Protective effect of canolol from oxidative stress-induced cell damage in ARPE-19 cells via an ERK mediated antioxidative pathway

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Purpose: Oxidative stress damage to retinal pigment epithelial (RPE) cells is thought to play a critical role in the pathogenesis of age-related macular degeneration (AMD). This study was conducted to investigate the protective effect of canolol against oxidative stress-induced cell death in ARPE-19 cells and its underlying mechanism.

Methods: ARPE-19 cells, a human retinal pigment epithelial cell line, were subjected to oxidative stress with 150 μM t-butyl hydroxide (t-BH) in the presence/absence of canolol in different concentrations. Cell viabilities were monitored by a 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay. The apoptosis was measured by flow cytometry using Annexin V-FITC and PI staining and intracellular reactive oxygen species (ROS) levels were measured by a fluorescence spectrophotometer. Gene expression of NF-E2-related factor (Nrf-2), heme oxygenase-1 (HO-1), catalase and glutathione S-transferase-pi (GST-pi) were measured by a reverse transcription polymerase chain reaction (RT–PCR) assay. Activation of the extracellular signal regulated kinase (ERK) protein was evaluated by western blot analysis.

Results: Canolol showed relatively high safety for ARPE-19 cells and recovered the cell death caused by t-BH dose-dependently at a concentration of 50–200 μM. Canolol also reduced t-BH-induced intracellular ROS generation and thus protected ARPE-19 cells from cell apoptosis. HO-1, catalase, GST-pi, and Nrf-2 were elevated in ARPE-19 cells after treatment with different concentrations of canolol for 24 h. Finally, canolol was found to activate extracellular signal regulated kinase (ERK) phosphorylation in ARPE-19 cells under the condition, with or without t-BH.

Conclusions: Canolol protected ARPE-19 cells from t-BH-induced oxidative damage and the protective mechanism was associated, at least partly, with the upregulation (activation) of antioxidative enzymes, probably through an ERK mediated pathway. This suggests that canolol offers a remarkable protective effect against oxidative damage of RPE cells and may have a therapeutic effect on AMD and other oxidative stress-related retinal diseases.

Age-related macular degeneration (AMD) is one of the most common causes of severe visual loss in the elderly in developed countries [1]. The number of patients with AMD is expected to increase from 1.75 million to 3 million in the next decade [2]. In AMD, pathologic changes in the retinal pigment epithelium (RPE) have been observed early in the disease process, and reactive oxygen intermediates (ROS) may mediate RPE cell dysfunction and contribute to the development of AMD [3,4]. The decline of the phagocytic protective functions of RPE cells has been implicated in the etiology of AMD [5]. Therefore, strategies for protecting RPE cells against oxidative damage may be particularly important in retarding AMD. Increasingly, research has focused on the protection of RPE cells from oxidative damage [6-8]. The Age-Related Eye Disease Study (AREDS) recently confirmed that increasing the body’s defenses against oxidative stress using specific antioxidants and mineral supplements can preserve vision in patients with macular degeneration and can reduce the rate of disease progression [9].

Canolol, 4-vinyl-2, 6-dimethoxyphenol, is a phenolic compound recently isolated from crude canola (rape seed) oil, which exhibits potent antioxidant activity [10]. The scavenging potency of canolol against alkylperoxyl radical (ROO•) is much higher than that of well known antioxidants, such as α-tocopherol, vitamin C, β-carotene, lutein, and quercetin [11]. Canolol can suppress the induction of iNOS and various inflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and cyclooxygenase-2 (COX-2) in H. pylori-infected Mongolian gerbils, and can thus markedly suppress gastric carcinogenesis [12]. These findings suggest the potential of canolol as an anti-oxidant, anti-cancer, and anti-inflammatory...
agent, though the mechanism of the effect mentioned above is not fully understood.

Following a similar line of analysis, we anticipated the protective effect offered by canolol in retina, and thus its therapeutic potential for AMD. In this study, we first examined the cytoprotective effect of canolol against t-butyl hydroxide (t-BH)-induced cell death in ARPE-19 cells and then discussed the possible mechanisms involved.

