Taxifolin protects RPE cells against oxidative stress-induced apoptosis

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Purpose: Oxidative stress-induced damage to RPE cells has been suggested to be an important factor in the pathogenesis of age-related macular degeneration. Taxifolin, a flavonol, has been shown to exhibit significant antioxidant properties. The purpose of this study was to investigate the potential protective effects of taxifolin on RPE cells cultured under oxidative stress conditions and to elucidate the underlying mechanisms.

Methods: Human RPE (ARPE-19) cells were treated with different concentrations of taxifolin and 0.4 mM of H₂O₂ for 24 h. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Apoptosis was quantitatively measured by annexin V/propidium iodide double staining, and the expression levels of poly (ADP-ribose) polymerase (PARP) were evaluated by western blotting. Reactive oxygen species (ROS) were measured using a commercially available ROS detection system. The expressions of phase II enzymes, including NAD(P)H quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and glutamate–cysteine ligase modifier (GCLM) and catalytic (GCLC) subunits, were examined using real-time PCR and western blotting. The nuclear localization of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) protein was detected by western blotting.

Results: Taxifolin clearly inhibited the decrease in H₂O₂-induced cell viability, cell apoptosis, and intracellular ROS generation. In addition, taxifolin inhibited the H₂O₂-induced PARP cleavage. Moreover, treatment with taxifolin activated mRNA and the protein expression of NRF2 by inducing the translocation of NRF2 to the nucleus. Consequently, the mRNA and protein levels of the phase II enzymes NQO1, HO-1, GCLM, and GCLC increased.

Conclusions: Taxifolin was shown to protect RPE cells against oxidative stress-induced apoptosis. The potential mechanism appears to involve the activation of NRF2 and the phase II antioxidant enzyme system.

Age-related macular degeneration (AMD) is a progressive eye disease caused by the degeneration of photoreceptors and adjacent RPE cells in the macula, the central portion of the retina. AMD is the leading cause of irreversible visual impairment and blindness among people aged 60 years and older [1,2]. It is a multifactorial late-onset disease, and oxidative stress-induced RPE cell damage is suggested to be an important factor of AMD [3-5]. Oxidative stress produces reactive oxygen species (ROS) and non-radical species, such as H₂O₂, which damage the cellular components of RPE cells, leading to apoptotic cell death [6-8]. Therefore, our studies have focused on methods for protecting RPE cells from oxidative stress-induced injury.

Taxifolin (3,5,7,3′,4′-pentahydroxy-flavanone or 2,3-dihydroquercetin), a type of flavonoid, is abundant in citrus fruits, grapes, olive oil, and onions [9-11]. As a common bioactive constituent of foods and herbs, taxifolin has been shown to exert a wide range of biochemical and pharmacological effects, including antitumor, anti-inflammatory, anti-diabetic, hepatoprotective, cardioprotective, and neuroprotective effects, and it contributes to the prevention of Alzheimer’s disease [12-20]. Importantly, taxifolin exerts significant antioxidant effects that are critical in preventing the onset of apoptosis [21]. Moreover, taxifolin has also been found to inhibit oxidative enzymes and the overproduction of ROS, thus ameliorating cerebral the ischemia–reperfusion injury [22].

However, the potential protective effects of taxifolin on AMD have not been studied. Therefore, in the present study, we investigated the cytoprotective effect of taxifolin on the oxidative stress induced by H₂O₂ in RPE cells and we explored the underlying mechanisms.
**METHODS**

*Cell culture and chemicals:* The RPE cell line, ARPE-19, was obtained from the American Type Culture Collection (Manassas, VA, Appendix 1). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; HyClone, Logan, UT) at 37 °C in a humidified atmosphere of 5% CO₂. Taxifolin, H₂O₂, 2',7'-dichlorodihydro-fluorescein diacetate (DCFDA), and all other routine chemicals were purchased from Sigma (St. Louis, MO).

