] Consequently, CoQ10 is often recommended as a supplement. However, studies have indicated that oral administration of CoQ10 may not effectively elevate cellular CoQ10 levels in certain organs, such as the brain, due to poor lipid uptake.[7,8] Hence, there is a pressing need to explore innovative methods for augmenting cellular CoQ10 levels beyond oral supplementation.

Resveratrol (RSV), found in a wide range of plants, has garnered attention as an anti-aging compound.[9] It has been associated with a variety of health-promoting effects and has demonstrated the potential to mitigate various diseases, including diabetes, neurodegeneration, and aging.[10–12] While the precise mechanisms underlying these functions are still debated, they may be attributed in part to their antioxidant properties and its ability to modulate mitochondrial functions.[13,14]

The biosynthesis of CoQ10 involves the formation of both the benzene ring and the isoprenyl side chain.[15] However, several questions still surround the mechanisms of CoQ10 biosynthesis, particularly regarding the production of hydroxybenzoic acid, considered a precursor to the benzene ring portion of CoQ10.[16] Notably, Xie *et al.*[17] previously reported that RSV can serve as a precursor for the aromatic ring in coenzyme Q biosynthesis. Their research employed aromatic $^{13}C_6$-ring-labeled compounds, specifically $^{13}C_6$-resveratrol, to investigate RSV's role as an aromatic ring precursor in CoQ10 biosynthesis in various organisms, including *Escherichia coli*, *Saccharomyces cerevisiae* (*S. cerevisiae*), and human and mouse cells. Their findings indicated that these organisms, when cultured with $^{13}C_6$-RSV, could synthesize $^{13}C_6$-CoQ10, implying that RSV can be converted to CoQ10 in human and mouse cells. Nevertheless, it should be noted that although $^{13}C_6$-CoQ10 was detected in *S. cerevisiae*, U251 cells, 3T3 cells, and 293T cells, total CoQ10 levels did not increase with RSV treatment in these cases.

In light of the fact that CoQ10 biosynthesis involves both the benzene ring and the isoprenyl side chain, and RSV serves as an aromatic ring precursor of CoQ10, we propose that augmenting intracellular CoQ10 content may be achievable by administering RSV to cells with a robust isoprenyl side chain biosynthesis rate and an ample supply of the side chain.[18] Importantly, the isoprenyl side chain moiety is synthesized from acetyl CoA via the mevalonate pathway, a pathway predominantly active in the liver. Therefore, this study aims to elucidate the effects of RSV administration on HepG2 cells that mevalonate pathway is activated.

Materials and Methods

Cell culture and treatment. The cultivation of HepG2 cells followed the method previously reported.[19] In brief, HepG2 cells were cultured in DMEM (High Glucose) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin, at 37°C under 5% CO_2 saturation. RSV was dissolved in ethanol to achieve concentrations of 10, 25, and 50 μM, and administered to the cells. Simvastatin was dissolved in DMSO to achieve a concentration of 10 μM and added to the cells. As previously reported, 4-nitrobenzoate (4-NB) was dissolved in DMSO to achieve a concentration of 5 mM and administered to the cells.[19] 4-hydroxybenzoate (4-HB) was also dissolved in DMSO to achieve a concentration of 25 μM. Each control group received an equivalent amount of ethanol and/or DMSO. Following administration, the cells were cultured for 48 h before measurement, if not stated otherwise.

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doi: 10.3164/jcbn.24-70

Isolation of mitochondria. Mitochondrial isolation was performed according to Wallace's method with minor modification.[20] In brief, cells were collected in isolation buffer [210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 0.5% BSA (fatty acid-free), 5 mM HEPES, pH 7.2]. Subsequently, the cell suspension was homogenized using a glass homogenizer and centrifuged at 1,000 × *g* for 10 min. The supernatant was collected and further centrifuged at 8,500 × *g* for 15 min at 4°C to pellet the mitochondrial fraction.

CoQ10 and free cholesterol (FC) analysis. CoQ10 and FC were analyzed by using HPLC, following a previously reported method with slight adjustments.[19] In brief, cells collected in 2-propanol (IPA), or the mitochondrial fraction dissolved in IPA, were centrifuged, and the resulting supernatant was injected into the HPLC system. Two separation columns (Ascentis® C8, 5 µm, 250 mm × 4.6 mm i.d. and Supelcosil™ LC-18, 3 µm, 5 cm × 4.6 mm i.d.; Supelco Japan, Tokyo, Japan) along with a reduction column (RC-10, 15 mm × 4 mm i.d.; IRICA, Kyoto, Japan) were utilized. The mobile phase for the separation columns consisted of 50 mM sodium $NaClO_4$ in methanol/IPA (85/15, v/v) and was delivered at a flow rate of 0.8 ml/min. The columns were maintained at 25°C. The measured amounts of CoQ10 and FC were normalized for total protein mass measured by using the BCA method.[21]

