INTRODUCTION
Aging is a physiological phenomenon that occurs in all eukaryotes. Cellular senescence manifests as a stable cell arrest with active metabolism, which plays an important role in aging. 

H2O2 (hydrogen peroxide) is one type of reactive oxygen species (ROS) which has been widely used to achieve oxidative stress-induced premature senescence within a short period of time. In our previous study, short H2O2 exposure led to a significant decrease in the cell population and mitochondrial respiratory capacity and resulted in an imbalance of mitochondrial fusion/fission. The regulation of aging also relies on ROS function in redox signaling, and the control of oxidative stress is essential.

Pyroloquinoline quinone (PQQ) was first reported in 1964 by Hauge et al. as a new coenzyme distinct from nicotinamide adenine dinucleotide (NADH) and flavin in glucose dehydrogenase (GDH), and its structure was determined by X-ray analysis in 1979. PQQ is contained in fruits and vegetables, such as kiwi fruit, parsley, cocoa powder, fermented soybeans (Japanese natto) and green peppers, and in human breast milk. PQQ is a novel biofactor of physiological importance, and has been reported to be involved in a variety of biological functions that are clearly beneficial to survival, such as growth and fertility in newborns. It has also been reported to protect against glutamate-induced cell apoptosis in primary cultured hippocampal neurons. PQQ is especially effective in neutralizing superoxide and hydroxyl radicals, which cause mitochondrial dysfunction, and it also antagonizes several types of oxidative stress-induced cell damage, including reoxygenation cardiac injury, chronic heart failure, ethanol-induced liver damage, and hyperoxia-induced cognitive deficits.

PQQ is also involved in the control of redox processes in the mitochondrial respiration chain, the attenuation of oxidative stress in mitochondria, and the protection of neurons. PQQ not only protects mitochondria from oxidative stress but also promotes mitochondrial biogenesis. Dietary supplementation of PQQ improves mitochondrial amounts and lipid metabolism in rats and has been shown to improve respiratory quotients by increasing the mitochondrial numbers and function in mice. PQQ prevents rotenone-induced neurotoxicity in Parkinson’s disease models by promoting mitochondrial function and regulating mitochondrial fission and fusion. In humans, dietary supplementation with PQQ restores the antioxidant potential, attenuates the inflammatory response, and increases urinary metabolites related to mitochondrial functions. Considering these findings, the protective effect of PQQ in mitochondrial biogenesis is also expected for inner ear cells.

Mitochondrial biogenesis is mediated by peroxisome proliferator-activated receptor coactivator-1α (PGC-1α) under the control of the histone deacetylase sirtuin 1 (SIRT1), which has emerged as a crucial regulator of mitochondrial function in vascular smooth muscle, liver, kidney, and heart. PQQ has positive effects on biological activities and neural functions in SK-N-SH cells, HepG2 cells, and in C57BL/6 mice by increasing PGC-1α expression. PQQ also stimulates mitochondrial biogenesis through phosphorylation of the cAMP response element-binding protein and an increase of PGC-1α expression in mouse hepatocytes. In humans, the supplementation of PQQ has been reported to improve peak oxygen consumption and impact mitochondrial biogenesis by elevating PGC-1α protein content. The SIRT1/PGC-1α signaling pathway modulation with the administration of PQQ may also play an important role in protecting mitochondrial function in the inner ear tissues.

This study aims to assess mitochondrial metabolic activity, mitochondrial network structure, mitochondrial motility, and the SIRT1/PGC-1α signaling pathway under PQQ treatment in immortalized Corti-derived auditory epithelial cell line HEI-OC1 using the H2O2-induced premature senescence model.

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RESULTS

High-concentration PQQ-induced cytotoxicity

We first evaluated the cytotoxicity of PQQ in HEI-OC1 cells to confirm the safety of PQQ as a medicine. To achieve this, we examined the cell population doubling rate, metabolic enzyme activity using WST-8 formazan, and the mitochondrial membrane potential (MMP) using the JC-1 fluorescence dye, techniques that have been previously reported for evaluating PQQ and similar compounds in cell lines 40, 44. The population doubling rate increased significantly compared to the control when the concentration of PQQ was 1.0 nM (P < 0.05), but it then decreased at higher concentrations; the population doubling rate was greater at 1.0 nM compared to that at concentrations greater than 10 nM (Fig. 1A) (P < 0.05). The metabolic enzyme activity was increased significantly compared to the control at PQQ concentrations of 0.1 nM and 1.0 nM, whereas there was no significant difference when the concentration was greater than 10 nM (Fig. 1B) (P < 0.05). The metabolic enzyme activity was increased significantly compared to the control at PQQ concentrations of 0.1 nM and 1.0 nM, whereas there was no significant difference when the concentration was greater than 10 nM (Fig. 1B) (P < 0.05). The MMP decreased significantly compared to the control when the concentration was 10 nM or greater (Fig. 1C). These results indicate that, for the HEI-OC1 auditory cells, the optimum concentration of PQQ determined during the evaluation of cell metabolism was 1.0 nM and that PQQ induces cytotoxicity when applied at higher concentrations. The optimum concentration of PQQ we found was similar to those reported previously 40, thus we decided to use PQQ concentrations of 0.1 nM and 1.0 nM in the following experiments.