**METHODS**

**Chemicals and reagents:** Canolol was purchased from Junsai Chemical Co (Tokyo, Japan) and was sealed using helium or nitrogen at −80 °C (Figure 1). Canolol was dissolved in ethanol and diluted in a serum-free medium immediately before the experiments. We obtained 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT), t-butyl hydroxide (t-BH), and U0126 from Sigma (St. Louis, MO). We purchased 2', 7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) from Molecular Probes (Eugene, OR). An Annexin V FITC-Propidium Iodide (PI) apoptosis kit was obtained from Becton Dickinson (Mountain View, CA). Primary bodies—rabbit polyclonal anti-phospho-ERK and anti-ERK—and secondary antibodies—horseradish peroxidase-conjugated anti-rabbit IgG—were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Cell culture:** The human retinal pigment epithelium cell line ARPE-19 was obtained from the American Type Culture Collection (ATCC, Mantissa, VA) [13], and was cultured in DMEM/Ham’s F12 medium (Invitrogen-Gibco, Grand Island, NY), supplemented with 10% heat-inactivated (56 °C, 0.5 h) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured at 5% CO$_2$ and 37 °C and were used at passage 10 to 15. They were seeded in a 100 mm culture dish (Falcon, Becton Dickinson) and were counted with a Casy cell counter (Schärfe System, Reutlingen, Germany) before seeding.

**Cell viability assay:** An 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide (MTT) assay, an assay of early mitochondrial dysfunction, was used to determine cell viability. Briefly, cells were plated at a density of 5×10$^4$ cells/well in 96-well microplates. After 24 h incubation, cells were pretreated with 25, 50, 100, and 200 µM canolol for 24 h. Then 150 µM t-BH was added to the cells and they were then cultured in combination for an additional 24 h, followed by MTT assay [14]. The absorbance of the samples was measured at 570 nm using a microplate reader (MTP-800; CORONA, Tokyo, Japan). The cell survival ratio was calculated as the percentage of the untreated control.

**Detection of intracellular ROS:** Intracellular accumulation of ROS was estimated using the fluorescent dye H$_2$DCFDA, which is converted to a membrane impermeable and highly fluorescent compound, dichlorofluorescin diacetate (DCF), in the cell in the presence of ROS [15]. The cells were pretreated with the vehicle (ethanol) or different concentrations of canolol for 24 h, and were then incubated with 150 µM t-BH for another 4 h. The cells were rinsed with a serum-free medium and were incubated in 5 µM H$_2$DCFDA for 60 min at 37 °C. The cells were then examined under a fluorescence microscope (CI-T-SM; Nikon, Tokyo, Japan). Then, the cells were collected and subjected to fluorescence spectrophotometer (F-2500; HITACHI, Tokyo, Japan) to detect the fluorescence of DCF inside cells (excitation=488 nm; emission=521 nm).

**Flow cytometric analysis using Annexin V-FITC and propidium iodide staining:** The cells were grown on a six-well plate at 1×10^5 cells per well and were pretreated with a vehicle (ethanol) or different concentrations of canolol for 24 h, before treatment with 150 µM t-BH for 6 h. The cells were washed and collected in PBS. Apoptosis staining was performed using an Annexin V FITC-PI apoptosis detection kit as per the manufacturer’s instructions. Stained cells were analyzed using FACS Calibur$^	ext{TM}$ Flow cytometry (BD Biosciences, San Jose, CA) with Cell-Quest software. Ten thousand events were collected for each sample.

**Semi-quantitative RT–PCR assay:** Total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized with 500 ng of DNA-free total RNA for each sample and an oligo-(dT) primer using TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Tokyo, Japan). The primers used are summarized in Table 1. The amplification was performed in the DNA Engine Opticon 2 System (Bio-Rad, Richmond, CA) under 32 cycles at 58 °C, 30 s; 62 °C, 30 s; and 94 °C, 30 s. PCR products were separated on 1.2% agarose gels and were visualized by staining with ethidium bromide. The intensity of the band was analyzed using image analyzing software (Eastman Kodak, Rochester, NY). GAPDH was used as an internal control for normalizing the relative abundance of each sample.
sample normalization. Data was expressed relative to the control subjects.

**Western blot analysis:** ARPE-19 cells (1×10⁶/10 cm dish) were cultured for 3 days. The cells were then incubated with 200 μM of canolol for 24 h with/without pretreatment with 10 μM U0126 for 30 min before 150 μM t-BH incubation for 1 h. After washing with ice-cold PBS, the cells were lysed in CellLytic M, Mammalian Cell Lysis / Extraction Reagent (Sigma, NY) with a protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan). The lysates were centrifuged (15,000× g for 10 min at 4 °C). The protein concentration was quantified by a Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Hampton, NH). The lysates (30 μg per lane) were separated by SDS-polyacrylamide gel (Precast 5%–20% gel, Atto Corp., Tokyo, Japan) and transferred to a Clear Blot Membrane-P (Atto Corp, Tokyo, Japan) at 30 V for 30 min. The membranes were blocked in 5% non-fat dried milk for 1 h at room temperature and were then incubated with primary antibodies (1:500 for the antibody of p-ERK and ERK, 1:2,000 for β-actin) in 5% nonfat dried milk overnight at 4 °C. After washing and blocking, the membranes were further incubated with horseradish peroxidase-linked secondary antibodies (1:10,000) for 1 h at room temperature. Signals were developed using the enhanced chemiluminescence system (ECL; GE Healthcare, Buckinghamshire, UK). Images were obtained and quantified using a Bio-Rad Versa Doc imaging system model 5000 (Bio-Rad, Hercules, CA) with Bio-Rad Quantity One software. The amount of protein expression was standardized against the amount of β-actin. The results were expressed relative to the untreated control.