HPLC equipped with an optimized time-of-flight mass spectrometry (LC/TOF-MS) analysis. HepG2 cells were seeded at a density of 6.25×10^4 cells/ml with treated for 10 µM Resveratrol-(4-hydroxyphenyl-$^{13}C_6$) (#711004; Sigma-Aldrich Japan, Tokyo, Japan)-containing medium (dissolved in 99.5% EtOH). The cells were treated with RSV-containing medium for an additional 48-h incubation period. After washing the cells twice with PBS, cells were centrifuged at 3,000 rpm for 10 min at 4°C, and the supernatant was removed. The pelleted samples were then suspended in 5 ml of hexane and 2 ml of methanol and vigorously agitated, followed by centrifugation at 3,500 rpm for 10 min at 4°C. The upper hexane layer was collected after centrifugation. The collected hexane was evaporated to dryness, and the residue was suspended in 450 µl of IPA for measurement. To obtain mass-to-charge ratio (*m*/*z*) value of CoQ10, an LC/TOF-MS (JMS-T100LC; JEOL Ltd., Tokyo, Japan) was used. Methanol as a mobile phase was delivered at 1.0 ml/min. Separation was performed with a C8 column (5 µm, 4.6 mm × 250 mm, Supelco; Sigma-Aldrich Japan K.K., Tokyo, Japan). Positive ionization was carried out at an ionization potential 2,500 V. Applied voltage to the ring lens, outer orifice, inner orifice, and ion guide were 10 V, 100 V, 10 V, and 1,500 V, respectively.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). The mRNA expression levels in HepG2 cells were assessed through reverse transcription PCR, following a previously described protocol.[19] In brief, total RNA was extracted using TRizol reagent (Thermo Fisher Scientific, Waltham, MA). RNA quality and concentration were evaluated using an Ultrospec 2100 pro spectrophotometer (Biochrom, Cambridge, UK). Reverse transcription was carried out to synthesize cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Venlo, The Netherlands). The expression levels of genes were quantified by qPCR, with detected gene and primer sequences listed in Table 1 and previously reported.[22] Housekeeping genes were selected *ACTB*. The qPCR was performed using a QuantStudio® instrument (Thermo Fisher Scientific), with cycling conditions of 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 72°C for 30 s, and a final extension step at 60°C for 30 s. Changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method.[23]

Mitochondrial DNA (mtDNA) isolation and quantification by quantitative PCR (qPCR). The quantification of mtDNA was conducted based on previously reported methods.[22] mtDNA isolation from HepG2 cells was performed using the NucleoSpin® Tissue kit (Takara Bio Inc., Shiga, Japan). For quantification, two nuclear DNA (nDNA) genes serpin family A member 1 (SERPINA1) and solute carrier organic anion transporter family member 2B1 (SLCO2B1) and two mtDNA genes mitochondrially encoded NADH dehydrogenase 1 (ND-1) and mitochondrially encoded NADH dehydrogenase 5 (ND-5) were utilized. The primer sequences used for qPCR are reported previously.[22] The qPCR procedure utilized PowerTrack™ SYBR Green Master Mix for qPCR on the QuantStudio® platform following the recommended protocol (Thermo Fisher Scientific). The change in mtDNA levels was determined using the mtDNA_Copy_Number_Calculation method (#7246, Human Mitochondrial DNA Monitoring Primer Set; Takara Bio Inc.).

Table 1. List of forward (F) and reverse (R) primer sequences used in qPCR assays

PDSS1	F	5'-TCTGTTCTAGGATGTCCCGA-3'
	R	5'-CAAACAGGACAGGACCAGTG-3'
PDSS2	F	5'-CCCACTGGAATCAGGTAGTG-3'
	R	5'-TCCAGCTGTCATGTACAAGC-3'
Coq2	F	5'-CGTTACTTGGATGGTCTGCT-3'
	R	5'-CCAGCATTGCAACACTGAAG-3'
Coq3	F	5'-TACTTCCCAAACCACTGTCG-3'
	R	5'-CACAGCCAACGTCAAGAATC-3'
Coq4	F	5'-CGTGAAACCGAAAGTCTGTC-3'
	R	5'-GCATTTACTATGTGCCAGGC-3'
Coq5	F	5'-TGAAGAAGATTCCTTGGGCG-3'
	R	5'-TGACATTCCGGATCCCAAAG-3'
Coq6	F	5'-GAGATTTCTTCCCTCTGGGC-3'
	R	5'-GCACTTGGAAACTCTCAAGC-3'
Coq7	F	5'-TTGTGACCTCCATCCCAAAG-3'
	R	5'-GTTTGCTCCATATTCGCCTG-3'
Coq8	F	5'-CGCTGGTCTGTAGAACTCTC-3'
	R	5'-GCCTCTAGCATCTCAGGAAC-3'
Coq9	F	5'-TACCACTTCGTGTCCCAAAG-3'
	R	5'-GCCACTGAAACAGAAACTGG-3'

