Quercetin attenuates the effects of H_2O_2 on endoplasmic reticulum morphology and tyrosinase export from the endoplasmic reticulum in melanocytes

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Received January 10, 2014; Accepted October 24, 2014

DOI: 10.3892/mmr.2015.3242

Abstract. Swollen endoplasmic reticulum (ER) is commonly observed in the melanocytes of vitiligo patients; however, the cause and proteins involved in this remain to be elucidated. Oxidative stress has been reported to be involved in the pathogenesis of vitiligo and previous studies have demonstrated that hydrogen peroxide (H_2O_2) induced melanocyte apoptosis, whereas quercetin exhibited cytoprotective activities against the effects of H_2O_2. The aim of the present study was to further investigate the role of H_2O_2 in the ER of melanocytes as well as its role in the export of tyrosinase from ER; in addition, the present study aimed to determine the mechanism by which quercetin protects against the effects of H_2O_2. The results demonstrated that melanocyte cells treated with H_2O_2 presented with swollen ER; however, a normal ER configuration was observed in untreated cells as well as quercetin/H_2O_2-treated cells. Furthermore, H_2O_2 inhibited tyrosinase export from the ER and decreased expression levels of tyrosinase; however, quercetin was found to attenuate the effects induced by H_2O_2. In conclusion, the results of the present study confirmed the hypothesis that H_2O_2 induced ER dilation and hindered functional tyrosinase export from the ER of melanocytes. It was also found that quercetin significantly weakened these effects mediated by H_2O_2, therefore it may have the potential for use in the treatment of vitiligo.

Introduction

Vitiligo is a prevalent disorder, affecting 0.5-1% of the population worldwide, which results in depigmented areas of the skin (1). The absence of melanocytes in the skin lesions was previously reported to be the key event in the pathogenesis of vitiligo (2); however, the aetiology of vitiligo remains to be elucidated. Previous studies have suggested that oxidative stress may result in the loss of melanocytes (3,4); increased intracellular reactive oxygen species (ROS) production was observed in the epidermis of vitiligo patients (5), which therefore indicates the presences of systemic oxidative stress in vitiligo (6,7). Accumulated oxidative stress leads to DNA damage, lipid and protein peroxidation and cell death (8,9). Hydrogen peroxide (H_2O_2)-mediated oxidation was reported to result in inhibition of tyrosinase (10) and the significant decrease in acetylcholine esterase (AChE) activity (11). A previous study showed that antioxidants may be resistant to cell death mediated by oxidative stress. Green tea extract and quercetin were demonstrated to have potent cytoprotective effects on H_2O_2-induced cell death (12); in addition, another study showed that quercetin inhibited H_2O_2-induced melanocyte apoptosis (13). Furthermore, a double-blind placebo controlled trial revealed that oral supplementation with an antioxidant pool (AP)containing α-lipoic acid prior to and during narrowband ultraviolet B (NB-UVB) exposure significantly improves the clinical effectiveness of NB-UVB, reducing vitiligo-associated oxidative stress (14).

Extensive dilation of the rough endoplasmic reticulum (RER) was observed in numerous vitiligo patients; however, the cause and the proteins involved remain to be elucidated (15). A previous study demonstrated that swollen ER were present in melanocytes transfected with *FBXO11* siRNA; tyrosinase, the rate-limiting enzyme for melanin synthesis, was also reported to be regulated by the *FBXO11* gene (16). Furthermore, H_2O_2 was found to induce partially damaged plasma membranes, swollen RER and swollen or deformed mitochondria with ruptured cristae (17). Therefore, it was suggested that H_2O_2 may induce dilated ER and melanocyte dysfunction.

The present study aimed to evaluate the effects of H_2O_2 on the morphology of melanocyte ER and the export of tyrosinase from the ER, as well as to determine the mechanisms underlying the protective role of quercetin against the effects of H_2O_2.