PQQ pretreatment before H2O2 exposure increased cell proliferation and cell metabolism in HEI-OC1 cells but decreased the mitochondrial membrane potential

H2O2 is widely utilized as an agent to induce premature cellular senescence in cell lines 45–47 including HEI-OC1 cells 48. We have previously shown that premature cellular senescence was induced in HEI-OC1 cells by exposing them to H2O2 at a concentration of 100 μM for 1 h 5. We evaluated the protective effect of pretreatments of 0.1 nM and 1.0 nM PQQ for 1 day on auditory cells with H2O2-induced premature senescence using population doubling rate analysis and metabolic enzyme activity analysis. The cell counts significantly increased in H2O2-exposed cells pretreated with 1.0 mM PQQ, although they remained unchanged in the other conditions (Fig. 2A). There was no significant decrease of viability in H2O2-exposed cells pretreated with PQQ (0.1 mM and 1.0 mM) (Fig. 2B). The population doubling rate decreased significantly in the H2O2-exposed group (with no PQQ pretreatment), whereas pretreatment with 0.1 nM or 1.0 nM PQQ significantly ameliorated the decrease induced by H2O2 exposure (Fig. 2C, D). These results indicate that H2O2 exposure induced premature senescence in HEI-OC1 cells and PQQ pretreatment had a protective effect against H2O2 exposure. H2O2 exposure decreased the metabolic activity of HEI-OC1 cells even when they were pretreated with PQQ, but the decrease in metabolic activity was significantly ameliorated when the cells were pretreated with 1.0 nM PQQ (Fig. 2E).

We also evaluated the expression of cell cycle regulators (Cdkn1a (p21WAF1/CIP1), Cdkn2a (p16INK4A), and Trp53 (p53)),
senescence-associated β-galactosidase (SA-β-Gal), and the dipeptidyl peptidase DPP4 (CD26) cell surface protein as cellular senescence biomarkers. The expression of the primary senescence markers p21, p16, and p53 are associated with cell cycle arrest in mediating senescence and the senescent phenotype\textsuperscript{49,50}. SA-β-Gal is widely utilized as a senescence marker in aging research\textsuperscript{51–53} and has also been previously evaluated in HEI-OC1 cells\textsuperscript{2,54}. CD26 is recently reported as a senescence marker\textsuperscript{55,56} and is emerging as a therapeutic target\textsuperscript{57}. While H\textsubscript{2}O\textsubscript{2} exposure increased the expression of p21 and the activity of SA-β-Gal and CD26, the
expression of p21, p16, and p53 were all alleviated by 0.1 nM PQQ pretreatment (Fig. 2F-I). These results also support the theory that H2O2 exposure induces premature senescence and that PQQ pretreatment protects HEI-OC1 cells from H2O2-induced premature senescence.

The MMP was decreased by exposure to H2O2 and more significantly decreased when the HEI-OC1 cells were pretreated with PQQ (Fig. 2K, L). These results suggest that PQQ pretreatment protects HEI-OC1 cells from H2O2-induced premature senescence and that PQQ has a mitochondrial uncoupling effect. The mitochondrial reactive oxygen species (ROS) evaluated with MitoSOX Red was not decreased by exposure to H2O2 whereas PQQ pretreatment significantly increased the ROS level (Supplementary Data). These results indicate that PQQ mildly increases the ROS levels, which can help to maintain the regulation of ROS levels in HEI-OC1 cells and so protect against premature senescence.

PPQ alleviated ultrastructural damage of mitochondria induced by exposure to H2O2 in HEI-OC1 cells

We also evaluated the protective effect of PQQ pretreatment on auditory cells with H2O2-induced premature senescence using transmission electron microscopy (TEM) image analysis. The shape of the mitochondria under TEM in the control cells was normal, but the mitochondria in the H2O2-exposed cells showed ultrastructural damage, including typical matrix swelling, the loss of cristae under TEM in the control cells was normal, but the mitochondria in the H2O2-exposed cells showed ultrastructural damage, including typical matrix swelling, the loss of cristae in the majority of mitochondria, and the increased formation of autophagosomes (Fig. 3A). The mitochondrial damage was alleviated by PQQ pretreatment before H2O2 exposure; PQQ pretreatment improved the internal heterogeneity and the swollen shape of the mitochondria and increased the number of endosomes. Image analysis of the area of endosomes showed that the increase in the number of endosomes and lysosomes was significant in the 0.1 nM PQQ-pretreated group compared to the H2O2-exposed group with no PQQ pretreatment (Fig. 3B). In contrast, the number of autophagosomes was significantly increased in the H2O2-exposed group, whereas PQQ pretreatment suppressed this increase. These morphological findings indicate that PQQ alleviates ultrastructural damage to the mitochondria of HEI-OC1 cells induced by a short exposure to H2O2. In addition, PQQ pretreatment increased the number of both endosomes and lysosomes, which implies an acceleration of autophagy or the inhibition of lysosome function (Fig. 3B).

The area of total mitochondria including healthy mitochondria and damaged mitochondria showed no significant difference between groups (Fig. 3B). The copy numbers of the mitochondrial DNA did not show a significant difference between groups (Supplementary Data), which indicated that the changes under the exposure to H2O2 and the treatment of PQQ mainly causes morphological and structural changes in mitochondria but not the proliferation of mitochondria in the timeframe of this study. These results indicate that H2O2 exposure or PQQ pretreatment does not alter mitochondrial proliferation but causes damages or protection in mitochondrial structure.