Statistical analysis. Statistical analysis was conducted using Student's *t* test and one-way analysis of variances (ANOVA). BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) was utilized for statistical analysis. Group differences were considered statistically significant at the following levels: $*p \leq 0.05$, $**p \leq 0.01$, and $***p \leq 0.001$.

Results

Effect of RSV treatment on cellular levels of CoQ10 in HepG2 cell. As shown in Fig. 1A, the administration of 10 µM RSV to HepG2 cells did not significantly impact the protein levels in the culture dish. This suggests that the cell growth rate and cell viability were not significantly affected by the 10 µM RSV administration. However, the administration of 25 µM RSV slightly reduced the protein content in the culture dish, while 50 µM RSV administration substantially decreased protein content in the well, indicating that 50 µM RSV exhibited toxicity to HepG2 cells.

Figure 1B demonstrates that RSV administration led to a dose-dependent increase in cellular CoQ10 level. Notably, the administration of RSV significantly elevated cellular CoQ10 levels by approximately twofold. Administration of RSV elevated both reduced and oxidized forms of CoQ10. Given that cholesterol is also synthesized from acetyl CoA via the mevalonate pathway, similar to CoQ10, we assessed the impact of RSV on cellular cholesterol level. As shown in Fig. 1C, cellular cholesterol levels standardized by protein content remained unchanged following RSV administration.