Materials and methods

Reagents. Culture medium and supplements were obtained from Gibco-BRL (Carlsbad, CA, USA), with the exception

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Key words: quercetin, hydrogen peroxide, endoplasmic reticulum, tyrosinase

of recombinant human basic fibroblast growth factor (bFGF), isobutylmethylxanthine (IBMX) and cholera toxin (CT), which were purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). H_2O_2 and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-tyrosinase and rabbit polyclonal anti-calreticulin antibodies were purchased from Abcam (Cambridge, MA, USA), anti-β-actin mouse monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), Alexa Fluor® 488 donkey anti-mouse Immunoglobulin G (IgG; heavy and light chain, H+L) and Alexa Fluor® 594 donkey anti-rabbit IgG (H+L) from Life Technologies (Grand Island, NY, USA) and IRDye 680RD goat anti-mouse IgG highly cross adsorbed was from LI-COR Biosciences (Lincoln, NE, USA).

Cell culture. Human epidermal melanocytes were obtained from normal foreskins as previously described (18). Written informed consent was obtained from the patients and the study was approved by the ethics committee of the Third People's Hospital of Hangzhou (Hangzhou, China). In brief, the samples were incubated in a solution of 0.25% trypsin and 0.2% ethylenediamine tetraacetic acid for 20 h; trypsinization was terminated by the addition of Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% fetal bovine serum (FBS; Gibco-BRL). Melanocytes were then scraped from the epidermis, washed with phosphate-buffered saline (PBS) and centrifuged at 100 x g for 5 min. The pellet was resuspended in Hu16 medium (F12 medium with 20 ng/ml bFGF, 20 μg/ml IBMX, 10 ng/ml CT, 50 μg/ml gentamicin and 10% FBS). Cells were incubated in a humidified 95% air/5% CO_2 atmosphere at 37˚C. Geneticin was added to the medium (100 μg/ml) three days later in order to eliminate contaminating cells. Primary cultures were stored until they reached 80% confluence and then the melanocytes were detached using 0.125% trypsin/0.01 M EDTA solution, centrifuged at 100 x g for 5 min, resuspended and then seeded into culture flasks for subculture. Melanocytes used in the present study were of passage 2-3.

Exposure to H_2O_2. In order to evaluate effect of H_2O_2 on melanocytes, cultured cells were seeded at $1x10^4$ per well in 96-well plates at different H_2O_2 concentrations (0-400 μM) for 24 h. Cell viability was then assessed using an MTT assay kit (Promega, Sunnyvale, CA, USA).

Treatment of quercetin. In order to investigate the cytoprotective activity of quercetin, cells ($2x10^5$/well) were seeded onto six-well plates. Following serum starvation for 24 h, cells were pretreated with quercetin (0-200 μM dissolved in NaOH) for 24 h, H_2O_2 was then added to each well and incubated for 24 h. Cell viabilities were determined using an MTT assay.

Cell viability assay. MTT assays were used to assess cell viability in melanocytes following H_2O_2 and quercetin treatment. Following treatment, 10 μl MTT (10 mg/ml) was added to cells seeded in 96-well plates and incubated for 4 h, then 100 μl dimethyl sulfoxide was added for 15 min to dissolve. The absorbance value was measured at 490 nm using a microplate spectrophotometer (SoftMax Pro5; Molecular Devices, LLC, Sunnyvale, CA, USA).

Intracellular ROS measurement. ROS levels were determined by measuring the oxidative conversion of 2',7'-dichlorofluorescin diacetate (DCFH-DA) into the fluorescent compound dichlorofluorescin (DCF) using a ROS assay kit (Beyotime Insitute of Biotechnology, Jiangsu, China). In brief, $1x10^4$ cells/well were seeded into a 96-well plate and then the medium was replaced with serum-free medium for 24 h starvation. Cells from each well were then incubated with 10 μM DCFH-DA for 20 min at 37˚C. Cells were treated with quercetin or NaOH for 30 min and then H_2O_2 was added to each well, except those containing the untreated group, and the cells were incubated for 30 min. For the estimation of intracellular ROS, DCF fluorescence was determined at 485 nm excitation and 520 nm emission using a flow cytometer (BD FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA). Three independent experiments were performed.

Electron microscopy observation. Cells were seeded into a six-well plate and the medium was replaced with serum-free medium for 24 h starvation. Cells were pretreated with 0.01 μM NaOH or 25 μM quercetin for 24 h at 37˚C; 200 μM H_2O_2 was then added to each well, except the untreated group, and incubated for 24 h. ER configuration was observed using electron microscopy as previously described (16). In brief, cells were collected and fixed with 2.5% glutaraldehyde in 0.1 M PBS at 4˚C overnight; cells were then post-fixed with 1% OsO_4 in 0.1 M PBS at 4˚C for 1 h. Cells were then embedded in 5% agarose (Sigma-Aldrich) and cut into 2-3-mm^2 blocks, dehydrated in a graded series of ethanol and embedded in epoxy resin (West System, Bay City, MI, USA). Ultrathin sections were stained with uranyl acetate (Sigma-Aldrich) and lead citrate (Sigma-Aldrich) and were examined using a transmission electron microscope (JEM-1230; JEOL, Ltd., Tokyo, Japan).

