Reduced prosaposin levels in HepG2 cells with long-term coenzyme Q10 deficiency

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Glycosphingolipids are involved in intercellular signaling, adhesion, proliferation, and differentiation. Saposins A, B, C, and D are cofactors required for glycosphingolipid hydrolysis. Saposins A–D are present in series in a common precursor protein, prosaposin. Thus, glycosphingolipids amounts depend on prosaposin cellular levels. We previously reported that prosaposin and saposin B bind coenzyme Q10 in human cells. Coenzyme Q10 is an essential lipid of the mitochondrial electron transport system, and its reduced form is an important antioxidant. Coenzyme Q10 level decreases in aging and in various progressive diseases. Therefore, it is interesting to understand the cellular response to long-term coenzyme Q10 deficiency. We established a long-term coenzyme Q10 deficient cell model by using the coenzyme Q10 biosynthesis inhibitor, 4-nitrobenzoate. The levels of coenzyme Q10 were reduced by 4-nitrobenzoate in HepG2 cells. Administration of 4-nitrobenzoate also decreased prosaposin protein and mRNA levels. The cellular levels of coenzyme Q10 and prosaposin were recovered by treatment with 4-hydroxybenzoquinone, a substrate for coenzyme Q10 synthesis that counteracts the effect of 4-nitrobenzoate. Furthermore, the ganglioside levels were altered in 4-nitrobenzoate treated cells. These results imply that long-term coenzyme Q10 deficiency reduces cellular prosaposin levels and disturbs glycosphingolipid metabolism.

Key Words: 4-nitrobenzoate, coenzyme Q10, glycosphingolipids, prosaposin

Coenzyme Q10 (CoQ10) is a key component of the mitochondrial electron transfer chain.1 The reduced form of CoQ10, ubiquinol, is one of the most important lipid-soluble antioxidants.2 CoQ10 cellular levels decrease with aging.3 Moreover, several diseases are associated with low CoQ10 levels, such as Parkinson’s disease.4,5 Interestingly, these disorders, as well as aging, progress over several years. To elucidate the physiological relevance of CoQ10, the cellular response after long-term CoQ10 deficiency needs to be understood.

CoQ10 is synthesized in vivo from acetyl-CoA. Starting from acetyl-CoA, a series of reactions comprising the mevalonate pathway produces farnesyl-PP, the cholesterol precursor, CoQ10, dolichol, and isoprenylated proteins.6 The quinone part derives from a tyrosine. The hydroxybenzoic acid produced from tyrosine is bound to the side chain by the CoQ2 enzyme. This reaction is blocked by a competitive inhibitor, namely, 4-nitrobenzoate (4-NB).7,8 Administration of 4-NB has been shown to reduce cellular CoQ10 levels.7,8

We previously reported that CoQ10 is, at least to some extent, bound to saposin B and its precursor, prosaposin (Psap), in human cells.9 Psap is a highly conserved multifunctional glycoprotein and is the lysosomal precursor of four small sphingolipid activator proteins, known as saposins A, B, C, and D. These four saposins are homologous and contain six conserved cysteines and one common glycosylation site.10 The mature saposins activate several lysosomal hydrolases involved in the metabolism of various sphingolipids and ceramides. Saposins act as essential cofactors for sphingolipid hydrolases and/or destabilize the lipids.10 We reported that the loss of Psap in HepG2 and Caco-2 cells decreases CoQ10 cellular levels.11,12 Psap and its derived proteins, saposins A–D, are implicated in the sphingolipid metabolism.13 Psap is important to maintain normal glycosphingolipid levels.10,14 Glycosphingolipids are involved in intercellular signaling,15 adhesion,16 proliferation,17 and differentiation.18 Therefore, it is essential to maintain normal sphingolipid levels.