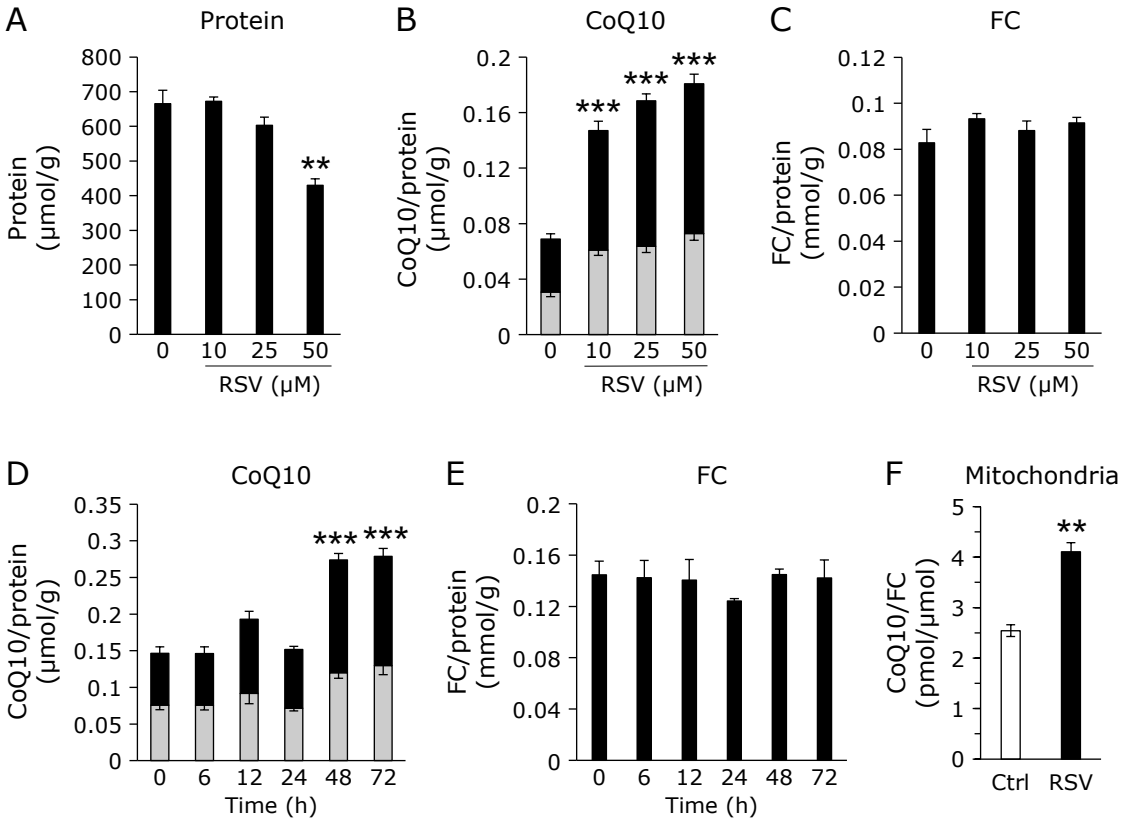

Fig. 1. RSV treatment increase cellular CoQ10 levels in HepG2 cells. (A–C) Effects of RSV (0, 10, 25, 50 μM) treatment on HepG2 cells: (A) Total protein levels, (B) Total CoQ10 levels corrected by total protein levels, and (C) FC levels corrected by total protein levels. Total CoQ10 levels are presented for both oxidized CoQ10 (gray) and reduced CoQ10 (black). (D, E) Time course of Total CoQ10 levels (D) and FC levels (E) following treatment with 25 μM RSV, corrected by total protein levels. Total CoQ10 levels are presented for both oxidized CoQ10 (gray) and reduced CoQ10 (black). (F) Mitochondrial CoQ10 levels corrected by FC with and without 25 μM RSV treatment. Statistical analysis was conducted by *t* test or ANOVA. $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$.

We then examined the time course of cellular CoQ10 levels following the administration of 25 μM RSV. In light of the considerable perturbation observed with the administration of 50 μM RSV, subsequent experiments employed 25 μM RSV. Figure 1D illustrates that cellular CoQ10 levels increased significantly after 48 and 72 h post-RSV administration. Figure 1E shows cellular FC level after administration of RSV. FC level did not change.

Since CoQ10 is an important lipid in mitochondrial electron transport chain, we next assess the effect of administration of RSV on mitochondrial CoQ10 level. As shown in Fig. 1F, level of CoQ10 in mitochondria also increased by the administration of RSV.

Effect of RSV administration on cellular CoQ10 synthesis genes expressions. Figure 2A illustrates the scheme how CoQ10 is synthesized *in vivo*. We first elucidate the level of mRNA expressions profiles of various CoQ10 synthesis genes (Fig. 2B). mRNA expression levels of PDSS2, coq5, and coq7 were increased at 12 h after RSV treatment and those of coq3, coq6, coq8, and coq9 were decreased at 24 h and 48 h and thereafter. However, these changes were not particularly substantial. These data suggest that the elevation of CoQ10 levels due to RSV treatment may not be primarily attributed to the upregulation or enhancement of CoQ10 synthesis genes.

We previously reported that prosaposin (PSAP) is a CoQ10 binding-protein.[24,25] We also reported that upregulation of PSAP increased and downregulation of PSAP decreased cellular CoQ10 level in HepG2 cells.[26,27] Therefore, we next measured the mRNA expression level of PSAP. As shown in Fig. 3A, mRNA expression level of PSAP did not change.

Effect of RSV on the expressions of mitochondria-related genes. As the final step of CoQ10 biosynthesis occurs within the mitochondria, we evaluated the expression levels of various mitochondria-related genes with and without RSV treatment. We examined the levels of SIRT1, PGC-1alpha, NRF1, and NRF2 (Fig. 3). SIRT1, PGC-1alpha, and NRF1 exhibited decrease was modest. In contrast, NRF2 expression showed a slight increase. Additionally, we analyzed mitochondrial DNA copy number (mtDNAcn) following RSV administration, as shown in Fig. 3B, and found no significant differences. Therefore, the mechanism by which administered RSV increase intercellular CoQ10 is presumably not due to upregulation of various CoQ10 synthesis genes nor changes in mitochondrial mass itself, but because they are substrate for the benzene ring portion of CoQ10.

Effect of $^{13}C_6$-RSV treatment on cellular levels of $^{13}C_6$-CoQ10 in HepG2 cell. Previously, Xie *et al.*[17] reported that RSV serves as a ring precursor for coenzyme Q biosynthesis. In our present study, we observed an increase in intracellular CoQ10 in HepG2 cells following RSV administration. We aimed to investigate whether RSV was incorporated into CoQ10 as part of this increase, utilizing RSV labeled with $^{13}C_6$ in benzene rings for analysis. As shown in Fig. 4, CoQ10 generated in response to this treatment was analyzed via LC-MS, confirming the presence of a peak at position +6. This indicates that the administered RSV was taken up by the cells and utilized as a component of CoQ10.