Immunofluorescence assay. In order to examine the co-localization of tyrosinase and calreticulin, cells were grown in six-well plates containing coverslips, with various treatments. Cells were then fixed with 4% formaldehyde in PBS for 30 min. Anti-tyrosinase and anti-calreticulin antibodies were used at 10 μg/ml in buffer (0.5% BSA in PBS) and incubated with the coverslips for 1 h. Cells were then incubated with the secondary antibodies, Alexa Fluor® 488 donkey anti-mouse IgG (H+L) for tyrosinase and Alexa Fluor® 594 donkey anti-rabbit IgG (H+L) for calreticulin, at a dilution of 1:500 in PBS for 30 min at room temperature. Cover slips were washed three times in PBS for 5 mins each, mounted using a mounting medium and observed with confocal laser scanning microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany).

Western blot analysis of tyrosinase. Total proteins were isolated from cells and separated on a 10% polyacrylamide gel. Proteins were measured using western blot analysis with mouse antibodies against tyrosinase, followed by incubation with IRDye 680RD goat anti-mouse IgG highly cross adsorbed (1:10,000) for 1 h. β-actin was used as the internal control. An infrared imaging system, Licor Odyssey (LI-COR Biosciences), was used to visualize the protein bands and the relative intensities of bands were quantified using Image Studio for Licor Odyssey CLx and Classic (LI-COR Biosciences).

Statistical analysis. Values are presented as the mean ± standard deviation. Comparisons among groups were analyzed by a one-way analysis of variance using SPSS Version 13.0 (SPSS Inc., Chicago, IL, USA). P<0.05 and P<0.01 were considered to indicate a statistically significant difference.

Results

Cell viability in the presence and absence of H_2O_2. Human melanocytes and H_2O_2 were used as a model for oxidative stress in order to investigate cell viability using an MTT assay. Cells were treated with different concentrations of H_2O_2 (0-400 μM) for 24 h, and cell viability was measured. As shown in Fig. 1, the viability of H_2O_2-treated cells significantly decreased in a dose-dependent manner. Following treatment with 200 μM H_2O_2, cell viability was reduced by ~50%, this was considered to be the optimum concentration of H_2O_2 and was used for subsequent experiments.

Quercetin protects against H_2O_2-induced cell death. In order to investigate the effect of quercetin on H_2O_2-induced oxidative stress, H_2O_2-treated cells were pretreated with different concentrations of quercetin. In contrast to the H_2O_2-treated cells, quercetin was observed to have a minor, but not significant, protective effect at concentrations of 6.25 and 12.5 μM; however, quercetin concentrations of 25-200 μM exhibited significant protective effects against H_2O_2-induced cell death (Fig. 2). Therefore, 25 μM quercetin was selected to be used for following experiments.

ROS production in melanocytes under different conditions. ROS production was evaluated in melanocytes following exposure to quercetin or NaOH in the presence or absence of H_2O_2. As shown in Fig. 3, H_2O_2 and NaOH/H_2O_2 treatments resulted in an 0.5 fold increase in ROS production compared with that of the untreated control levels (P<0.01); however, no significant difference was identified between these two groups (P=0.123). In addition, ROS levels in the quercetin/H_2O_2 group demonstrated a significant decrease compared with those of the untreated control group (P=0.022).

Effect of quercetin and H_2O_2 on ER configuration. ER modality was analyzed using electron microscopy in order to observe the effects of different conditions on melanoctyes. As shown in Fig. 4, dilated ER was observed in H_2O_2- and NaOH/H_2O_2-treated cells. In untreated cells, ER configuration was shown to be normal. Notably, normal ER configuration was also observed in quercetin/H_2O_2-treated cells. This therefore suggested that quercetin was able to prevent H_2O_2-induced ER dilation.