**Figure 1.** Taxifolin prevented the decrease in retinal pigment epithelial (RPE) cell viability induced by H₂O₂. A: ARPE-19 cells were incubated with different concentrations of taxifolin (0, 10, 20, 50, and 100 µg/ml) for 24 h. B: ARPE-19 cells were treated with different concentrations of H₂O₂ (0, 50, 100, 200, and 400 µM) for 24 h. C: ARPE-19 cells were treated with 0.4 mM H₂O₂ for 24 h in the presence of different concentrations of taxifolin. Data are shown as the mean ± SD (n = 3); *p<0.05, **p<0.01, and ***p<0.001.
Cell viability assays: The viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Briefly, cells were plated at a density of $3 \times 10^4$ cells/well into 96-well plates. (This density leads to >90% confluence for 24 h). After 24 h of incubation, a fresh medium containing 10% FBS and 20 μl of an MTT solution (5 mg/ml) was added to each well. The plate was incubated for an additional 4 h at 37 °C, and absorbance was measured at 540 nm using a microwell plate reader (BioTek, Winooski, VT). Each individual measurement was repeated three times.

Apoptosis assay: Cells were stained using FITC Annexin V apoptosis detection kit (556,547, BD Biosciences, Franklin Lakes, NJ) according to the manufacturer’s instructions and they were subjected to analysis by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ). The results are presented as the mean values from three independent determinations.

Measurement of cellular ROS: Intracellular ROS were measured by flow cytometry using a ROS detection kit (S0033, Beyotime, Shanghai, China). Briefly, cells were washed with phosphate-buffered saline (PBS) after treatment. Then, the cells were incubated with 15 μM DCFDA for 30 min at 37 °C. The cells were subsequently subjected to analysis using a FACSCalibur flow cytometer (BD Biosciences). Intracellular ROS levels are expressed as the mean DCFDA fluorescence intensity of the cells.

Western blotting assay: After treatment with taxifolin, cells were harvested by centrifugation. Cellular extracts were prepared by washing the cells with PBS and lysing them in a lysis buffer containing a protease inhibitor. Protein concentrations were measured using a BCA protein assay kit (P0009, Beyotime). Equal amounts of protein were loaded, separated by sodium dodecyl sulfate PAGE, and then transferred to a polyvinylidene difluoride membrane. After blocking with 5% milk for 1 h at room temperature, the membranes were incubated with primary antibodies, followed by incubation with...
horseradish peroxidase-conjugated secondary antibodies. The protein bands were visualized using an enhanced chemiluminescent substrate (Thermo Scientific, Rockford, IL).

RNA extraction and quantitative real-time PCR: Total RNA was extracted using the TRizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The amount and purity of total RNA were analyzed with an ultraviolet spectrophotometer at 260 nm. In addition, cDNA was reverse-transcribed from total RNA using the SuperScript III kit (12,574, Invitrogen). Quantitative real-time PCR was performed using an ABI PRISM 7000 fluorescent quantitative PCR system (Applied Biosystems, Foster City, CA). Each sample was measured in triplicate.

Silencing of Nrf2: Nrf2 levels were downregulated by transiently transfecting ARPE-19 cells with Nrf2 siRNA (5′-CACACTGGATCAGACAGGAGGATAT-3′; Genepharm, Shanghai) and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. As a negative control, cells were transfected with negative control siRNA (si-NC; Genepharm). The efficiency of downregulation was evaluated by western blot.

Statistical analysis: All statistical analyses were performed using the SPSS 18.0 software (SPSS, Chicago, IL). Data from at least three independent experiments, each performed in triplicate, are presented as the mean ± standard deviation (SD). The significance of the differences between groups was
estimated using the one-way ANOVA. Values were considered statistically significantly different when p<0.05.

RESULTS

Taxifolin inhibits a decrease in hydrogen peroxide-induced RPE cell viability: To determine whether taxifolin has cytotoxic effects on human RPE cells, we incubated ARPE-19 cells with different concentrations of taxifolin alone for 24 h. Figure 1A shows there was no significant difference in cell viability between the non-treated and taxifolin-treated groups, even at a high concentration of taxifolin (100 μg/ml). H₂O₂ has been shown to model the oxidative damage to cells [23]. Therefore, we incubated ARPE-19 cells for 24 h with different concentrations of H₂O₂. Figure 1B shows that H₂O₂ dose-dependently reduced the viability of human ARPE-19 cells, with an especially strong inhibition observed at a high concentration (0.4 mM) of H₂O₂. Therefore, 0.4 mM H₂O₂ was used in the following experiments. As shown in Figure 1C, the viability of ARPE-19 cells significantly and dose-dependently increased when the cells were incubated with 0.4 mM H₂O₂ in the presence of different concentrations of taxifolin for 24 h. Together, these results demonstrate that taxifolin inhibits the H₂O₂-induced decrease in RPE cell viability in a dose-dependent manner.