Effect of simvastatin on the CoQ10 level increase by RSV administration. These results shown above suggest that administered RSV integrate into the benzene ring part of CoQ10.

doi: 10.3164/jcbn.24-70

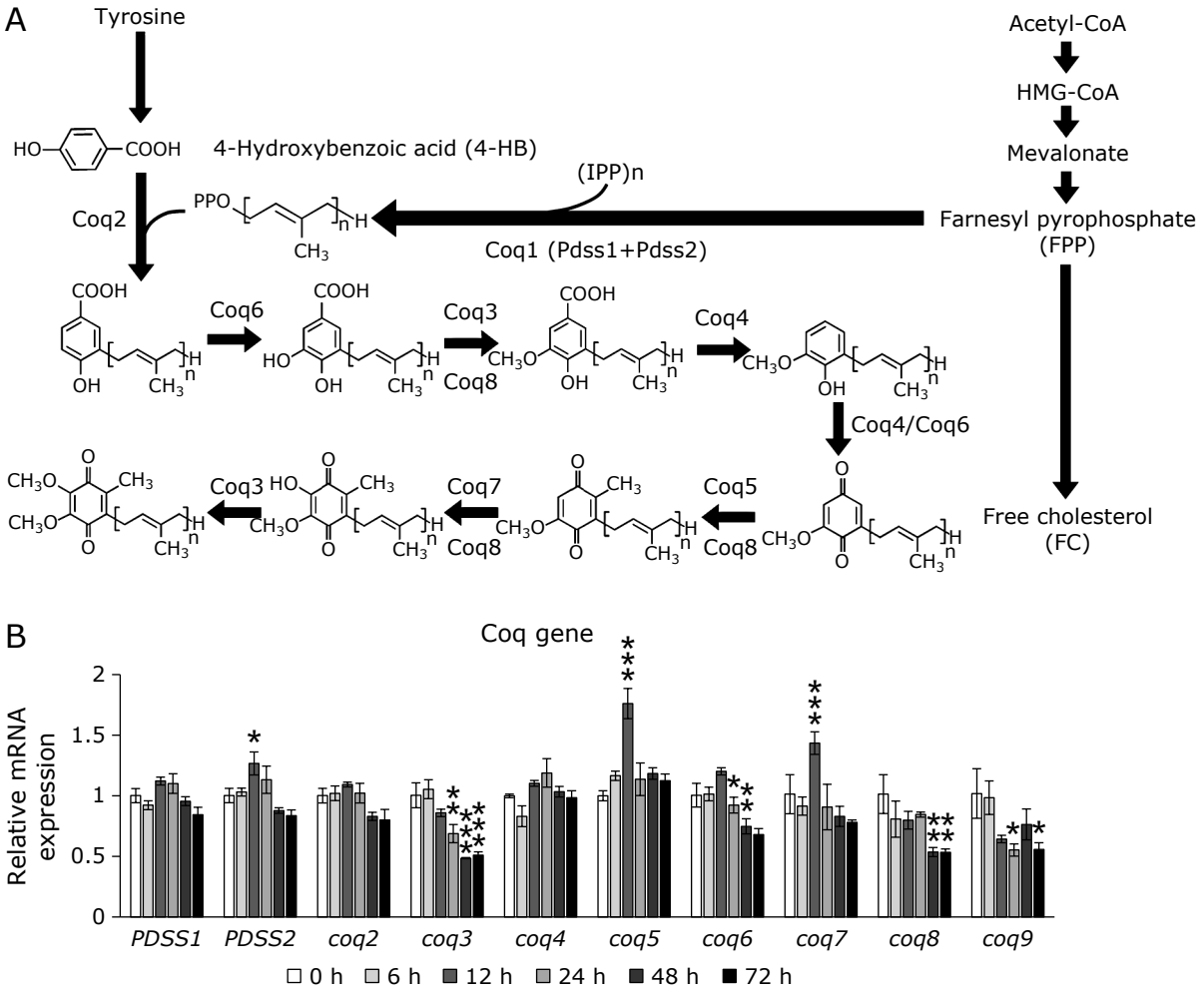

Fig. 2. Outline of the CoQ synthesis pathway and expression levels of each gene. (A) Illustrated schemes of CoQ and cholesterol biosynthetic pathways. (B) Time course of gene expression levels of CoQ10 synthesis enzymes genes following treatment with 25 μM RSV. Each gene was corrected by *ACTB*. The mean expression level was normalized to that of each control ($2^{-\Delta\Delta Ct}$ method). Statistical analysis was conducted by ANOVA. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