Here, we analyzed the levels of the CoQ10 binding protein Psap in cells with long-term CoQ10 deficiency. CoQ10 is a lipid synthesized from acetyl-CoA together with cholesterol. The liver is a key organ to control lipid metabolism in the body. We used HepG2 cells, which are hepatoma cell line. We established 4-NB treated HepG2 cell models. In the previous studies using 4-NB,7,8 4-NB is usually treated for a few days to 1 week. To elucidate the Psap level in chronic CoQ10 deficiency, we created a cell model which is treated 4-NB for more than a month, and we call these cell model as a long-term CoQ deficient cell model in this study. We found that long-term CoQ10 deficiency induced reduced Psap levels and modulated the lipid metabolism in cells.

Materials and Methods

Preparation of HepG2 cells. HepG2 cells (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) were cultured in Dulbecco’s MEM (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (HyClone; Thermo Scientific, Waltham, MA), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Cell treatment with 4-NB. 4-NB (Wako, Osaka, Japan) was dissolved in DMSO before use. We treated the cells with 4-NB at 0.1, 0.5, 1, 5, and 10 mM and determined the optimal concentration for generating CoQ10-deficient cells over 96 h. The cells were collected every 24 h to quantify the amount of CoQ10. In the following experiments, 4-NB-treated cells were kept in culture medium containing 5 mM 4-NB. The control cells were incubated with DMSO at the corresponding concentration. 4-NB treated cells and control cells were cultured for the same treatment time. It should be noted that we did not see any morpho-

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logical changes such as hypertrophy in DMSO treated control cells in these periods.

To demonstrate that the effects of 5 mM 4-NB were mediated by the CoQ10 deficiency rather than by side effects of the compound, cells were cotreated with 5 mM 4-NB and 25 μM 4-hydroxybenzoate (4-HB; Wako) or were incubated with normal medium.

**Western blot analysis.** Western blotting analysis was performed as reported previously with minor modification.\(^{(12)}\)

Briefly, each dish of HepG2 cells was incubated with lysis buffer (150 mM NaCl, 50 mM Tris–HCl pH 7.4, 0.1% nonidet P-40 (Nacalai Tesque, Tokyo, Japan), 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, pepstatin A, 1 μg/ml 5-nitrosyl-L-phenylalanine chloromethyl ketone, and 1 μg/ml 5-nitrosyl-L-lysyl chloromethyl ketone) for 1 h. Then, the samples were collected and centrifuged at 15,000 × g for 10 min. The protein concentration was measured in the supernatants with a Pierce BCA Protein Assay Kit (Thermo Scientific). The same protein amount (10 μg) was loaded to each well. Samples were separated by electrophoresis through a 7.5% or 10% SDS/polyacrylamide gel. After electrophoresis, proteins were transferred to polyvinylidene di-fluoride membranes. The membranes were incubated with mouse anti-saposin B IgGs (monoclonal antibody generated previously)\(^{(9)}\) or mouse anti-acrolein IgGs (NOF Co., Tokyo, Japan) for 1 h at room temperature. Proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Japan, Tokyo, Japan) for 1 h at room temperature. The protein bands were visualized using EzWestBlue (#AE-1490; ATTO, Tokyo, Japan) for 5 min at room temperature and were imaged by the GNU Image Manipulation Program 2.8. The imaged protein bands analyzed using the ImageJ software.

**RNA isolation and quantitative PCR (qPCR).** The mRNA expression levels in HepG2 cells were determined by reverse transcription PCR as reported previously.\(^{(19)}\) Briefly, cells were seeded into 6-well plates (7.5 × 10^5 cells/well), and total RNA was extracted using TRizol reagent (Thermo Fisher Scientific). The RNA quality and concentration were assessed using a Pierce BCA Protein Assay Kit.

