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Quercetin phospholipid complex significantly protects against oxidative injury in ARPE-19 cells associated with activation of Nrf2 pathway

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ABSTRACT

Age-related macular degeneration (AMD) is a major cause of blindness worldwide. Oxidative stress plays a crucial role in the pathogenesis of dry AMD. Quercetin has potent anti-oxidative activities, but poor bioavailability limits its therapeutic application. Herein, we prepared the phospholipid complex of quercetin (quercetin-PC), characterized its structure by differential scanning calorimetry, infrared spectrum and x-ray diffraction. Quercetin-PC had equilibrium solubility of 38.36 and 1351.27 μg/ml in water and chloroform, respectively, which was remarkably higher than those of quercetin alone. Then we established hydrogen peroxide (H_2O_2)-induced oxidative injury model in human ARPE-19 cells to examine the effects of quercetin-PC. Quercetin-PC, stronger than quercetin, promoted cell proliferation, and the proliferation rate was increased to be 78.89% when treated with Quercetin-PC at 400 μM. Moreover, quercetin-PC effectively prevented ARPE-19 cells from apoptosis, and the apoptotic rate was reduced to be 3.1% when treated with Quercetin-PC at 200 μM. In addition, quercetin-PC at 200 μM significantly increased the activities of SOD, CAT and GSH-PX, and reduced the levels of reactive oxygen species and MDA in H_2O_2-treated ARPE-19 cells, but quercetin at 200 μM failed to do so. Molecular examinations revealed that quercetin-PC at 200 μM significantly activated Nrf2 nuclear translocation and significantly enhanced the expression of target genes HO-1, NQO-1 and GCL by different folds at both mRNA and protein levels. Our current data collectively indicated that quercetin-PC had stronger protective effects against oxidative-induced damages in ARPE-19 cells, which was associated with activation of Nrf2 pathway and its target genes implicated in antioxidant defense.

1. Introduction

Age-related macular degeneration (AMD) is a leading irreversible blindness in developed countries, accounting for 8.7% of blindness worldwide. Currently, there are about 30 million AMD patients, of which more than 0.5 million loss their vision due to AMD (Klein and Klein, 2013). AMD is commonly divided into two categories: dry AMD and wet AMD. The former is characterized by drusen and/or pigmentary abnormalities, geographic atrophy occurs in its advanced stage, leading to decreased visual acuity or, sometimes, progressing to wet AMD (Buschini et al., 2015). The wet AMD is pathologically featured by choriodal neovascularization (CNV), resulting in retinal exudation and hemorrhage and eventually serious impairment of vision (Ishikawa et al., 2015). At present, much attention has been attached to the therapeutic treatment for the wet AMD and several anti-angiogenic agents such as pegaptanib, ranibizumab and conbercept have been demonstrated to effectively prevent the progressing vision loss in patients with wet AMD (Hernandez-Pastor et al., 2010; Li et al., 2014). However, little is known about the mechanisms underlying dry AMD and effective therapeutic options for dry AMD are urgently needed.

It has been generally accepted that RPE cell senescence, metabolism disorder, and impairment of functions are key initiating factors for the pathogenesis of AMD. A variety of aetiologies can damage RPE cells by optical radiation, oxidative stress, lipofuscin deposition, and immune and inflammatory reactions (Mettu et al., 2012). Nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant responsive element (ARE) system is the most important defensive signaling pathway for combating oxidative stress in mammals. Nrf2 is located in cytoplasm binding to Keap1 and subjected to rapid degradation by ubiquitin proteasome pathway.

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When exposed to reactive oxygen, Nrf2 dissociates with Keap1 and translocates into nucleus, where it forms a heterodimer with Maf and then binds to ARE, regulating the transcription activity of genes encoding phase II metabolizing enzymes and antioxidases, including heme oxygenase-1 (HO-1), quinone oxidoreductase-1 (NQO-1), glutathione peroxidase (GSH-PX), glutamate cysteine ligase (GCL), glutathione, epoxide hydrolase, etc (Itoh et al., 2010; Zhang, 2006). These enzymes protect against the damages caused by reactive or poisonous substances, and modulating cell proliferation and death. Activation of Nrf2/ARE pathway can enhance the capacity of scavenging of reactive oxygen species, maintain the equilibrium of redox state, and attenuate oxidative damage within cells by induction of phase II metabolizing enzymes and antioxidases (Ma, 2013).

Quercetin is a widespread flavonoid compound in natural sources. Mounting evidence suggests that quercetin has pharmacological activities of anti-oxidation, reactive oxygen species scavenging, anti-inflammation, and anti-cancer (Ishizawa et al., 2011). However, quercetin has poor water solubility and fat solubility with low bioavailability (Cai et al., 2013). Phospholipid complex of active components derived from medicinal herbs can significantly improve the physicochemical properties of the original compound, increasing the absorption and action duration, eliminating adverse reactions, and enhancing pharmacological effects (Choi et al., 2009). Therefore, we herein prepared the phospholipid complex of quercetin (quercetin-PC) and characterized its physicochemical properties. Furthermore, we examined the protective effects of quercetin-PC on oxidative stress-induced damages in ARPE-19 cells, and explored the roles of Nrf2 pathway and the downstream phase II metabolizing enzymes and antioxidases in quercetin-PC effects.