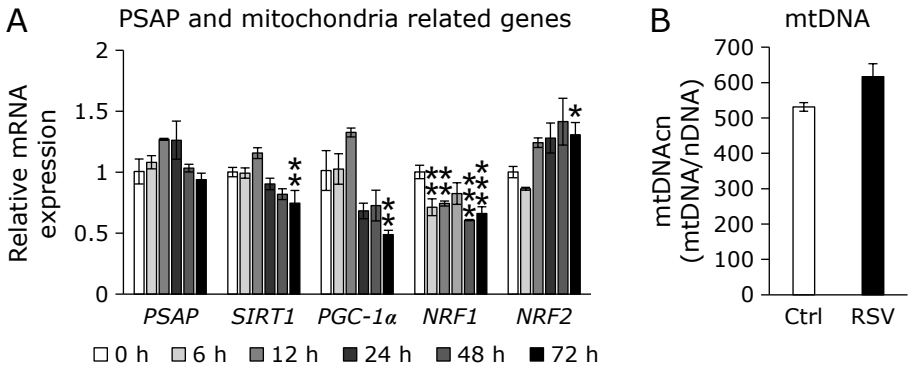

Fig. 3. Time course of gene expression levels of PSAP and mitochondrial biogenesis-related genes following RSV treatment. (A) Time course of gene expression levels of PSAP and mitochondrial biogenesis-related genes following treatment with 25 μM RSV. Expression levels of each gene was corrected by *ACTB*. The mean expression level was normalized to that of each control ($2^{-\Delta\Delta Ct}$ method). (B) Effect of RSV treatment on mtDNAcn. Cells were treated with 25 μM RSV for 48 h. Statistical analysis was conducted by *t* test or ANOVA. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

Thus, we next elucidate whether the administration of simvastatin, an inhibitor of mevalonate pathway, would alter the effect of RSV administration.[28] As shown in Fig. 5A, simvastatin administration inhibited the increase in CoQ10 induced by RSV. Additionally, administration of simvastatin slightly reduced cellular FC level, while, consistent with previous experiments, RSV administration had no effect on cholesterol levels (Fig. 5B). Subsequently, we investigated the effect of administering 4-HB