**Statistical analysis:** All assays were performed using at least three separate experiments and in triplicate, data were expressed as mean±standard error (SEM). Statistical analysis of the data was performed using an unpaired, two-tailed Student t-test. A p value <0.05 was considered to be statistically significant.

**RESULTS**

**Canolol shows no obvious cytotoxicity on ARPE-19 cells:** We performed a cell viability assay to examine the cytotoxicity of canolol on ARPE-19 cells. The data indicated that because canolol is an extract of a natural product, it is safe for use in ARPE-19 cells up to 200 μM (Figure 2A). Therefore 50–200 μM of canolol was used in the following experiments.

**Canolol inhibits t-BH-induced ARPE-19 cell death:** Initial experiments were performed with an oxidative stress model using t-BH in ARPE-19 cells. It is known that t-BH exhibited cytotoxicity on ARPE-19 cells [16]. We confirmed this in our study. We exposed cultured ARPE-19 cells to increasing doses of t-BH (100, 120, 150, and 200 μM) for 24 h. Cell viability was determined using MTT assay. As shown in Figure 2B, ARPE-19 cells underwent cell death in a dose-dependent manner (p<0.01). At 150 μM, t-BH caused an approximate 50% loss in cell viability, and this dosage was used in our study to investigate the protective effect of canolol on t-BH-induced cell death.

The protective effects of canolol against t-BH-induced cell death in ARPE-19 cells are shown in Figure 2C. In a dose-dependent manner and with a concentration of 50–200 μM, canolol recovered the cell death caused by t-BH (p<0.01), with almost 100% of the cells surviving at a dose of 200 μM, whereas cell viability was about 50% without canolol treatment. Consistent with these results, the morphology of the cells pretreated with canolol was similar to that of the normal controls without t-BH (Figure 2D). Moreover, the protective effect of canolol was better than that of N-acetyl cysteine (NAC), a well recognized antioxidant. These observations

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**Table 1. Primers for RT–PCR assay.**

| Primers | Sequences |
|---------|-----------|
| **HO-1** | F: ATGGAGCGTCCGCAACCCGA<br>R: GCATAAGGCCTACAGCAAC |
| **Catalase** | F: AGCACAGTGGAGCAGTTTCAAGGG<br>R: CATCAGCTCTTACCAAAGGGTTTGGCC |
| **Nrf-2** | F: CGGTATGCAACAGGGACATTG<br>R: ACTGGTTGGGGCTTCTGATG |
| **GST-pi** | F: TGAATGACGGCGTGAGAG<br>R: CCCTCAGTTTTCCCGGATTGC |
| **GAPDH** | F: GAAGGTGAAGAGTCGGAGTC<br>R: GAAGATGGTGTATTGGATTTC |
Canolol inhibits t-BH-induced ARPE-19 cell damage: It has been reported that ROS generated by t-BH is responsible for cell damage in cultured ARPE-19 cells [17]. We therefore tested whether the cytoprotective effect of canolol in ARPE-19 cells against t-BH results from the reduction of ROS, by using H₂DCFDA, a fluorescent ROS indicator. The results showed that the mean fluorescence of intracellular ROS production was increased by about 2.3 fold in t-BH-induced cells relative to unexposed control cells. However, the increase in intracellular ROS was prevented by pretreatment with canolol in a concentration-dependent manner (p<0.01). At a canolol concentration of 200 μM, the intracellular ROS level decreased to a near normal level without t-BH treatment (Figure 3A,B). The antioxidative effect of canolol was more remarkable than that of NAC (Figure 3A), which was consistent with the results from the MTT assay. The data indicated that canolol can prevent intracellular ROS production in ARPE-19 cells challenged by t-BH.