2. Materials and methods

2.1. Regents and antibodies

Reagent grade soy lecithin (purity ≥ 97%) was purchased from Shanghai Guyan Industrial Co., Ltd. (Shanghai, China). Hydrogen peroxide (H_2O_2) and quercetin were obtained from Sigma Chemical (St. Louis, MO, USA). The primary antibodies used in western blot analyses against Nrf2, HO-1, and GCL were from Abcam (Cambridge, UK). The primary antibody against NQO-1 and the secondary antibody Goat Anti-Rabbit IgG/HRP were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents were all high grade for experiments.

2.2. Preparation of quercetin-PC

We used the solvent method to prepare quercetin-PC. Briefly, quercetin and soy lecithin (mole ratio 1:1) were dissolved in ethanol of adequate volume stirring for 1 h. Decompression distillation was carried out to remove ethanol. After 12 h vacuum drying, the resulting precipitates were grinded and sieved through 100 mesh sieve, and stored in a desiccator for experiments. The physical mixture of quercetin and soy lecithin was prepared by evenly grinding the two components (mole ratio 1:1) in a mortar.

2.3. Structural characterization of quercetin-PC

Firstly, differential scanning calorimetry was performed to determine the temperature of phase transformation point of quercetin, soy lecithin, physical mixture of quercetin and soy lecithin, and quercetin-PC. The testing conditions were 20–500 °C temperature range and 10 °C/min heating rate. Secondly, each sample of 1.5 mg and KBr of 200 mg were compressed and subjected to infrared spectra analyses (scanning range 4000–400 cm^{-1}). Thirdly, the four kinds of samples were also subjected to x-ray diffraction analysis and the detection conditions were as follows: copper target, temperature 50 °C, voltage 40 kV, current 100 mA, scanning rate 4 °C/min, and scanning range 5–50 °C.

2.4. Determination of quercetin-PC solubility

We firstly determined the standard curve. Quercetin of 10 mg was dissolved in ethanol and volumed in 50 ml volumetric flask, and thereby the quercetin control solution at a concentration of 200 mg/l was obtained. Anhydrous ethanol was used to dilute to control solution to yield a series of solutions at 10, 20, 40, 60, 80, and 100 mg/l. Each solution of 10 μl was injected into high performance liquid chromatograph (HPLC) for measurement of peak area. The chromatographic conditions were Zorbax SB C_{18} column, methanol-0.4% H_3PO_4 solution (v/v 50:50) as the mobile phase, 370 nm wavelength, 1.0 ml/min flow velocity, and 30 °C column temperature. Concentrations of quercetin ([*C*]) were marked at the horizontal ordinate and peak areas (*A*) at the longitudinal coordinate giving the standard curve equation: $A=4926.3\times[C]-10.4029$ ($R=0.9999$), linear range 2.08–93.5 μg/ml. We then performed precision test. Control solution samples of 10 μl was precisely subjected to HPLC analyses for 6 repeated times. Measurements of quercetin peak areas showed that the relative standard deviation was 0.92%, indicating favorable precision. Thirdly, quercetin, physical mixture and quercetin-PC (all containing 20 mg quercetin) were precisely weighted and added into 100 ml conical flask, and then distilled water/chloroform of 20 ml was added. The solutions were incubated in a 25 °C thermostatic oscillator for 6 h. Each sample of 5 ml was filtered through 0.45 μm microporous membrane. The successive filtrate of 10 μl was subjected to HPLC analyses. Control solution of 10 μl at 40 mg/l was spontaneously analyzed by HPLC. The equilibrium solubility of the three kinds of samples in water and chloroform was measured respectively.

2.5. Cell culture

Human RPE cell line ARPE-19 was purchased from the American Type Culture Collection (USA). These cells are isolated from human retinal pigmented epithelium, and form stable monolayers, which exhibit morphological and functional polarity. They express the RPE-specific markers CRALBP and RPE-65. ARPE-19 cells were cultured in Dulbecco's modified eagle medium (DMEM; Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown in a 95% air and 5% CO2 humidified atmosphere at 37 °C.

2.6. Cell proliferation assay

ARPE-19 cells were seeded in 96-well plates (1×10^4/well) and cultured in DMEM with 10% FBS for 24 h. Cells were treated with vehicle (DMSO), quercetin and quercetin-PC at indicated concentrations for 6 h, and then additionally treated with H_2O_2 (200 μM) for 24 h. Then the medium was replaced with 100 μl phosphate buffered saline (PBS) containing 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) incubating at 37 °C for 4 h. Next, the crystals were dissolved with 200 μl DMSO. The spectrophotometric absorbance at 490 nm was measured by a SPECTRAmax™ microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Results were from three independent experiments and each experiment had six replicates.