Co-localization of tyrosinase and calreticulum. Confocal laser scanning microscopy was performed in order to assess the co-localization of tyrosinase, the rate-limiting enzyme for melanin synthesis, and calreticulin, an ER marker protein. In untreated cells and quercetin/H_2O_2-treated cells, tyrosinase fluorescence was beyond the fluorescence marked by calreticulin, which indicated that tyrosinase was effectively exported from the ER. However, in H_2O_2- and NaOH/H_2O_2-treated cells, the distribution of tyrosinase fluorescence was pronounced in

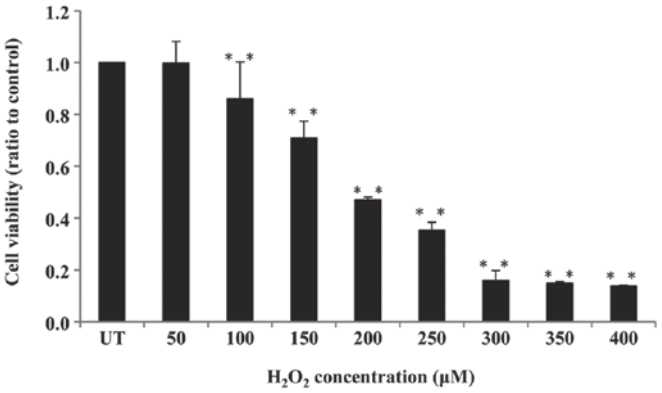

Figure 1. Effect of H_2O_2 on cell viability in human melanocytes. Human melanocytes were incubated for 24 h with various concentrations of H_2O_2 (0-400 μM) and cell viability was determined using an MTT assay. Treatment with 200 μM H_2O_2 reduced cell viability by ~50% and was used for subsequent experiments. Values are presented as the mean ± standard deviation (n=3). **P<0.01 vs. UT. H_2O_2, hydrogen peroxide; UT, untreated group.

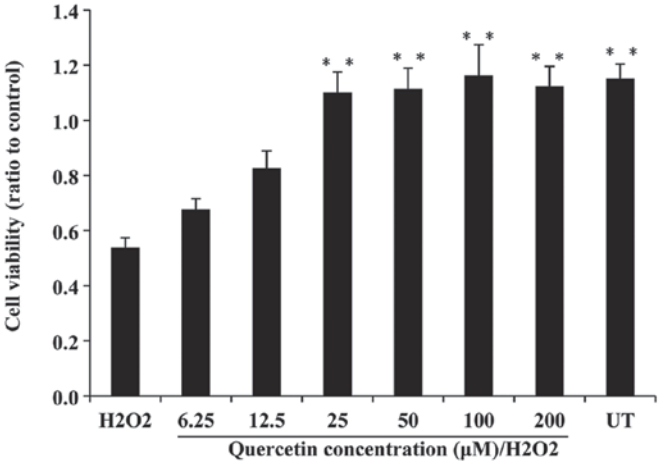

Figure 2. Protective effect of quercetin against H_2O_2 on melanocyte viability. Cells were incubated for 24 h with various concentrations of quercetin (0-200 μM) following serum starvation. Significant protective effects were observed at 25 μM quercetin, therefore this concentration was used for subsequent experiments. Values are presented as the mean ± standard deviation (n=3). *P<0.01 vs. H_2O_2-treated cells. H_2O_2, hydrogen peroxide.

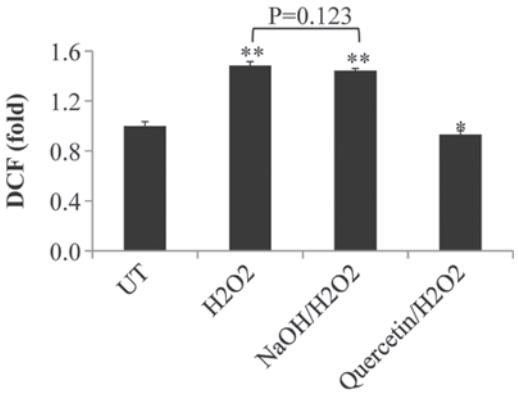

Figure 3. Detection of intracellular ROS levels. Human melanocytes were treated with 25 μM quercetin or NaOH in the presence or absence of 200 μM H_2O_2. ROS levels were determined by measuring the oxidative conversion of 2',7'-dichlorofluorescin diacetate into DCF. ROS levels were normalized to that of untreated cells. *P<0.05 vs. UT, **P<0.01 vs. UT. DCF, dichlorofluorescin; UT, untreated group; H_2O_2, hydrogen peroxide.