PPQ protected against the decline of mitochondrial respiratory capacity in the oxidative stress-induced premature senescence of HEI-OC1 cells

In order to confirm the protective effect of PQQ pretreatment in mitochondrial biogenesis and metabolic function in HEI-OC1 cells, we evaluated the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) using a XF24 extracellular flux analyzer, which measures the fundamental parameters of the electron transport chain (ETC), basal OCR, ATP-linked respiration, maximal OCR, spare respiratory capacity and proton leak (Fig. 4A, B). The basal ATP production speed did not differ significantly between groups; however, the maximal respiration measured following the injection of a mitochondrial uncoupling agent (cytochrome c oxidase-4 (trifluoromethoxy) phenylhydrazone (FCCP)) decreased significantly in the H2O2-exposed group compared to the control group, while it was protected significantly in both the 0.1 nM and 1.0 nM PQQ-pretreated groups compared to the H2O2-exposed group (Fig. 4C). ECAR tended to increase only in the H2O2-exposed group, although the change was not significant (Fig. 4B, D). ATP production speed represented by OCR, ECAR, and phosphate/oxygen ratio (P/O ratio) showed a slight increase of glycolytic ATP production under H2O2 exposure which was alleviated by PQQ pretreatment, although the difference was not significant between groups (Fig. 4D). These results indicate that PQQ prevents the decline of mitochondrial respiratory capacity under H2O2 exposure in HEI-OC1 cells.

The maximal respiration after the injection of a mitochondrial uncoupling agent indicates the mitochondrial respiratory capacity or reserve capacity, which works effectively in response to high-energy demand or oxidative stress.58,59 The mechanism of improvement in respiratory capacity mainly concerns the ETC because the medium in this assay contains glucose and pyruvic acid, but not glutamate or fatty acid (Supplementary Information). To further confirm the substrates on which HEI-OC1 cells depend, the metabolism regarding fatty acid oxidation (FAO) and glutaminolysis were analyzed. The palmitic acid supplementation increased the OCR whereas respiratory capacity was decreased; indicating that fatty acid worked as an uncoupler rather than an oxidation substrate, and fatty acid was not mainly utilized in HEI-OC1 cells (Fig. 5A). Carnitine palmitoyltransferase (CPT) inhibitor, Etomoxir, decreased respiratory capacity without PQQ pretreatment, but the decrease was not significantly different compared to the group with PQQ treatment, indicating that FAO was not the main pathway in the recovery of respiratory capacity (Fig. 5B). Glutamine supplementation increased the OCR and also respiratory capacity, although the protective effect of PQQ was still present under glutamine supplementation (Fig. 5C). Glutaminase (GLS) inhibitor, (bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES), disabled the protective effect by PQQ pretreatment (Fig. 5D), indicating that glutaminolysis is one of the alternative substrate pathways to protect damaged mitochondrial in HEI-OC1 cells. Other mechanisms including mitochondrial structural changes, however, may underlie in respiratory capacity recovery effect with PQQ pretreatment, because glutamine

Fig. 2 Cell proliferation, cell metabolic activity, senescence markers, and mitochondrial membrane potential (MMP) in HEI-OC1 cells with premature cellular senescence induced by H2O2 exposure with and without PQQ pretreatment. The effects of PQQ under H2O2 exposure are evaluated using multiple assays in the same manner as shown in Fig. 1. A Cell count 1 day after H2O2 exposure. B Cell viability 1 day after H2O2 exposure. C Cell proliferation rate using total cell count for 3 days after H2O2 exposure. D Cell proliferation rate using live-cell count for 3 days after H2O2 exposure. E Cell metabolic activity measured with WST-8 formazan assay. F Relative mRNA expressions of Cdk1α (p21), Cdkn2a (p16), and Trp53 (p53) using SPiDER-βGal staining in flow cytometry analysis. G The changes of senescence-associated β-galactosidase (SA-β-Gal) using SPiDER-βGal staining in flow cytometry analysis. H The changes of dipeptidyl peptidase DPP4 (CD26) surface protein marker in flow cytometry analysis. I The increased ratio in positivity rate over control of SPiDER-βGal positive cells. J The increased ratio in positivity rate over control of CD26 positive cells. K Imaging of mitochondria stained with JC-1. Green signal indicates low MMP and red signal indicates high MMP. L MMP measured with JC-1. (A-D, L: n = 6 per group, E, I, J: n = 5 per group, F: n = 3 per group). Box plot shows statistical parameters as follows; central line: median; box limits: first and third quartile; whiskers: minimum and maximum. RQ data are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Fig. 3  Mitochondrial ultrastructure in HEI-OC1 cells exposed to H$_2$O$_2$ (100 µM) with and without PQQ pretreatments (0.1 nM and 1.0 nM), and control cells. A Short exposure to H$_2$O$_2$ induces fine structural damage to mitochondria, which is alleviated with PQQ pretreatment. From left to right: control cells, cells exposed to H$_2$O$_2$, cells exposed to H$_2$O$_2$ after 0.1 nM PQQ pretreatment, and cells exposed to H$_2$O$_2$ after 1.0 nM PQQ pretreatment. AP autophagosome, DM damaged mitochondria, E endosome, ER endoplasmic reticulum, GL Golgi body, L lysosome, M mitochondria, N nucleus. Scale bar, 500 nm in top row, 200 nm bottom row. B The numerical analysis of TEM images. The endosome ratio, damaged mitochondria number, autophagosome number, and lysosome number were calculated by visual inspection ($n = 5$ per group). Box plot shows statistical parameters as follows; central line: median; box limits: first and third quartile; whiskers: minimum and maximum. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
supplementation did not fully recover respiratory capacity. The expression of CPT1C (Carnitine palmitoyltransferase 1C) was increased significantly under the exposure to H2O2 compared to the control group, which indicates that the energy metabolism was promoted to FAO from main glucose metabolism under the exposure to H2O2, whereas these expressions were nearly completely recovered to control level by PQQ pretreatment (Fig. 5E). The expression of glutaminase 2 (GLS2) was also increased significantly under the exposure to H2O2 compared to the control group, but the increase of GLS2 under PQQ pretreatment did not significantly different compared to the control group (Fig. 5E). The increase in GLS2 expression did not result in an increase in OCR under glutamine treatment, suggesting that the increase in GLS2 expression may indicate stress to the cells (Fig. 5F). These results indicate that the metabolic shift from glucose substrate-dependent pathway to FAO or glutaminolysis was induced under the exposure to H2O2 regarding the mitochondrial damage, whereas the promotion was