### Table 1. List of primer sequences used in the qPCR assays

| Gene | Forward | Reverse |
|------|---------|---------|
| NFYB | 5'-GGTGCATCAAGAAACGG-3' | 5'-GACTGCTCCAAATCCC-3' |
| PSAP | 5'-CTTCCGAAACCGAATGTGG-3' | 5'-GACCTTATTGGAATCCGCT-3' |
| ACTB | 5'-ATGGCCGACAGGTCGCAA-3' | 5'-GCTGATCCACATCTGGA-3' |

Fig. 1. Levels of CoQ10 in HepG2 cells treated or not with 4-NB. (A) Measurement of CoQ10 levels over 96 h of treatment with various concentrations of 4-NB. (B) Measurement of FC levels over 96 h of treatment with various concentrations of 4-NB. (C) Total CoQ10 levels normalized to the control values (%). Statistical analysis was conducted by one-way analysis of variances (ANOVA) against the level in control (A, B), and using the Student’s t test (C). *p<0.05, **p<0.01, ***p<0.001 vs control.
A reverse transcription was performed to synthesize cDNA using QuantiTect Reverse Transcription Kit (QIAGEN, Venlo, The Netherlands). The expression levels of the following genes were measured by qPCR: Psap, the nuclear transcription factor Y subunit beta (NP-YB), and beta-actin (ACTB). The sequences of the primers are presented in Table I. A QuantStudio® 5 (Thermo Fisher Scientific) was used to perform the qPCR (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). The changes in gene expression were calculated with the 2^−ΔΔCT method.20

CoQ10 and free cholesterol (FC) analysis. The cellular concentrations of CoQ10 and free cholesterol (FC) were determined by HPLC, as reported previously with minor modifications.19,21 Briefly, cells collected in 2-propanol were centrifuged, and the 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, Tokyo, Japan) and a reduction column (RC-10, 15 mm × 4 mm i.d.; IRICA, Kyoto, Japan) were employed. The mobile phase for the separation columns was 50 mM sodium NaClO4 in methanol/2-propanol (85:15, v/v) and was delivered at a flow rate of 0.8 ml/min. The columns were maintained at 25°C.

Ganglioside analysis. Gangliosides were isolated as described previously22 and analyzed by thin-layer chromatography (TLC). The TLC solvent system was CHCl3/MeOH/CaCl2 (0.02%) (11/9/2, v/v/v). Samples were separated on a plate similar to pure ganglioside GM3 standard (Funakoshi Co. Ltd., Tokyo, Japan). The ganglioside bands were detected with a resorcinol thiosulfuric acid coloring reagent. The TLC plate was sprayed with the reagent and developed at 100°C for 30 min. The ImageJ software was used to view and analyze the blot images.

Statistical analysis. Statistical significance was assessed using Student’s t test and one-way analysis of variances (ANOVA). Statistical analysis was performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). Generally, group differences at the level of *p<0.05, **p<0.01, and ***p<0.001 were considered statistically significant.

Results

Effect of 4-NB treatment on CoQ10 cellular levels. As shown in Fig. 1A, the CoQ10 cellular levels were reduced by 4-NB treatment in a dose-dependent manner. In our previous study,11,12 we corrected the CoQ values with the amount of FC that would be extracted into isopropyl alcohol as well as CoQ. It should be noted that FC s also produced from acetylCoA via malonate pathway, as CoQ. Figure 1B shows the FC level measured in each culture dish well. As shown in this figure, 10 mM 4-NB administration reduced not only CoQ level in each well but also FC level in the well. This result suggests that the administration of 10 mM 4-NB markedly inhibited cell proliferation. Thus, we used 5 mM 4-NB in the following experiments. We administrated 4-NB for 3 days to up to 16 months. CoQ10 cellular levels were significantly decreased compared with those of controls after 3 days of 4-NB treatment. These levels were also low after 6 and 12 months of 4-NB treatment (Fig. 1C).

