Isorhamnetin prevents H_2O_2-induced oxidative stress in human retinal pigment epithelial cells

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Abstract. Isorhamnetin, a 3-O-methylated metabolite of quercetin, exhibits antioxidant effects. However, to the best of our knowledge, no study to date has focused on the effects of isorhamnetin on retinal pigment epithelium (RPE) cells, and its underlying molecular mechanisms. Therefore, the present study aimed to examine the potential protective effect of isorhamnetin against oxidative stress in human RPE cells. The results demonstrated that pretreatment of RPE cells with isorhamnetin significantly protected cell viability against oxidative stress. In addition, isorhamnetin pretreatment inhibited hydrogen peroxide (H_2O_2)-induced reactive oxygen species (ROS) production and caspase-3 activation in RPE cells. Furthermore, isorhamnetin pretreatment significantly increased the phosphorylation of phosphoinositide 3-kinase (PI3K) and AKT serine/threonine kinase 1 (Akt) in RPE cells exposed to H_2O_2, compared with cells treated with H_2O_2 alone. Taken together, the present results demonstrated that isorhamnetin protected human RPE cells from oxidative stress-induced cell death, and this effect was associated with activation of the PI3K/Akt signaling pathway. Thus, isorhamnetin may be considered as a potential antioxidant useful for the prevention of age-related macular degeneration.

Introduction

Age-related macular degeneration (AMD) is a leading cause of visual impairment in the elderly in developed countries (1). Epidemiological studies have demonstrated that the prevalence of AMD in the aged population is increasing steadily every year (2). The retinal pigment epithelium (RPE), which forms a blood-retinal barrier between the neural retina and choriocapillaris, serves a pathophysiological role in the process of AMD (3). Oxidative stress is crucial in the development of AMD (4,5). Previous studies have reported that RPE cells are susceptible to oxidative damage, and oxidative stress from hydrogen peroxide (H_2O_2) leads to RPE cell death by causing preferential damage to mitochondrial DNA (6,7). Thus, strategies for protecting RPE cells against oxidative damage may be helpful for preventing the progression of AMD.

Isorhamnetin is one of the major active components isolated from herbal medicinal plants, such as *Hippophae rhamnoides* and *Ginkgo biloba* (8). Previous studies have indicated that isorhamnetin exhibits anti-inflammatory and antitumor effects, and that it protects ventricular myocytes from ischemia and reperfusion injury (9-11). In addition, isorhamnetin has been reported to exhibit antioxidant properties (12-14). Choi (15) reported that isorhamnetin reverses H_2O_2-induced growth inhibition and exhibits scavenging activity against intracellular reactive oxygen species (ROS) in mouse-derived C2C12 myoblasts (15). However, the effects of isorhamnetin on RPE cells and the underlying molecular mechanism remain unclear. Therefore, the aim of the present study was to examine the effect of isorhamnetin against oxidative stress in human RPE cells.

Materials and methods

Reagents and antibodies. Isorhamnetin (purity >98%) was purchased from Shanghai Tongtian Biotechnology Co., Ltd. (Shanghai, China). Antibodies against phosphoinositide 3-kinase (PI3K; 1:2,000; cat no. sc-365290), phosphorylated (p)-PI3K (1:2,000; cat no. sc-293115), AKT serine/threonine kinase 1 (Akt; 1:2,000; cat no. sc-5298), p-Akt (1:2,000; cat no. sc-52940), GAPDH (1:1,000; cat no. sc-365062) and horseradish peroxidase-conjugated secondary antibodies (1;3,000; cat no. sc-2370) were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Cell culture. The human RPE cell line ARPE-19 was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/Nutrient Mixture F-12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA),

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supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin, in a 37˚C incubator under a humidified atmosphere containing 5% CO_2.

Cell viability assay. Cell viability was evaluated using an MTT assay. Briefly, ARPE-19 cells ($4x10^4$ cells/well) were pretreated with various concentrations of isorhamnetin (25, 50 and 100 μM) for 24 h prior to exposure to 250 μM H_2O_2 for 24 h. The medium was then replaced with fresh medium containing 0.5 mg/ml MTT for 4 h at 37˚C. The MTT formazan crystals were dissolved in dimethyl sulfoxide, and the absorbance was measured at 450 nm with a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA). The relative cell viability was defined as the absorbance of treated cells divided by that of the control untreated cells.