Fig. 4 Mitochondrial biogenesis in the premature cellular senescence model with and without PQQ treatment. The effects of PQQ pretreatment in HEI-OC1 cells exposed to H2O2 were evaluated using a XF24 flux analyzer. A Oxygen consumption rate (OCR) diagram of the XF assay, normalized by the number of cells. B Extracellular acidification rate (ECAR) diagram of the XF assay. C ATP production rate quantified with OCR measured following the injection of oligomycin, maximal respiration rates measured following injection of FCCP, and cell energy phenotype profile of the XF assay. D Basal ATP production rate and ATP rate index of the XF assay (n = 5 per group). Data are shown as mean ± standard deviation. *P < 0.05.
Fig. 5  Substrate-specific mitochondrial biogenesis in the premature cellular senescence model with and without PQQ treatment. The substrate-specific effects of PQQ pretreatment in HEI-OC1 cells exposed to H2O2 were evaluated using a XF24 flux analyzer; A Oxygen consumption rate (OCR) under palmitic acid supplementation. B OCR under Cpt (Carnitine palmitoyltransferase) inhibition by Etomoxir. C OCR under glutamine supplementation. D OCR under Gls (Glutaminase) inhibition by BPTES (bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide). E Relative mRNA expressions of Cpt1a, Cpt1c, Cpt2, Gls1, and Gls2 using β-actin as an internal control. F OCR under glutamine supplementation in HEI-OC1 cells in the control group or H2O2-exposed group (n = 5 per group). Data are shown as mean ± standard deviation. *P < 0.05.
not enough to compensate respiratory capacity decrease and was rather a marker of \( \text{H}_2\text{O}_2 \)-exposed stress, and this shift was held to the nearly normal state with PQQ pretreatment protection.

**PQQ has the potential to protect against morphological and dynamic deteriorations in HEI-OC1 cells with short exposures to \( \text{H}_2\text{O}_2 \)**

We evaluated the structure and dynamic motility of the mitochondria to confirm the improvements in mitochondrial function under PQQ pretreatment. The HEI-OC1 cells were stained with TMRE fluorescent dye, which accumulates in mitochondria due to the MMP, and were observed under confocal microscopy. The early alterations in mitochondrial shape and motility represent the dynamic function of mitochondria in auditory cells. The control group had a good balance of mitochondrial fusion and fission, while fragmented mitochondria with a balloon-like shape or hyperperfused mitochondria with an enlarged shape were observed in the \( \text{H}_2\text{O}_2 \)-exposed group (Fig. 6A). In the 0.1 nM or 1.0 nM PQQ-pretreated groups, the mitochondrial shapes had partly recovered to the normal balance (Fig. 6A). In order to quantify the recovery of the mitochondrial structures, we processed the mitochondrial images and analyzed the specific parameters of the mitochondrial network nodes (Fig. 6B). The mitochondrial dynamics include different types of fusion and fission mechanisms of the network nodes (tip-to-tip, tip-to-side, or side-to-side) (Fig. 6C), and the specific parameters show the condition of mitochondrial dynamics (Fig. 6D). The number of branches, the number of junctions and the average branch length all increased significantly in 0.1 nM and 1.0 nM PQQ-pretreated groups compared to the \( \text{H}_2\text{O}_2 \)-exposed group; however, mitochondrial dynamics biased toward the fusion process was observed in the 0.1 nM or 1.0 nM PQQ-pretreated groups (Fig. 6E). These results indicate that PQQ pretreatment assists in the recovery from morphological damage caused to the mitochondria by \( \text{H}_2\text{O}_2 \) exposure and also aids the recovery of mitochondrial dynamics in HEI-OC1 cells.

For the analysis of mitochondrial motility, particle tracking was derived from spatiotemporal image sequences, and then the kinetics of the moving particles were evaluated quantitatively using the TrackMate plugin in ImageJ/FIJI and @msdanalyzer on the MATLAB platform (Fig. 7A, B, E). The mean square displacement (MSD) was calculated from the tracking of the mitochondria, and the area under the curve (AUC) of the MSD plot of each group (Fig. 7C, D). The mitochondrial motility in the short-exposure \( \text{H}_2\text{O}_2 \) group tended to decrease compared to the control group, while it increased in the PQQ-pretreated groups. The AUC of the MSD plot showed significant increases in the PQQ-pretreated groups compared to the short-exposure \( \text{H}_2\text{O}_2 \) group (Fig. 7D). These results also support our proposal that PQQ pretreatment protects mitochondrial dynamics under \( \text{H}_2\text{O}_2 \) exposure in HEI-OC1 cells.