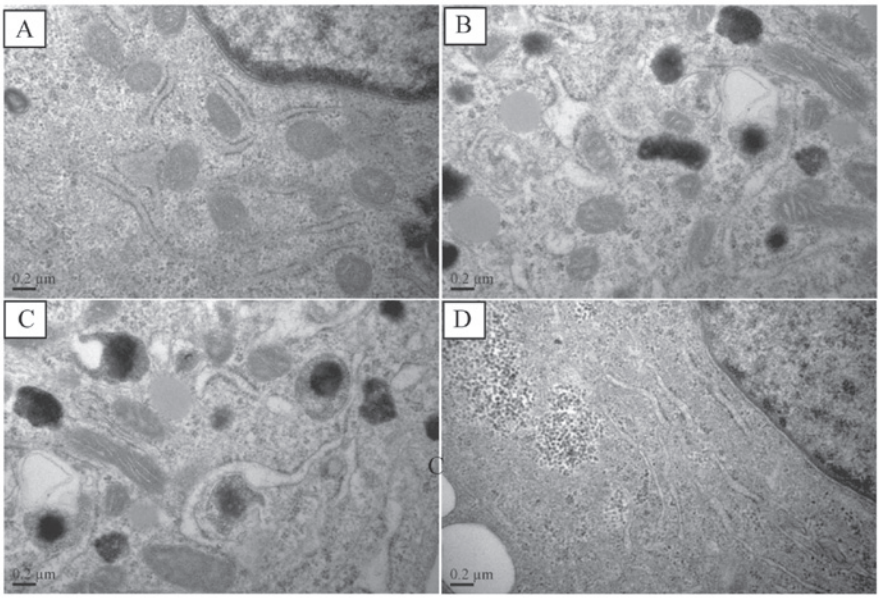

Figure 4. Morphological observations of the ER using electron microscopy. (A) Untreated cells, (B) H_2O_2-treated cells, (C) NaOH/H_2O_2-treated cells and (D) quercetin/H_2O_2-treated cells. ER dilation was observed in H_2O_2-treated and NaOH/H_2O_2-treated cells, while normal ER configuration was present in untreated and quercetin/H_2O_2-treated cells (magnification, x50,000). ER, endoplasmic reticulum; H_2O_2, hydrogen peroxide.