To investigate the effects of 4-NB on cellular oxidative stress, we measured the cellular levels of acrolein-bound proteins (Fig. 2A). It can be seen that the amount of high molecular weight acrolein-bound protein has increased in cells treated with 4-NB for 15 months. We have not been able to identify the type of these proteins. Figure 2B shows the proportion of oxidized CoQ10 (%CoQ10) after 3 days and 6 months of 4-NB treatment. This value is an indicator of the oxidative stress and is calculated as follows: [oxidized form of CoQ10]/[oxidized form of CoQ10 + reduced form of CoQ10] × 100. As shown in this figure, %CoQ10 increased after 3 days and 6 months of 4-NB treatment.
essential proteins metabolizing glycosphingolipids. Therefore, we analyzed ganglioside cellular levels in 4-NB-treated cells. Figure 5A shows the results of the TLC analysis. The amount of lipid linked to sialic acid was altered by 4-NB administration. Lipids positively stained with sialic acid were detected in extracts from control and 4-NB-treated cells. When GM3 was used as a marker, a positively stained band was present at the same position as GM3. The intensity of this band was reduced in 4-NB-treated cell extracts. TLC analysis of GM1, GM2 and GD under the same conditions showed staining at a lower position than that of GM3 (data not shown). Further analysis is necessary but this result suggests that the cellular levels of lipids bound to sialic acid are reduced in 4-NB-treated cell lines (Fig. 5B).

Cellular levels of nuclear transcription factor Y subunit beta (NF-YB) 4-NB-treated cells. Tharyan et al.\(^{(23)}\) previously reported that NFYB-1 regulates the expression of Psap. Indeed, the inhibition of NFYB-1 expression caused an upregulation of Psap mRNA expression. Since we found that Psap mRNA levels were reduced in 4-NB-treated cells, we investigated the expression of NF-YB mRNA. NF-YB is a human homologue of the NFYB-1 gene in C. elegans. As shown in Fig. 6, NF-YB mRNA levels were not increased, but rather reduced, by the 4-NB treatment. Therefore, it is likely that NF-YB is not involved in the decrease of Psap mRNA mediated by 4-NB.

Discussion

As shown above, long-term CoQ10 deficiency reduced Psap cellular levels. In cells treated with 4-NB for 3 days, CoQ10 levels were decreased, but there was no change in Psap quantities. However, Psap levels were diminished after 3 months of 4-NB treatment and remained low after several months of 4-NB treatment. These results imply that Psap levels are decreased if CoQ10 levels are maintained chronically low, and not immediately after CoQ10 decrease. In addition, the administration of 4-HB, a substrate used for CoQ10 synthesis, suppressed the Psap decrease, suggesting that the latter was not due to a mere toxic effect of 4-NB administration, but rather to the decrease in CoQ10 levels.

Reduced cellular CoQ10 levels lowered Psap cellular amounts and perturbed glycosphingolipid cellular content. Psap plays an important role in the metabolism of sphingolipids. A disease caused by mutations in the Psap gene, resulting in a lack of Psap, has been reported in at least four families worldwide.\(^{(24-27)}\) In all reported cases, severe neurological symptoms, such as generalized convulsive seizures and marked hepatosplenomegaly, were observed immediately after birth or in early neonatal and infantile stages. The clinical features are similar to those of Gaucher disease type II (acute neurological type). Fetal death has also been reported. In those cases, the liver was found to contain ceramide, glucosylerceramide, lactosylce-
Thus, a decrease in Psap disrupts the lipid homeostasis. Here, we measured sialic acid containing lipids and showed that it was reduced in 4-NB-treated cells. A lipid positively stained for sialic acid was detected at a similar position than GM3, which was used as a marker. Therefore, we considered this band as derived from GM3. The intensity of this band was reduced by the administration of 4-NB. The analysis of the effects of 4-NB treatment on other lipids will be required in the future.

The present study showed that the long-term CoQ10 decrease reduces the amount of Psap in cells. Indeed, Psap mRNA levels were decreased in CoQ10-deficient cells. Tharyan et al.\(^\text{(23)}\) reported that Nf-YB-1, a highly conserved histone-like transcription factor, represses the expression of Psap. In our long-term CoQ10-deficient cell model, NF-YB, NFYB-1 homolog in human, expression levels were rather decreased (Fig. 5). Therefore, the reduction of Psap mRNA levels is likely caused by mechanism(s) independent of NF-YB. Oxidative stress has been shown to increase Psap protein levels.\(^\text{(20,29)}\) The oxidative stress was increased in 4-NB treated cells as judged from the levels of