**Pretreatment with PQQ increased the expression of SIRT1 and PGC-1α in \( \text{H}_2\text{O}_2 \) short exposure HEI-OC1 cells, inducing deacetylation of PGC-1α**

To explore the pathways by which PQQ pretreatment led to the improvement of mitochondrial function, we investigated changes in the expression of SIRT1 and PGC-1α involving the regulation of mitochondrial metabolism. The expression of SIRT1 and PGC-1α decreased significantly in the \( \text{H}_2\text{O}_2 \) short-exposure group, while it recovered significantly in the 1.0 nM PQQ-pretreated group (Fig. 8A–C, G). In addition, the acetylation of PGC-1α increased significantly in the \( \text{H}_2\text{O}_2 \) short-exposure group, while it attenuated significantly in the 1.0 nM PQQ-pretreated group (Fig. 8D, E). No significant change was observed in the mRNA expression levels of SIRT1 and PGC-1α, and there was no correlation seen between the protein and mRNA expression (Fig. 8F). These results indicate that other post-translational modification factors including microRNA (miRNA) might regulate SIRT1 and PGC-1α at the transcriptional level\(^{50–63}\). These results indicate that \( \text{H}_2\text{O}_2 \) short exposure decreases the protein expression of SIRT1 and PGC-1α, while PQQ pretreatment increases the protein expression of SIRT1 and PGC-1α under \( \text{H}_2\text{O}_2 \) short exposure in HEI-OC1 cells, inducing the deacetylation of PGC-1α.

**DISCUSSION**

In this study, we demonstrated that PQQ has the potential to protect against oxidative-stress-induced premature cellular senescence in auditory cells by enabling the recovery of mitochondrial function by restoring the regulation of SIRT1/PGC-1α, the protein expression of SIRT1, and the deacetylation of PGC-1α, and by facilitating the recovery of mitochondrial biogenesis including the ATP production rate and maximum respiration rate. Restored SIRT1 leads to the mediation of PGC-1α and mitochondrial biogenesis in the auditory cells with oxidative-stress-induced premature senescence (Fig. 9). To our knowledge, this is the first report which indicates the effect of PQQ in protecting against the oxidative-stress-induced premature cellular senescence of auditory cells.

First, we confirmed the mitochondrial morphology and dynamics in auditory cells for evaluating the effect of PQQ pretreatment because these changes represent the condition of the mitochondrial quality control system\(^{64}\), and previous reports indicate that PQQ improves reproduction, neonatal development, and mitochondrial function in rats by mechanisms that involve mitochondrial-related signaling pathways\(^{28}\). The acceleration of autophagy function was indicated in the PQQ-pretreated groups by TEM images. In addition, the increase of the fusion of autophagosome and lysosome in the degradation step of autophagy, leading to the formation of autolysosome, was also presumed by TEM image analysis. PQQ could ameliorate autophagy-dependent apoptosis via the lysosome-mitochondria axis in vascular endothelial cells\(^{65}\), but because these functions interact closely with each other, it is difficult to precisely separate them and evaluate their functions, so further research is needed. In addition, we focused on mitochondrial motility in auditory cells because it decreases with age in vascular smooth muscle at the same time as mitochondrial dysfunction\(^{66}\). In this study, we confirmed the improvement of mitochondrial morphology as well as mitochondrial motility with PQQ pretreatment in the oxidative-stress-induced premature senescence of auditory cells, which supports the protective aspect of PQQ in the mitochondrial quality control system.

Mitochondrial function is dependent on mitochondrial structure and mitochondrial dynamics, including fission, fusion, and motility\(^{67}\). The mitochondrial transport to discrete subcellular regions may contribute to the accumulation of reactive oxygen species (ROS) in the nucleus, and the oxidative-based signaling function\(^{68}\). The fusion of mitochondria is dependent on the mitochondrial inner membrane potential and independent of microtubules or actin in neurons, and it directly reflects the mitochondrial condition\(^{69}\). The anterograde mitochondrial transport delivers healthy mitochondria to peripheral sites, while the retrograde mitochondrial transport returns damaged mitochondria to central sites, maintaining mitochondrial quality control, and the mitochondrial trafficking is crucial for neural survival\(^{70}\). Molecular pathways that control mitochondrial movement can be reduced to their effects on the balance of forces that act on mitochondria, driving and opposing movement\(^{71}\). The immobilization of mitochondria to microtubules is mediated by syntaphilin, an axonal molecule that facilitates the increase in mitochondrial volume in demyelinated axons, facilitating their survival and protecting against axonal degeneration in the central nervous system\(^{72}\). Mitochondrial motility and dynamics both regulate and...
reflect mitochondrial function in auditory cells. The morphological and dynamic deteriorations caused by H_{2}O_{2} exposure were alleviated by PQQ pretreatment in HEI-OC1 cells, which indicates that PQQ works protectively in terms of the mitochondrial function and the mitochondrial condition. The recovery of mitochondrial morphology and dynamics is the first step in the protection of mitochondrial metabolic activity in auditory cells.

The increase of expressions of FAO and glutaminolysis was observed under the exposure to H_{2}O_{2}, which was not enough to recover mitochondrial biogenesis, and this increase was reverted...
to nearly normal state by PQQ pretreatment. The induction of 
FAO or glutaminolysis is reported to be one of the phenotype of 
cellular senescence; CPT1A is overexpressed in senescent placenta-derived mesenchymal stem cells, the inhibition of 
CPT1A induced a change in energy metabolism and reversed 
senescence23, and GLS1 was identified as an essential gene for 
the survival of senescent cells56. The increase of expressions in 
FAO or glutaminolysis-related genes can draw the condition of 
H₂O₂-induced premature cellular senescence. This increase was 
inhibited by PQQ pretreatment, which indicates that PQQ have 
the protective function on mitochondria. Glutaminolysis was one 
of the alternative substrate pathways to protect damaged 
mitochondrial in HEI-OC1 cells under PQQ pretreatment, 
although other mechanisms including structural changes of 
mitochondria may underlie in respiratory capacity recovery effect 
with PQQ pretreatment. It has been reported that changes in 
mitochondrial morphology are related to the respiratory capacity 
protection. The mitochondrial respiratory capacity reserve capacity 
respond to high-energy demand or oxidative stress and acts as a buffer 
to protect the cells58,59.