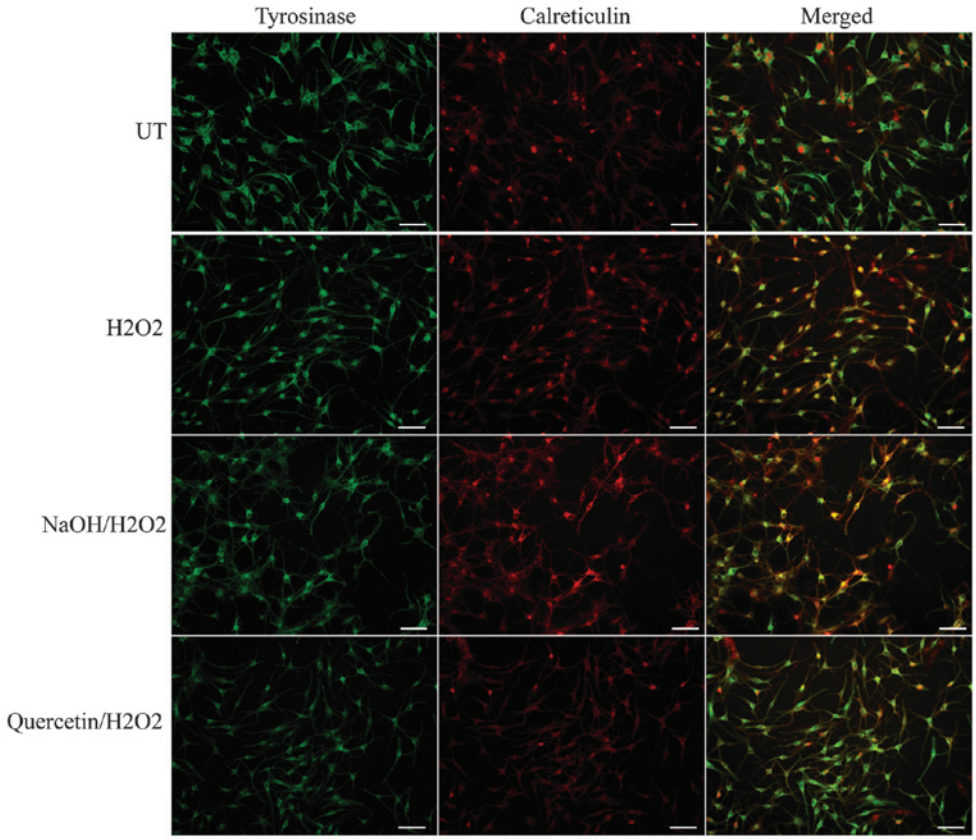

Figure 5. Characterization of tyrosinase and calreticulin co-localization using confocal laser scan microscopy. Merged images are shown in the last column (magnification, x400). Tyrosinase (green fluorescence), a key enzyme for melanin synthesis, and calreticulin (red fluorescence), an ER marker protein, are co-localized in the ER of the H_2O_2 and NaOH/H_2O_2 groups. Expression location of tyrosinase is beyond the endoplasmic reticulum marked by calreticulin. ER, endoplasmic reticulum; UT, untreated group; H_2O_2, hydrogen peroxide.

ER marked by calreticulinin, which suggested that the export of tyrosinase from the ER was disordered (Fig. 5); this therefore indicated that H_2O_2 interfered with the functional export of tyrosinase from the ER.

Quercetin attenuates H_2O_2-induced inhibition of tyrosinase expression. Western blot analysis was used to determined the expression levels of tyrosinase in melanocytes. As shown in Fig. 6, tyrosinase expression was significantly increased in

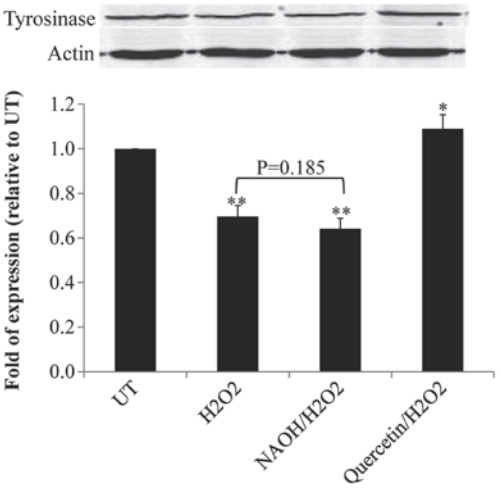

Figure 6. Quercetin protects melanocytes against H_2O_2-induced inhibition of tyrosinase expression. Following 24 h starvation, cell lysates were collected from melanocytes and western blot analysis was used to determine protein expression levels of tyrosinase and the internal control (β-actin). Values are presented as the mean ± standard deviation. *P<0.05 vs. UT, **P<0.01 vs. UT. UT, untreated group; H_2O_2, hydrogen peroxide.

the quercetin/H_2O_2 group compared with that of the untreated group (P=0.046). By contrast, tyrosinase expression was significantly decreased in the H_2O_2 (P<0.01) and NaOH/ H_2O_2 groups (P<0.01) compared with that of the control; however, no significant differences were observed between these two groups (P=0.185). This therefore indicated that quercetin attenuated the H_2O_2-induced inhibition of tyrosinase expression.

Discussion

H_2O_2 was previously demonstrated to inhibit tyrosinase expression and melanocyte viability; in addition, quercetin was found to protect melanocytes from H_2O_2-mediated oxidative stress (13). This previous study primarily focused on melanocyte viability; however, in the present study, the effects of H_2O_2 on the tyrosinase export from the ER and morphology of the ER, as well as the attenuation of H_2O_2-induced oxidative stress by quercetin, were further investigated.