Measurement of intracellular ROS generation. Intracellular ROS production was measured using the 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe (Sigma-Aldrich; Merck KGaA). Briefly, following treatment, ARPE-19 cells were washed twice with PBS, and then incubated with 10 mM H_2DCFDA for 30 min at 37˚C in the dark. Then, the cells were harvested and fluorescence of the resulting dichlorofluorescein (DCF) was measured using an F-2500 spectrophotometer (Hitachi, Ltd., Tokyo, Japan) at 488 nm excitation and 525 nm emission. The results were expressed as the fluorescence intensity of DCF of each sample relative to the control.

Caspase-3 activity assay. Caspase-3 activity was detected using a caspase-3 cellular activity assay kit (Merck KGaA), as per the manufacturer's instructions. Briefly, following treatment, ARPE-19 cells were harvested and then suspended in the cell lysis buffer. Cell lysates were incubated in the presence or absence of 5 μl Asp-Glu-Val-Asp-p-nitroanilide at 37˚C for 1 h. Following washing, the fluorescence released by active caspase-3 was measured using a microplate reader at 405 nm wavelength.

Western blot analysis. ARPE-19 cells were harvested, washed twice with PBS, and lysed in radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride (both from Shenneng Bocai Biotechnology Co., Ltd., Shanghai, China) on ice for 10 min. Cell lysates were centrifuged at 13,000 x g for 15 min at 4˚C. The supernatants were collected, and the protein content of each lysate was measured by BCA protein assay (Pierce; Thermo Fisher Scientific, Inc.). Protein (30 μg/lane) was separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked in 5% non-fat dry milk and TBST buffer (5 mM Tris-HCl, pH 7.4, 136 mM NaCl and 0.1% Tween-20) for 1 h at room temperature, and incubated with specific antibodies (p-PI3K, PI3K, p-Akt, Akt, and GAPDH) overnight at 4˚C. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) for another 1 h at room temperature. The blots were visualized with an enhanced chemiluminescence system (GE Healthcare Life Sciences, Chalfont, UK) and grey intensity analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

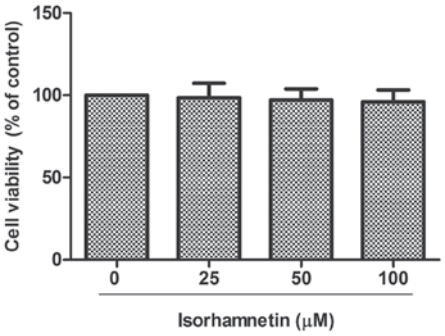

Figure 1. Effect of isorhamnetin treatment on ARPE-19 cell viability. ARPE-19 cells were pretreated with various concentrations of isorhamnetin (0, 25, 50 and 100 μM) for 24 h, and cell viability was determined by MTT assay. Data are expressed as mean ± standard deviation of three independent experiments.

Statistical analysis. The SPSS software package (version 17.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are expressed as mean ± standard deviation of three independent experiments performed in triplicate. Significant differences were determined using a Student's t-test or one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Isorhamnetin is not cytotoxic in ARPE-19 cells. First, the potential cytotoxicity of isorhamnetin was examined on human ARPE-19 cells using the MTT assay. As demonstrated in Fig. 1, isorhamnetin did not significantly affect the viability of ARPE-19 cells in doses up to 100 μM. Therefore, 25-100 μM of isorhamnetin was used for treatments in the following experiments.

Isorhamnetin treatment protects human RPE cells against oxidative stress. The effect of isorhamnetin on the cell viability of H_2O_2-treated ARPE-19 cells was examined by MTT assay. The results demonstrated that H_2O_2 treatment resulted in a significant decrease in cell viability compared with untreated cells, whereas pretreatment with isorhamnetin significantly reversed this decrease in a dose-dependent manner (Fig. 2).