The speed of glucose consumption varies between cell lines, 
and the drift of the OCR in this timeframe is also observed in 
normal condition, indicating that the metabolism of HEI-OC1 cells 
is very high to decrease the glucose concentration and OCR in this 
timeframe. The glucose concentration in the DMEM medium 
during the culture of HEI-OC1 cells decreases from the initial 
450 mg/dL to about 250 mg/dL within 5–6 days (Supplementary 
Information). This corresponds to a decrease of about 50% in 
120 h and about 2% in 4 h. During the analysis of XF24, where the 
medium volume is very low, this decrease in glucose concentration 
and OCR becomes more prominent and is likely to lead to a 
进一步 decrease in OCR over time during the analysis timeframe.

Importantly, the PQQ pretreatment led to the restoration of the 
expression of SIRT1 and PGC-1α and the deactivation of PGC-1α in 
the oxidative stress-induced premature senescence of auditory 
cells. These results suggest that PQQ regulates mitochondrial 
homeostasis via SIRT1 and the deactivation of PGC-1α in auditory 
cells, preventing premature senescence25. Recent studies suggest 
that PQQ activates SIRT1 and SIRT3 genes and increases NAD+ 
activity in the human hepatocyte cell line HepG226, induces 
deacetylation of PGC-1α and enhancement of mitochondrial 
activity in NIH3T3 cells after treatment with a SIRT1 selective 
inhibitor76 and protects skeletal muscles from denervationinduced atrophy by activating PGC-1α and improving the energy 
metabolic profile in C57BL6/J mice77. These effects of PQQ 
activating the SIRT1/PGC-1α signaling pathway in various cells 
support our data, including the protective effect of PQQ for the 
inner ear. In addition, the age-related decrease in the expression 
of SIRT1 and PGC-1α in cochlear tissue is observed in C57BL/6 
mice, and the overexpression of SIRT1 suppressed apoptosis and 
promoted cell proliferation in HEI-OC1 cells78. The activation of 
PGC-1α promoted mitochondrial biogenesis and protected them 
against cisplatin (CDDP)-induced ototoxicity in HEI-OC1 cells79.

Even caloric restriction, which has an anti-aging effect, induces 
SIRT1 expression and improves PGC-1α expression, which can 
regulate energy homeostasis and extend lifespan82. The mRNA expression levels of SIRT1 and PGC-1α did not correlate with the 
protein expression levels in this study, indicating post-translational 
modification factors in the protective pathway of PQQ pretreat-
ment. We consider that the expression of SIRT1 might be also 
regulated by microRNA (miRNA) in auditory cells, based on the 
results of previous studies40–63.

In this study, we confirmed that PQQ pretreatment had a 
mitochondrial uncoupling effect (the MMP decrease) in the high-
concentration condition, and it increased cell viability and worked 
protectively under H₂O₂ oxidative stress (Fig. 3C, D). Indeed, the 
decrease of MMP by PQQ has been reported in tumor cell lines at 
high doses of over 15 μM84. The optimum concentration analysis is 
crucial in evaluating the protective effect of PQQ, and from the 
aspect of the drug screening targeting the mitochondrial 
protection the mitochondrial uncoupling effect is one of the key 
factors in reducing damage and protecting the mitochondria in 
relation to ROS. We selected the optimum concentration of PQQ in 
this study by determining the concentration at which there was 
no decrease in cell numbers and no decrease in mitochondrial 
membrane potential. In a study evaluating the cardioprotective 
effect of FCCP (a mitochondrial uncoupler) in the post-ischemic 
functional recovery of rat hearts, the optimum concentration was 
also determined based on the mitochondrial oxidation without 
mitochondrial membrane depolarization81, and the protective 
way was ROS-dependent82. Generally, the MMP shows the 
condition of the mitochondria, and low membrane potential is 
normally considered to reflect the damaged condition; however, 
the MMP uncoupling effect of compounds should be interpreted 
carefully. The mitochondrial uncouplers including intrinsic uncou-
pling proteins, natural uncoupling compounds or newly devel-
oped agents which are reported to have a protective effect in 
mitochondria against ROS damage83. UCP2 and UCP3, homo-
logues of the endogenous mitochondrial uncouplers expressed in 
the heart, also protect against mitochondrial oxidative damage 
induced with cardiac ischemia-reperfusion by reducing the 
production of ROS84. FCCP reduces brain edema, decreases 
nitroinflammation, and improves neurological deficits following 
intracerebral hemorrhage by activating AMPK85. The newly 
discovered agent Ppc-1 functions as a mitochondrial uncoupler, 
and stimulates adipocytes to release fatty acids, thus acting as 
an anti-obesity agent86,87. Based on these previous studies, the 
higher respiratory flux leads to consumption of the membrane 
potential component of the proton motive force by ATP synthase 
which leads to the generation of ATP producing a mitochondrial 
membrane depolarization which can be one of the key factors 
underlying the protective mechanisms of PQQ under H₂O₂ exposure. Elevated mitochondrial ROS production promotes 
insulin resistance and obesity88, and the anti-obesity effect is also 
important in considering ROS protective agents. Low concentra-
tion of a mitochondrial uncoupler induces mild ROS production 
and PQQ extends the lifespan by increasing endogenous ROS 
levels89. Quercetin, one of the antioxidant polyphenols, is also 
known to protect neural and cardiac tissues through its mild 
mitochondrial uncoupling function90–92. ROS can act as essential
Fig. 7 The dynamic evaluation of mitochondrial motility in the premature cellular senescence model under PQQ pretreatment. The changes in mitochondrial dynamics with PQQ pretreatment in HEI-OC1 cells exposed to H\textsubscript{2}O\textsubscript{2} to induce premature cellular senescence were evaluated using microscopic imaging and image processing. A Image preprocessing flow of the motility analysis. B Motility analysis of the mitochondria. C The average of the mean square displacement (MSD) of each group. D The area under curve (AUC) of the MSD of each group. E Mitochondrial motion tracking video. The mitochondrial particle tracking lasted for 3 min in 3 s of intervals under ImageJ (D: n = 5 per group; repeated experiments). Box plot shows statistical parameters as follows; central line: median; box limits: first and third quartile; whiskers: minimum and maximum. *P < 0.05.
Fig. 8 Analysis of protein expression and protein acetylation in the premature cellular senescence model under PQQ pretreatment.