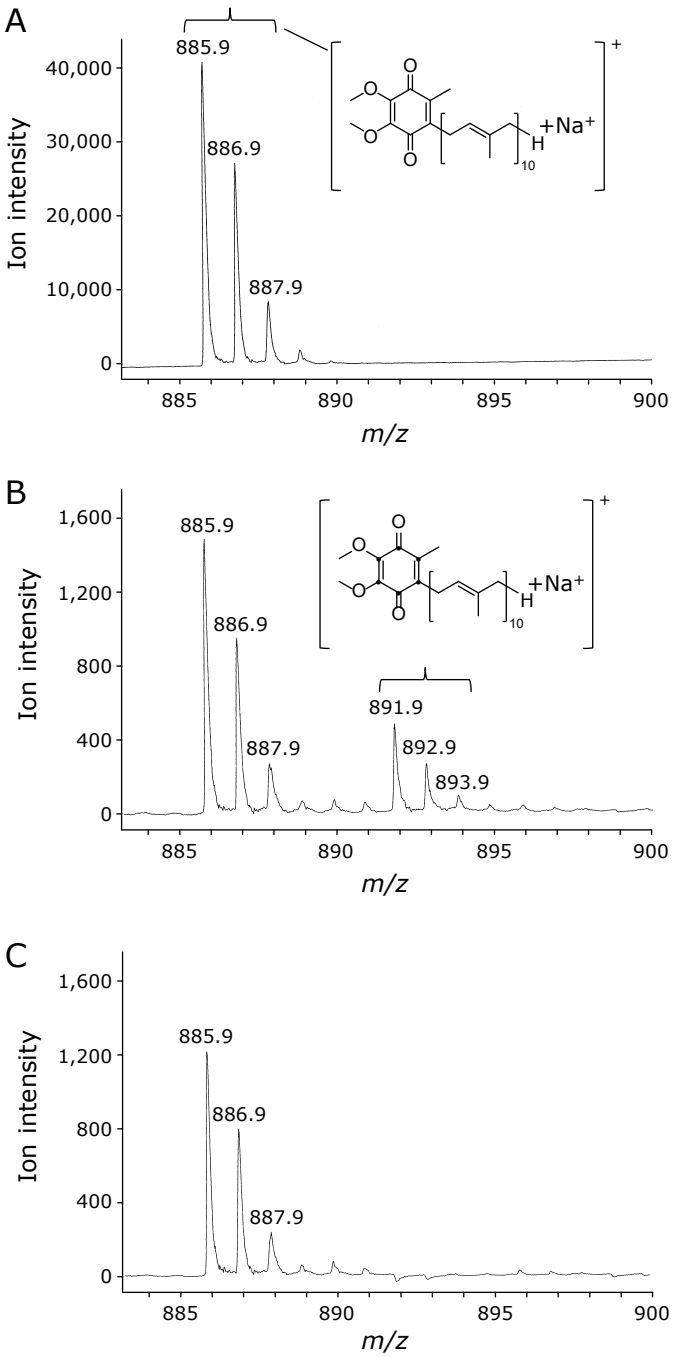

Fig. 4. MS spectra measured with an optimized TOF-MS of CoQ10. Authentic standard (A), extracted from HepG2 cell treated with $^{13}C(\bullet)$-labeled (B), and non-labeled (C) resveratrol. Positive ESI ionization was carried out with a potential of 2,500 V.

alongside RSV. As shown in Fig. 5C, although administration of 4-HB or RSV individually increased intracellular CoQ10 levels, their simultaneous administration did not produce an additive effect (Fig. 5C). Furthermore, Fig. 5E demonstrates the effect of 4-NB on RSV administration. 4-NB acts as a competitive inhibitor, suppressing CoQ10 biosynthesis by inhibiting the reaction catalyzed by coq2.[29,30] In the presence of 4-NB, the effect of RSV administration was diminished. In these experiments, FC levels were not altered (Fig. 5D and F).

Discussion

In HepG2 cells, RSV treatment increased intracellular CoQ10 levels. Addition of an inhibitor of the mevalonate pathway (simvastatin) suppressed this increase in CoQ10. These results suggest that sufficient activity of the mevalonic acid pathway is required for RSV administration to increase the amount of CoQ10 in the cells. Although 4-HB and RSV increased CoQ10 levels, there was no additive effect when they were administered simultaneously. 4-HB is bound to the isoprenoid side chain by the enzyme COQ2 to form the ring portion of CoQ10, and the lack of additive effects between 4-HB and RSV administration suggests that RSV may be a similar substrate to 4-HB. Administration of 4-NB, a competitive inhibitor of COQ2, a protein that condenses the benzene ring and side chains of CoQ10 synthase, reduced the effect of RSV on increasing CoQ10 levels. Considering these results, it is likely that RSV binds to the side chain of CoQ10, which is synthesized through the mevalonic acid pathway, as the benzene ring moiety of CoQ10, resulting in an increase in the amount of CoQ10.

Indeed, mass spectral analysis of cells treated with labeled RSV ($^{13}C_6$-resveratrol) showed an increase in the amount of labeled CoQ10 ($^{13}C_6$-CoQ10) in the cells. The benzene ring of the administered RSV was directly converted to the benzene ring of CoQ10. This is consistent with a previous report by Xie *et al.*[17] It is unclear how RSV is converted to 4-HB or other CoQ10 precursor substances.

RSV is known to have anti-aging effects, while the precise mechanisms underlying these functions are still under debate. The activation of the SIRT gene has attracted much attention as one of the effects of RSV.[31] In addition, RSV is known to have antioxidant activity and mitochondrial protection.[32] It is possible that some of these effects of RSV may be related to the conversion of RSV to CoQ10, which in turn may have antioxidant and mitochondrial function-enhancing effects as CoQ10.

There have been many searches for substances that can serve as ring precursors for CoQ10. Not only 4-HB, which is a known substrate, but also Kaempferol and Vanillic acid have been reported to be ring precursors for CoQ10.[33] Fernández-del-Río *et al.*[33] reported that the effect of Kaempferol on the biosynthesis of CoQ10 was more pronounced in kidney cells. It is possible that the type of effective ring precursors may vary depending on the cell type. It would be interesting to further investigate the type of ring precursor and its mechanism of action for each organ in which CoQ10 should be increased.

It will be interesting to see if CoQ10 actually increases at the individual level when RSV is taken. There have been reports in previous studies that RSV modulates COQ genes in metabolic syndrome model mice.[34] Nevertheless, Levels of CoQ9 and CoQ10 in liver from the RSV treated animal did not change. Future studies are expected to examine whether CoQ10 levels increase when the concentration of RSV or the duration of administration is varied.

Author Contributions

MK, YY, and AF conceived the project and designed the experiments. RO, RH, MO, SS, KS, YA, and AN performed the experiments. MK, MO, KS, and AF wrote the paper. MK coordinated and directed the project.