Isorhamnetin treatment inhibits H_2O_2-induced ROS production in human RPE cells. Increased ROS levels result in oxidative stress and are hypothesized to be critical in the pathogenesis of AMD. Therefore, the effect of isorhamnetin on ROS generation was examined in ARPE-19 cells exposed to H_2O_2. As indicated in Fig. 3, treatment with H_2O_2 for 24 h dramatically increased the production of ROS compared with control untreated cells. However, pretreatment with isorhamnetin significantly suppressed the H_2O_2-induced ROS production in a dose-dependent manner (Fig. 3).

Isorhamnetin treatment inhibits H_2O_2-induced caspase-3 activity in human RPE cells. Caspase-3 is a main executor and

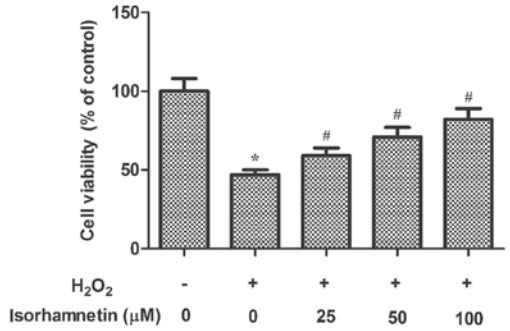

Figure 2. Isorhamnetin treatment protects human retinal pigment epithelium cells against oxidative stress. ARPE-19 cells were pretreated with various concentrations of isorhamnetin (0, 25, 50 and 100 μM) for 24 h, prior to exposure to 250 μM H_2O_2 for 24 h. Cell viability was determined by MTT assay. Data are expressed as mean ± standard deviation of three independent experiments. *P<0.05 vs. control untreated group; #P<0.05 vs. H_2O_2 group. H_2O_2, hydrogen peroxide.

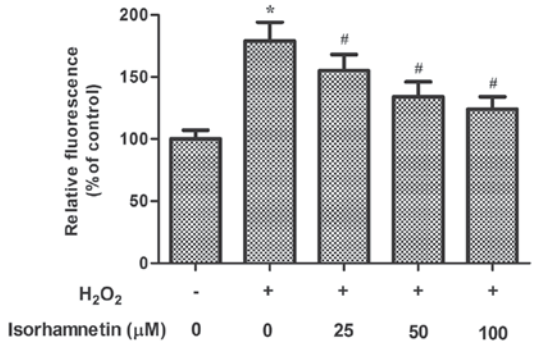

Figure 3. Isorhamnetin treatment inhibits H_2O_2-induced ROS production in human retinal pigment epithelium cells. ARPE-19 cells were pretreated with various concentrations of isorhamnetin (0, 25, 50 and 100 μM) for 24 h prior to exposure to 250 μM H_2O_2 for 24 h. Intracellular ROS production was measured using a 2',7'-dichlorodihydrofluorescein diacetate probe. Data are expressed as mean ± standard deviation of three independent experiments. *P<0.05 vs. control untreated group; #P<0.05 vs. H_2O_2 group. H_2O_2, hydrogen peroxide.

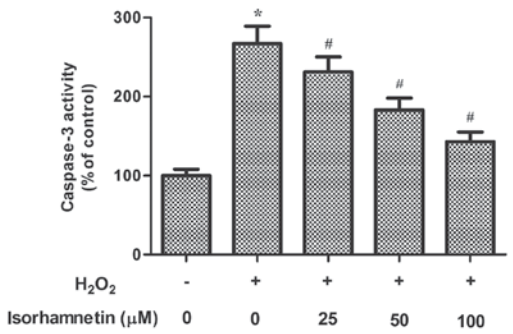

Figure 4. Isorhamnetin treatment inhibits H_2O_2-induced caspase-3 activation in retinal pigment epithelium cells. ARPE-19 cells were pretreated with various concentrations of isorhamnetin (0, 25, 50 and 100 μM) for 24 h prior to exposure to 250 μM H_2O_2 for 24 h. Caspase-3 activity was measured using a caspase-3 cellular activity assay kit. Data are expressed as mean ± standard deviation of three independent experiments. *P<0.05 vs. control untreated group; #P<0.05 vs. H_2O_2 group. H_2O_2, hydrogen peroxide.