Taxifolin inhibits hydrogen peroxide-induced RPE cell apoptosis: After we demonstrated that taxifolin inhibited the H₂O₂-induced decrease in ARPE-19 cell viability, we investigated whether apoptosis was involved in the process and whether taxifolin inhibited H₂O₂-induced apoptosis. We divided the ARPE-19 cells into four groups: a control group, taxifolin-treated group, H₂O₂ (0.4 mM)-treated group, and H₂O₂ (0.4 mM) plus taxifolin (100 μg/ml)-treated group. After incubation for 24 h, the four groups were stained with annexin V/propidium iodide (PI), and flow cytometry was used to determine the apoptosis rate. Figure 2A, B shows that H₂O₂ (0.4 mM) induced ARPE-19 cell apoptosis, with more than 5% of cells showing early apoptotic signs (PI−/Annexin V+/+) and another 50% of cells showing late apoptotic signs (PI+/Annexin V+/+). Further, Figure 2C shows that taxifolin decreased H₂O₂-induced poly(ADP-ribose) polymerase (PARP) cleavage. Meanwhile, there was no significant difference between the control and taxifolin (100 μg/ml)-treated groups. These results demonstrate that taxifolin inhibits H₂O₂-induced ARPE-19 cell apoptosis.

Taxifolin inhibits hydrogen peroxide-induced ROS production: Next, we tested whether taxifolin affects H₂O₂-induced ROS production. ARPE-19 cells were divided into three groups: a control group, H₂O₂-treated group, and H₂O₂ plus taxifolin-treated group. After incubation for 24 h, we used DCFDA fluorescence to record the production of intracellular ROS. Figure 3A shows that taxifolin treatment inhibited
dramatically the H$_2$O$_2$-induced ROS production in ARPE-19 cells. Moreover, Figure 3B shows that despite the increased concentrations of H$_2$O$_2$, taxifolin exhibited a dose-dependent inhibition of intracellular ROS production as determined by quantitative analysis.

**Taxifolin activates mRNA and the protein expressions of NRF2, HO-1, NQO1, and GCLM:** As shown above, taxifolin protected ARPE-19 cells against oxidative stress by inhibiting H$_2$O$_2$-induced ROS production. It has been reported that the master transcriptional factor nuclear factor (erythroid-derived 2)-like 2 (NRF2) plays an important role in antioxidant responses and activates, in response to ROS, the expressions of cytoprotective genes, such as those encoding phase II enzymes [24]. To investigate the antioxidative mechanisms of taxifolin further, we determined the expression levels of NRF2, heme oxygenase-1 (HO-1), NAD(P)H quinine oxidoreductase 1 (NQO1), and the glutamate–cysteine ligase modifier (GCLM) and catalytic (GCLC) subunits. As shown in Figure 4A, treatment with taxifolin for 24 h upregulated the NRF2, HO-1, NQO1, and GCLM protein expressions in ARPE-19 cells in a dose-dependent manner. Figure 4B shows that the NRF2, HO-1, NQO1, and GCLM mRNA expressions were also significantly upregulated by taxifolin treatment for 24 h. These results suggest taxifolin protects ARPE-19 cells against oxidative stress by activating NQO1, HO-1, GCLC, and GCLM.

**Nrf2 mediates the taxifolin-induced RPE cytoprotective effect against H$_2$O$_2$:** SiRNA was used to study whether taxifolin-induced Nrf2 activation protects cells from oxidative stress-induced apoptosis by H$_2$O$_2$. Nrf2 siRNA-treated cells showed significantly lower levels of Nrf2 compared to untreated cells and negative control siRNA (si-NC) transfected cells (Figure 5A). Under a non-stressed condition, the downregulation of Nrf2 leads to a slight decrease in cell viability. When cells were treated with H$_2$O$_2$, the silencing of Nrf2 had a much lower survival rate and higher apoptosis rate than si-NC transfected cells (Figure 5B). Furthermore, taxifolin’s decreasing of the H$_2$O$_2$-induced poly (ADP-ribose) polymerase (PARP) hydrolysis product, cleaved PARP (Figure 5C).
cleavage was also abolished after the silencing of Nrf2. These data indicate that taxifolin promotes cell survival via Nrf2 activation (Figure 5C).