A SIRT1 and PGC-1α protein expression was analyzed by Western blotting (upper, middle) using β-actin as a loading control (lower).

B Relative protein expression level of SIRT1 normalized by β-actin. n = 10 per group.

C Relative protein expression level of PGC-1α normalized by β-actin.

D Acetylation of PGC-1α analyzed by immunoprecipitation.

E Relative acetylation level of PGC-1α normalized by PGC-1α.

F Relative mRNA expressions of GAPDH, SIRT1, and PGC-1α using β-actin as an internal control.

G The changes of SIRT1 expression in flow cytometry analysis.

H The rate of SIRT1 positive cells (B, C, E, H; n = 5 per group; repeated experiments). Box plot shows statistical parameters as follows; central line: median; box limits: first and third quartile; whiskers: minimum and maximum. RQ data are shown as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, *****P < 0.00001.
were protected from H2O2-induced cellular senescence by mature cellular senescence. The cell numbers and cell viability function in HEI-OC1 auditory cells with stress-induced pre-auditory cells. way, in the oxidative stress-induced premature senescence of regulation of the uncoupling effect of PQQ can work protec-

signaling molecules to promote metabolic health and long-
evity. The mild mitochondrial uncoupling effect and the mild ROS production could be key factors in the protective pathway of PQQ. Based on these previous reports and the results in this study, we consider that the decrease in MMP dissipation at high doses of PQQ might be related to decreased cellular ATP levels and the enhanced regulation of ROS relating to apoptosis. Previous reports have also suggested that the mitochondrial membrane permeability transition is a critical step in the induction of intrinsic apoptosis and often represents the earliest apoptotic signal, and that the loss of mitochondrial ATP synthesis in apoptotic cells and the increase of inter-membrane creatine phosphate concentrations might be consequences of the loss of MMP caused by the translocation of proapoptotic BH3-only proteins to the mitochondria. Because ROS can alter the expression level of many miRNAs and SIRT1 expression is also regulated by the miRNA pathway in oxidative stress, ROS regulation of the uncoupling effect of PQQ can work protectively, through the miRNA post-translational modification pathway, in the oxidative stress-induced premature senescence of auditory cells. This study demonstrated that PQQ protected mitochondrial function in HEI-OC1 auditory cells with stress-induced premature cellular senescence. The cell numbers and cell viability were protected from H2O2-induced cellular senescence by PQQ, which attenuated both H2O2-induced mitochondrial respiration decreases, as well as H2O2-induced morphological and dynamic movement changes. The mechanism underlying...
times with distilled water at 37 °C in room air for 30 min, washed with HBSS two times and diluted in HBSS for flow cytometry analysis. In the analysis of CD26, the cells were washed with DPBS, harvested from the flask by trypsinization (0.05% trypsin, 0.53 mM EDTA for 2 min), washed with PBS two times, stained with 1 μg/100 μL CD26 antibody in PBS with 0.1% bovine serum albumin (BSA) and 0.1% NaN₃, washed with PBS three times and diluted in HBSS for flow cytometry analysis.

Flow cytometry analysis

In the staining of SIRT1, the cells were washed with DPBS, harvested from the flask by trypsinization (0.05% trypsin, 0.53 mM EDTA for 2 min), washed with PBS two times, pretreated with PQQ for 1 day followed by 100 μM Oligomycin A; (3) 2.5 μM FCCP. In the fatty acid oxidation (FAO) assay, the number of cells was counted after each XF24 analysis as follows; the cells in the XF24 plate was washed with PBS 1 time, fixed with 4% paraformaldehyde (PFA) (Nacalsal Tesque, Tokyo, Japan) for 30 min, washed with PBS one time, captured with a fluorescence microscope (BZ-X710, KEYENCE, Osaka, Japan) using the DAPI filter cube, and counted automatically using ImageJ (93,104) with Fiji (105).

Analysis of mitochondrial dynamics and morphology

Cells were dyed with tetramethylrhodamine, ethyl ester (TMRE) (200 nM, Biotium, USA) mitochondrial fluorescence and captured with confocal microscopy focusing on mitochondrial-rich regions beside the cell nucleus. Z stacks across the depth of the cell provide 3D information about mitochondrial morphology while the time-lapse images allow the study of changes in mitochondrial dynamics over time. Images were captured at 3 s intervals for a duration of 3 min. Supplementary Video 1 depicts a representation of a projection of 2 Z stacks of mitochondria in a single cell with a focus on the mitochondrial-rich regions. All image processing and analysis were performed using NIS-Elements (Nikon, Japan), ImageJ (93,104) with Fiji (105). The mean square displacement (MSD) and the area under curve (AUC) measurements were used for the analysis of the parameters of mitochondrial motion. The mitochondrial skeleton was vectorized to measure branches, junctions, and average branch lengths as described in our previous study (106).
protocol above. The membranes were incubated with acetyl-lysine HRP (1:200, (7F8) sc-81623 HRP, Santa Cruz) to stain at 4 °C overnight after blocking the transferred membrane. The membranes were then detected with the same procedure described above before being repropped with anti-PGC-1α HRP (1:200, (D-5) sc-518025 HRP, Santa Cruz) antibody and a secondary antibody (anti-mouse 1:1000 Mouse TrueBlot ULTRA, 18-8817-30, Rockland).