The present study determined that 200 μM H_2O_2 and 25 μM quercetin, with incubation for 24 h were the optimum parameters for the experiments performed. ROS levels in melanocytes of different treatment groups were determined using DCFH-DA. ROS production was found to increase 1.5 fold in the H_2O_2-treated group compared with that of the untreated group, whereas cells that underwent quercetin/H_2O_2 treatment demonstrated comparative ROS levels with those of the untreated group. This therefore suggested that quercetin attenuated the H_2O_2-induced increase in ROS levels. The effects of H_2O_2 on ER modality in melanocytes was observed using electron microscopy; markedly dilated ER were observed in the H_2O_2-treated group, whereas normal ER configuration was found in the quercetin/H_2O_2-treated and untreated groups. This therefore indicated H_2O_2 induced ER dilation in melanocytes, which was prevented by quercetin treatment. The ER is a vital and highly dynamic organelle present in all eukaryotic cells; a multitude of parameters inside the cell and in its microenvironment significantly influence the complex functions of ER. Factors including the availability of glucose (hypoglycemia), hyperthermia, calcium levels and the redox milieu, impact and disturb the proper functioning of the ER, resulting in ER stress, which results in improper protein folding in the lumen of the ER (19,20). Pronounced dilation of the ER lumen is a well established ultrastructural response to ER stress; under which mammalian cells have been reported to expand their ER volume several fold (21,22). The results of the present study demonstrated that ER volume of melanocytes increased 2.02±0.07 fold following H_2O_2 treatment, therefore indicating that H_2O_2 disturbed the proper function of the ER, while quercetin attenuated the effects of H_2O_2.

Tyrosinase is the core enzyme that catalyzes melanogenesis in melanocytes. Abnormalities in the post-translational processing of tyrosinase have been implicated in several depigmentation diseases (23). Stagnation of tyrosinase in ER was found to be relevant to the phenotype of pigment loss in melanoma (24). The dysfunctional transportation of tyrosinase from the golgi to melanosomes leads to different diseases, including generalized albinism types 2 and 4 as well as Hermansky-Pudlak syndrome (25-27). Therefore, in the present study, in order to evaluate protein processing and transport in the ER of melanocytes in different treatment groups, confocal laser scanning microscopy was performed to assess the co-localization of tyrosinase and calreticulin. The result demonstrated that tyrosinase and calreticulin expression were both localized in the ER of H_2O_2- and NaOH/H_2O_2-treated cells. A large amount of tyrosinase was not observed at the endoplasmic reticulum marked by calreticulin in untreated and quercetin/H_2O_2-treated cells. These results suggested that H_2O_2 hindered tyrosinase export from the ER; however, quercetin pretreatment enabled cells to maintain the effective export of tyrosinase from the ER. Furthermore, comparative expression levels of tyrosinase were observed in the quercetin/H_2O_2-treated and untreated groups; however, tyrosinase expression was significantly decreased in the H_2O_2 and NaOH/H_2O_2 groups.

Numerous studies have reported that antioxidants may protect melanocytes against oxidative stress; green tea extract was found to protect cellular membranes against t-butylhydroperoxide-induced oxidative damage (28), and a combination of vitamins C and E demonstrated a protective effect against ultraviolet radiation (29,30). Quercetin is found in a variety of plant-based foods, including red onions, red grapes and a certain berries (31). The potential chemopreventive effects of quercetin have been attributed to various mechanisms, including its antioxidative activity as well as its capacity to inhibit enzymes that activate carcinogens (resulting in the modification of signal transduction pathways) and interact with and regulate cell receptors and other proteins (32). The results of the present study demonstrated that quercetin protected melanocytes from the effects of H_2O_2 on the morphology of ER, tyrosinase export from the ER and tyrosinase expression.

In conclusion, the results of the present study showed that H_2O_2 induced the dilation of ER lumina and hindered the functional export of tyrosinase from the ER, while quercetin attenuated these effects induced by H_2O_2. To the best of our knowledge, the present study provided the first evidence that H_2O_2 has an important role on the ER morphology of melanocytes and functional export of tyrosinase from ER. These

results may aid in elucidating the potential effect of antioxidants on the ultrastructure of melanocytes.

Acknowledgements

The present study was supported partly by grants from the National Natural Science Foundation of China (grant nos. 30800563 and 81071294), the Zhejiang Provincial Natural Science Foundation of China (grant nos. Y2101132, Z2100973, LY12H11008 and LY12H11009), the Science Technology Department of Zhejiang Province (grant no. 2013C33093) and the Hangzhou Science and Technology project (grant no. 20092133W04). Additionally, the authors would like to acknowledge the financial support from the State Clinical Key Specialty Construction Project and Zhejiang Provincial Program for the Cultivation of High-level Health talents.

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