an established marker of cell apoptosis, so caspase-3 activity was measured as a surrogate for cell death initiation (16). As

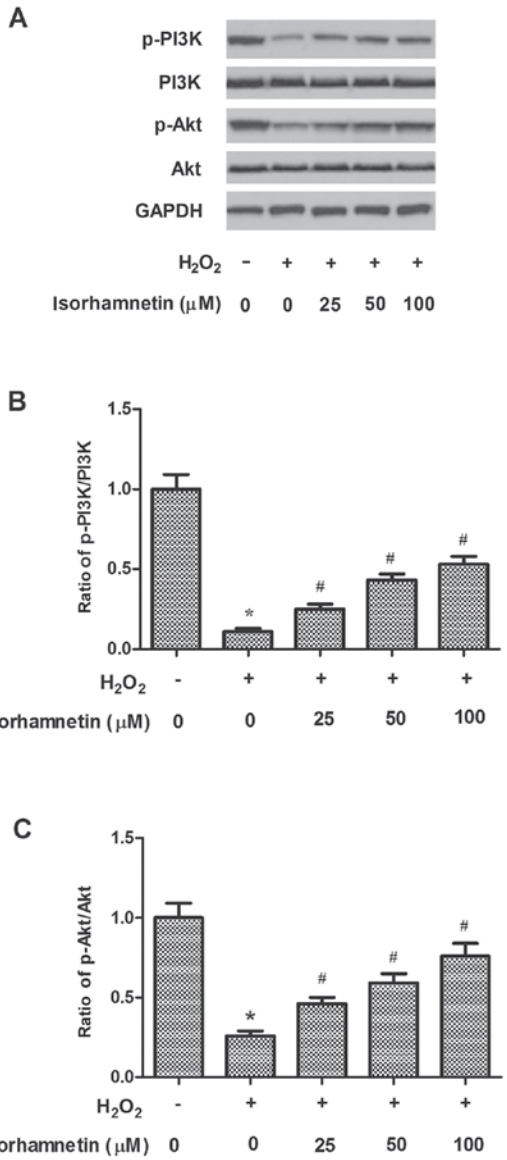

Figure 5. Isorhamnetin treatment results in PI3K/Akt pathway activation in retinal pigment epithelium cells. ARPE-19 cells were pretreated with various concentrations of isorhamnetin (0, 25, 50 and 100 μM) for 24 h prior to exposure to 250 μM H_2O_2 for 24 h. Protein expression levels of p-PI3K, total PI3K, p-Akt, total Akt and GAPDH (as a loading control) were examined by western blotting. (A) Representative images. (B) Quantitative analysis of p-PI3K/PI3K ratio. (C) Quantitative analysis of p-Akt/Akt ratio. Data are expressed as mean ± standard deviation of three independent experiments. *P<0.05 vs. control untreated group; #P<0.05 vs. H_2O_2 group. PI3K, phosphoinositide 3-kinase; Akt, AKT serine/threonine kinase 1; H_2O_2, hydrogen peroxide; p, phosphorylated.

demonstrated in Fig. 4, H_2O_2 treatment significantly increased caspase-3 activity, compared with untreated control. This H_2O_2-induced increase in caspase-3 activity, however, was dramatically attenuated by isorhamnetin pretreatment in a dose-dependent manner (Fig. 4).

Isorhamnetin treatment activates the PI3K/Akt pathway in human RPE cells. Activation of the PI3K/Akt signaling pathway is important in protecting RPE cells from oxidative stress (17). Therefore, the hypothesis that the PI3K/Akt pathway may be involved in the protective effects of isorhamnetin on

H_2O_2-induced cell death was tested in ARPE-19 cells. As demonstrated in Fig. 5, phosphorylation of PI3K and Akt were significantly decreased by H_2O_2 treatment, compared with the control group, as evident by the decreased p-PI3K/PI3K and p-Akt/Akt ratios. However, pretreatment with isorhamnetin for 24 h markedly increased phosphorylation of both PI3K and Akt in ARPE-19 cells exposed to H_2O_2, compared with cells treated with H_2O_2 alone (Fig. 5).