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**Table 1. Effects of 4-NB and 4-HB treatments on CoQ10 and Psap protein levels.**

| Time     | 5 mM 4-NB | 4-NB | 4-HB |
|----------|-----------|------|------|
| 0.0      | +         | -    | -    |
| 0.5      | +         | -    | +    |
| 2        | +         | -    | +    |
| 3        | +         | -    | +    |
| 4        | +         | -    | +    |
| 5        | +         | -    | +    |
| 6        | +         | -    | +    |
| 7        | +         | -    | +    |
| 8        | +         | -    | +    |
| 9        | +         | -    | +    |
| 10       | +         | -    | +    |
| 11       | +         | -    | +    |
| 12       | +         | -    | +    |
| 13       | +         | -    | +    |
| 14       | +         | -    | +    |
| 15       | +         | -    | +    |

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**Table 2. Effects of 4-NB and 4-HB treatments on Psap protein levels.**

| Time     | 5 mM 4-NB | 4-NB | 4-HB |
|----------|-----------|------|------|
| 0.0      | +         | -    | -    |
| 0.5      | +         | -    | +    |
| 2        | +         | -    | +    |
| 3        | +         | -    | +    |
| 4        | +         | -    | +    |
| 5        | +         | -    | +    |
| 6        | +         | -    | +    |
| 7        | +         | -    | +    |
| 8        | +         | -    | +    |
| 9        | +         | -    | +    |
| 10       | +         | -    | +    |
| 11       | +         | -    | +    |
| 12       | +         | -    | +    |
| 13       | +         | -    | +    |
| 14       | +         | -    | +    |
| 15       | +         | -    | +    |

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**Fig. 4.** Effect of 4-NB and 4-HB treatments on CoQ10 and Psap protein levels. Cells were treated or not with 4-NB for 4 months. Then, the cells were divided into three groups: cells treated with 4-NB, cells cotreated with 4-NB and 4-HB (4-NB + 4-HB), and 4-NB removed medium. (A) CoQ10 quantification in the cells. (B) Western blot analysis of Psap. (C) Quantification of Psap protein levels using ImageJ. Data are expressed as mean ± SD. The statistical analysis was conducted by one-way analysis of variances. *p<0.05, **p<0.001 vs control.

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**Fig. 5.** TLC analysis of lipids containing sialic acid. (A) Representative TLC plate. Lane 1: GM3 marker, Lane 2: control cell extracts, Lane 3: extract from 4-NB-treated cells. (B) Quantitative analysis of lipid sialic acid content by ImageJ. Data are expressed as mean ± SD (n = 2).

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**Fig. 6.** Expression level of NF-YB mRNA normalized to ACTB mRNA level. The gene expression in control (black bar) and 4-NB-treated cells (white bar) was measured by quantitative PCR. The results are shown as mean ± SD of triplicate measurements. The mean expression level was normalized to that of the control (2^(-ΔΔCt) method). The statistical analysis was conducted with the Student’s t test. *p<0.05.
acrolein-bound protein and the %CoQ10 value (Fig. 2). However, Psap mRNA and protein levels were reduced. Therefore, the mechanisms mediating the effects of 4-NB treatment on Psap levels need further investigation.

In conclusion, we established a long-term CoQ10-deficient cell model by treating the cells with a CoQ10 biosynthesis inhibitor, 4-NB. The amount of intracellular CoQ10-binding protein Psap decreased with prolonged reduction of CoQ10 levels. Psap is a protein involved in the metabolism of glycosphingolipids. The amount of gangliosides also changed in cells with decreased Psap induced by prolonged CoQ10 deficiency. Further investigations are needed to understand the effects of changes in the amounts of CoQ10 and its binding protein Psap on intracellular glycolipid metabolism.

Author Contributions

Study concept and design: AF, YY, and MK; acquisition of data: HT, KS, MOkamoto, AN, TT, YF, KI, HY, and AM; interpretation of data: MOkada, AM, AF, YY, and MK; drafting of manuscript: MOkamoto, AF, YY, and MK. All authors approved the final version of this manuscript to be published.

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