**Quantitative RT-PCR analysis**

Total RNA was isolated with NucleoSpin RNA (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. The concentration of extracted mRNA was measured using NanoDrop Lite (Thermo Fisher Scientific, USA) and complementary DNA (cDNA) was generated using RevertASe qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) according to the manufacturer’s instructions. The quality of mRNA was assessed by visualization of the 28S and 18S ribosomal RNA bands using DynaMark™ High Resolution Easy Electrophoresis (BioDynamics Laboratory, Japan). The primers of genes including SIRT1 (forward GGCTGACCTTCCGACGAC, reverse TCCTGCAACAGGGTTGCTC, final concentration 250 nM), PGC-1α (forward AATGGTGCTTAGCGACCACTG, reverse AAGTGGAGGCAATCCGTTCGA, final concentration 50 nM), Cdkn2a (p16) (forward CGCAAGCTTGGCTCAGTCT, reverse GTCTGCCAAGAGCCAGAGG, final concentration 500 nM), Cdkn1a (p21) (forward CCTGGTGATGGCGGAGCTG, reverse CCAACTCAGCCACATG, final concentration 500 nM), forward (GGCTAAAGGCTTGCAAGTG, reverse TTTTTTTGCGGGAAAAGTA GACTG, final concentration 500 nM), Cpt1b (forward GTCCTCCTAAGCTGTCG TATC, reverse CATGGCCACCTGTTGCTCTA, final concentration 1000 nM), Cpt1c (forward TCTTCTAGGCTTCCAGG, reverse AGGCACAGA TGCTTCTTTC, final concentration 125 nM), Cpt2 (forward CCTGCTCATACC AGCCGATAA, reverse CATACATGGAAGCCAAGCCA, final concentration 500 nM), Gls1 (forward TGGCCTCGGAGATGCTTAC, reverse CCAAGTCAGG TAAAGACCCCT, final concentration 500 nM), Gls2 (forward CGTGGCTAGTAC TACTTGCTGT, reverse GTGCCTCCTGAATGTAAGA, final concentration 250 nM), GAPDH (forward TGAAGGGGAGTGGTATGTCG, reverse CCAC AGTCCCTGGGCTGCGACG, final concentration 125 nM) and β-actin (forward CCTCTATGCACAGACACGCCG, reverse GTACCTGCTCCTGCTGTAC, final concentration 250 nM) were purchased from FASMAC (Kanagawa, Japan). The forward and reverse primers were mixed with 100 ng cDNA and quantitative RT-PCR was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany) or THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan). The following experimental run protocol was used: denaturation and activation program (50 °C for 120 s, 95 °C for 120 s), amplification and quantification program repeated 60 times (95 °C for 15 s, 60 °C for 60 s), melting curve program (95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s). Data collection was performed using QuantStudio 7 Flex Detection System (Thermo Fisher Scientific, USA). The 2-ΔΔCt method was applied to analyze the relative changes in gene expression. The mRNA expression level of each gene was normalized using β-actin as an internal control.

**Mitochondrial DNA copy number analysis**

Total DNA was isolated with NucleoSpin Tissue (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. The concentration of extracted DNA was measured using NanoDrop Lite (Thermo Fisher Scientific, USA). The primers of mitochondrial and nuclear genes including mitochondrial 16S RNA (forward CGCGCAAGGGAAAGATGAAAGAC, reverse TCCTGGTTGTGGCCTGGTTT, final concentration 1000 nM), mitochondrial ND1-3 adenine nucleohydrolase chain 1 (ND1) (forward CTAGACAGAAAACAAGGCAGG, reverse CCGGCTGCTGATTCAGCTG, final concentration 500 nM), mitochondrial G0T (forward CCAACACTCGTAGGGAAGC, reverse GTATCATTTGGAGGACTAGCTT, final concentration 1000 nM), nuclear hexokinase 2 (HK2) (forward GCCACGCTTCTGTTTTAGTT, reverse GGGAAACAAGAAGCTCCTTCTG, final concentration 500 nM) and nuclear thymidine kinase 1 (TK1) (forward GACCTGATTGAGGCCTACTG, reverse CATGCTGGGTGGACACAT, final concentration 500 nM). The copy number of mitochondrial DNA was calculated using the deviations of the cycle threshold (Ct) between mitochondrial and nuclear genes according to the previous studies.108,109

**Measurement of glucose concentration of the medium**

The glucose concentration of the medium was measured using the following glucometers: Accu-Chek Aviva Nano/Guide (Roche Diagnostics GmbH, Mannheim, Germany), OneTouch Verio Relius (Johnson & Johnson, New Jersey, USA), Freestyle Libre (Abbott Diabetes Care, Witney, U.K.) and NIPEO StatStrip XP2 (NIPEO, Osaka, Japan).

**Statistical analysis**

The statistical software GraphPad Prism and R version 4.1.0 software (R Core Team; R Foundation for Statistical Computing, Vienna, Austria, 2021) were used in data processing and statistical analysis. A one-way ANOVA with Bonferroni’s test was used to make multiple comparisons between groups. A value of P < 0.05 was considered significant. Box plot shows statistical parameters as follows; central line: median; box limits: first and third quartile; whiskers: minimum and maximum.

**Reporting summary**

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**DATA AVAILABILITY**

Data are available from the corresponding author on a reasonable request.

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