Discussion

The present study focused on the effect of isorhamnetin on oxidative stress in human RPE cells. The results provide the first evidence that pretreatment of RPE cells with isorhamnetin significantly protected cell viability against oxidative stress. Furthermore, isorhamnetin inhibited H_2O_2-induced ROS production and caspase-3 activation in ARPE-19 cells. This protective effect of isorhamnetin may be mediated through PI3K/Akt signaling in ARPE-19 cells.

H_2O_2 is a well-established model of oxidative stress in RPE cells because of its rapid membrane permeability (18). A vast array of studies have demonstrated that oxidative stress induced by chemical oxidants, such as H_2O_2, leads to RPE damage and cell death (5,19,20). For these reasons, H_2O_2 was selected in the present study as an inducer of oxidative stress in human RPE cells to investigate the effect of isorhamnetin. The results demonstrated that H_2O_2 treatment resulted in a significant decrease in viability of ARPE-19 cells. However, pretreatment with isorhamnetin significantly reversed this decrease in a dose-dependent manner, implying that isorhamnetin exhibits a protective effect against oxidative stress in RPE cells.

Oxidative damage is important in the pathogenesis of AMD. ROS levels are increased in retinas of patients with AMD, resulting in oxidization of key cellular components in local RPE cells and severe RPE damage (21). It has been reported that isorhamnetin pretreatment blocks tertiary-butyl hydroperoxide-induced ROS production in hepatocytes (12). Sun *et al* (22) reported that isorhamnetin significantly decreases ROS generation in H9c2 cardiomyocytes exposed to H_2O_2. In accordance with these previous reports, the present results demonstrated that H_2O_2 treatment significantly increased intracellular ROS production in human RPE cells compared with untreated cells, and that this effect was significantly inhibited by isorhamnetin pretreatment. These results suggest that isorhamnetin protected ARPE-19 cells from H_2O_2-induced cell damage through its antioxidant activity.

Caspase-3 activation is an initiative process of cell apoptosis (23). It has been reported that exposure of RPE cells to increased ROS results in mitochondrial DNA damage and induces caspase-3 activation (24). In the present study, pretreatment with isorhamnetin was demonstrated to significantly suppress H_2O_2-induced caspase-3 activation, compared with cells treated with H_2O_2 alone. These data suggest that isorhamnetin protected ARPE-19 cells from H_2O_2-induced cell damage via its anti-apoptotic effect.

There is substantial evidence that the PI3K/Akt signaling pathway is involved in regulating the antioxidant function in RPE cells (25-27). Akt becomes phosphorylated in a PI3K-dependent manner in response to oxidants, such as H_2O_2, in human RPE cells, and treatment with a PI3K inhibitor (LY294002) blocks H_2O_2-mediated Akt phosphorylation and significantly enhances caspase-associated RPE cell death (28); this result is different to that observed in the present study which, may be due to the length of H_2O_2 exposure. In the study by Yang *et al* (28), when RPE cells were exposed to H_2O_2 for 15 min, p-Akt levels increased. However, in the present study, cells were exposed to H_2O_2 for 24 h, and the phosphorylation of PI3K and Akt significantly decreased. A recent study reported that isorhamnetin attenuates atherosclerosis by suppressing apoptosis in THP-1-derived macrophages via PI3K/Akt activation (29). In the present study, it was demonstrated that pretreatment with isorhamnetin significantly enhanced phosphorylation of PI3K and Akt in RPE cells exposed to H_2O_2, compared to cells exposed to H_2O_2 alone. These data suggest that isorhamnetin may have protected human RPE cells from oxidative stress-induced cell death, through activation of the PI3K/Akt signaling pathway.

In summary, the present report demonstrated that isorhamnetin protected human RPE cells from oxidative stress-induced cell death, which may be associated with the activation of PI3K/Akt signaling. Thus, isorhamnetin may be considered as a potential antioxidant agent towards the prevention of AMD.

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