Abbreviations

CoQ	coenzyme Q
4-HB	4-hydroxybenzoate
IPA	2-propanol
LC/TOF-MS	HPLC equipped with an optimized time-of-flight mass spectrometry

doi: 10.3164/jcbn.24-70

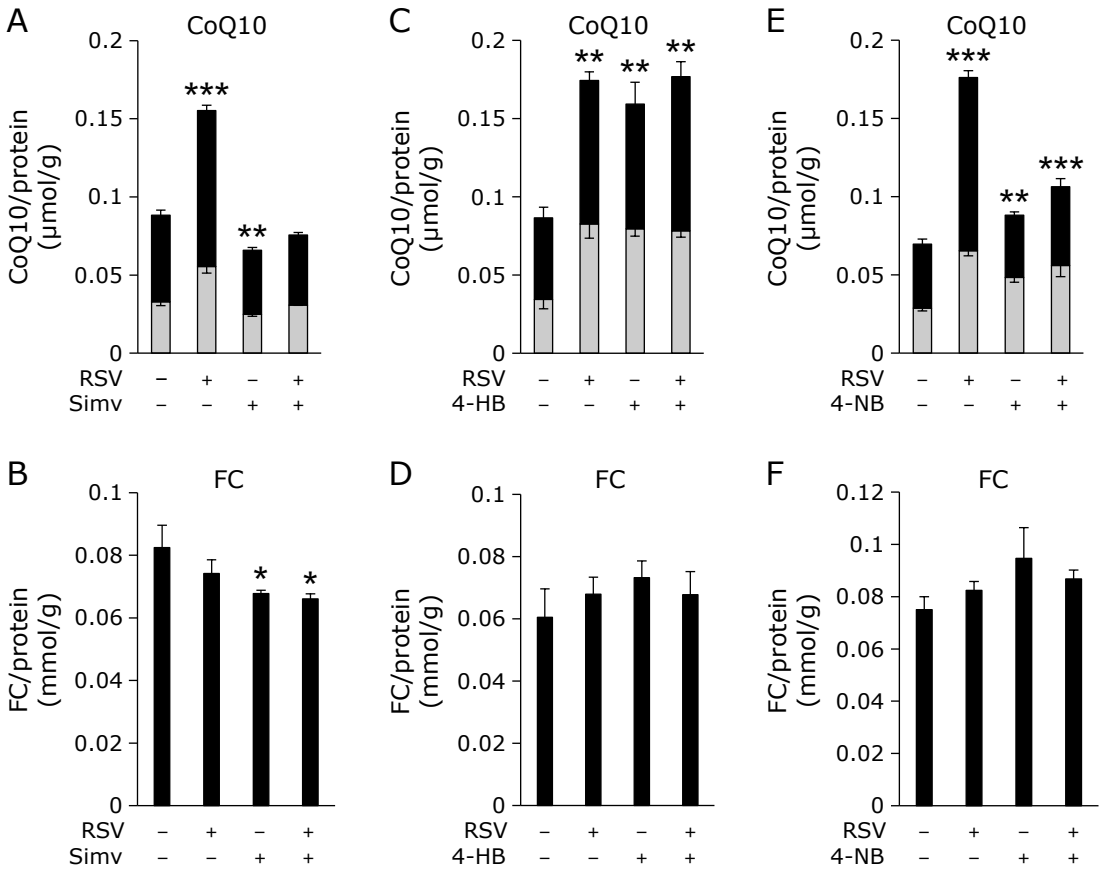

Fig. 5. Effect of various regents on the CoQ10-enhancing effect of RSV. (A, B) CoQ10 (A) and FC (B) levels in HepG2 cells with and without 25 μM RSV and 10 μM simvastatin. Total CoQ10 levels are presented for both oxidized CoQ10 (gray) and reduced CoQ10 (black). The respective measurements were corrected by total protein. (C, D) CoQ10 (C) and FC (D) in HepG2 cells with and without 25 μM RSV and 25 μM 4-HB. Total CoQ10 levels are presented for both oxidized CoQ10 (gray) and reduced CoQ10 (black). The respective measurements were corrected by total protein. (E, F) CoQ10 (E) and FC (F) in HepG2 cells with and without 25 μM RSV and 5 mM 4-NB. Total CoQ10 levels are presented for both oxidized CoQ10 (gray) and reduced CoQ10 (black). The respective measurements were corrected by total protein. Statistical analysis was conducted by ANOVA. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.

mtDNA	mitochondrial DNA
mtDNAcn	mitochondrial DNA copy number
4-NB	4-nitrobenzoate
nDNA	nuclear DNA
PSAP	prosaposin
qPCR	quantitative PCR
RSV	resveratrol

Conflict of Interest

No potential conflicts of interest were disclosed.

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doi: 10.3164/jcbn.24-70