**DISCUSSION**

AMD is one of the most important causes of blindness among the elderly [25], and oxidative stress has been suggested to play an important role in the pathophysiology of AMD [9]. Most risk factors for AMD, except genetic predisposition, such as an age above 65 years, cigarette smoking, obesity, exposure to blue light, and bright irises, lead to oxidative stress in the retina [26-28]. Additionally, oxidative stress is aggravated by the presence of lipofuscin [29]. Therefore, protecting RPE cells against oxidative damage is an important consideration for treating AMD. In this study, we found that taxifolin exhibited protective effects against oxidative damage in cultured ARPE-19 cells, which indicates its potential clinical significance as a new effective therapeutic treatment for AMD.

Taxifolin is a flavanonol derivative of flavonoids, which are abundant in foods and herbs [30]. It exhibits a wide range of bioactive effects, among which the antioxidant activity is quite distinct [9]. In the present study, we first revealed that taxifolin protected ARPE-19 cells against oxidative stress-induced apoptosis. We adopted a classical model, which includes the addition of H2O2 to cultured cells, to test the susceptibility of ARPE-19 cells to oxidative stress and the antioxidant efficacy of taxifolin and we demonstrated that H2O2 induced a decrease in the viability of ARPE-19 cells. However, treatment with taxifolin inhibited a decrease in cell viability in a dose-dependent manner. It has been reported that oxidative stress plays an important role in cell apoptosis [31,32]. In this study, we found that H2O2 induced ARPE-19 cell apoptosis, which was inhibited by treatment with taxifolin. In addition, we detected the cleavage of PARP, which is often associated with apoptosis and has served as one of the hallmarks of apoptosis and caspase activation [33]. Western blot analysis showed that taxifolin decreased the H2O2-induced PARP cleavage. Moreover, using DCFDA fluorescence, we detected that taxifolin inhibited the H2O2-induced intracellular generation of ROS. Consequently, these results demonstrate that taxifolin protects ARPE-19 cells from H2O2-induced cell damage via its anti-apoptotic and antioxidative effects.

We investigated further the potential antioxidative mechanisms of taxifolin. It has been reported that the master transcriptional factor Nrf2 activates the expressions of cytoprotective genes in response to ROS and attenuates oxidative stress [34-36]. In a dormant state, Nrf2 is subjected to ubiquitin-mediated proteasomal degradation through interactions with its component, Kelch-like epithorohydrin (ECH)-associated protein 1 (KEAP1) [37]. Upon activation by oxidative stimuli, Nrf2 dissociates from KEAP1 and then translocates to the nucleus, where it activates the transcription of target genes and upregulates the levels of phase II defense enzymes, such as HO-1, NQO1, and GCLM [38-41]. In addition, studies have shown that taxifolin upregulated the Nrf2 pathway to curb the nuclear factor-kB-mediated Wnt/β-catenin signaling in colon carcinogenesis [42]. In this study, we investigated whether taxifolin targets the Nrf2 pathway and promotes the expression of phase II enzymes in ARPE-19 cells. The western blot results showed that treatment with taxifolin increased the protein levels and mRNA expressions of Nrf2, HO-1, NQO1, and GCLM in a dose-dependent manner. In addition, we found that ARPE-19 cells died despite taxifolin treatment after the downregulation of Nrf2 in the presence of H2O2. Therefore, these results suggested that the protective effects of taxifolin likely involve the upregulation of Nrf2 and the enhancement of the phase II antioxidant enzyme system.

In conclusion, the present study revealed a novel function of taxifolin, to protect RPE cells against oxidative stress by inhibiting the H2O2-induced decrease in cell viability, cell apoptosis, and the intracellular generation of ROS. The potential mechanism appears to involve the activation of Nrf2 and the upregulation of the phase II antioxidant enzyme system. Our results provide important information on taxifolin as a potential therapeutic agent for the prevention and therapy of AMD.

**APPENDIX 1. STR ANALYSIS**

To access these data, click or select the words "Appendix 1".

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