Canolol inhibits t-BH-induced apoptosis in ARPE-19 cells: Apoptosis is the major consequence of cells exposed to t-BH [18]. To examine whether canolol protects against t-BH-induced apoptosis, the ARPE-19 cells were incubated with 100 μM and 200 μM of canolol for 24 h and were then treated with 150 μM t-BH for 6 h. A flow cytometric analysis was used to quantify the rate of cell apoptosis using double staining of Annexin V-FITC and PI. As shown in Figure 4, the lower right field (high Annexin V, low PI staining) indicates the apoptotic cells. Similar to previous reports, the cells exposed to 150 μM t-BH showed a 15-fold increase in the apoptosis rate compared to untreated control cells. Importantly, pretreatment with canolol significantly reduced the percentage of apoptotic cells in a dose-dependent manner: with 100 μM and 200 μM doses of canolol before t-BH exposure, the percentage of apoptotic cells reduced to 12.69±1.02% and 4.60±0.56%, respectively (p<0.01).

Canolol induces oxidative stress-related enzymes: To clarify the antioxidative mechanisms of canolol against t-BH-induced cell damage in ARPE-19 cells, we evaluated the expression levels of a variety of antioxidative enzymes, namely heme oxygenase (HO-1), catalase, and glutathione S-transferase-pi.
As shown in Figure 5, after incubation with different concentrations of canolol (0, 50, 100, and 200 μM) for 24 h, HO-1 and catalase mRNA levels were elevated compared to untreated control cells, which was consistent with the concentration of canolol, and GST-Pi was also observed though no dose-dependency was found (p<0.05). To better understand the protective mechanism of canolol on ARPE-19 cells, we examined the mRNA expression of NF-E2–related factor (Nrf-2). Nrf-2 expression was significantly induced by treatment with canolol, which was consistent with the expression of various antioxidative enzymes, i.e., HO-1, catalase (Figure 5). These results indicate that HO-1, catalase, and GST-pi may play an important role in the protective action of canolol.

Canolol activates ERK phosphorylation: Previous studies suggest that the overactivation of extracellular signal regulated kinase (ERK) may participate in the defense signaling against oxidative stress damage in cells [19]. To better understand the protective mechanism of canolol on ARPE-19 cells, we examined phosphorylation of the ERK protein using a western blot analysis. As shown in Figure 6A, canolol moderately activated phosphorylated ERK, both with and without t-BH, though no change in the total ERK protein level was observed (p<0.05). Furthermore, pre-treatment for 30 min with U0126 (10 μM), a specific inhibitor of the ERK kinase, abolished the ERK phosphorylation induced by canolol (Figure 6A). These data indicated that canolol could activate the ERK pathway in ARPE-19 cells.

We next addressed whether the enhanced cell viability of human RPE cells by canolol under oxidative stress was closely related to ERK phosphorylation. Data from the MTT assay showed that canolol effectively prevented ARPE-19 cells from t-BH-induced cell death, which was, however,
partially inhibited by U0126 (p<0.01). In addition, U0126 used in this study (10 μM) did not show apparent cytotoxicity in the cells (Figure 6B). In cells treated exclusively with t-BH, pretreatment with U0126 actually enhanced t-BH-induced ARPE-19 cell death (by 29%; Figure 6B), which demonstrates that activation of the ERK signaling pathway could protect cells from oxidative cell damage.

**DISCUSSION**

Ample studies have shown that canolol is an ROS scavenger in extracellular environments [7,8] and in several types of cells it has a protective effect against oxidative stress-induced cell damage. However, whether canolol possesses similar activity in retina is not known. In the present work, we first demonstrated that canolol could reduce t-BH-induced cell death, intracellular ROS production, and apoptosis in ARPE-19 cells, and that the mechanism of canolol induced cytoprotection was probably related to protective enzymes through the ERK pathway. This data thus suggested that canolol may have a potential therapeutic role in the prevention of early AMD.

Oxidative damage is thought to play an important role in the pathogenesis of AMD. Due to its unique location and function, RPE provides an ideal environment for the accumulation of ROS, which in turn leads to mitochondrial dysfunction and apoptosis [3-5]. Regarding the possible mechanism exposure to t-BH, cell viability was measured by an MTT assay. Data was expressed as a percentage of the control. Each value represents the mean±SEM (n=3) of three independent experiments. The single asterisk indicates p<0.05 and the double asterisk indicates p<0.01.
by which canolol inhibits t-BH-induced RPE cell death, our results showed that it may act through its antioxidative and anti-inflammatory effects. T-BH, a pro-oxidative stressor, has been reported to induce apoptosis in several cell types including RPE cells [18]. Therefore, we used t-BH as the oxidant model for classical oxidative stress. In our experiment, t-BH treated cells showed increased production of intracellular ROS (Figure 3), a consequent high incidence of apoptosis (Figure 4), and decreased cell viability (Figure 2). Canolol pretreatment significantly increased cell survival after t-BH incubation, which is consistent with the fact that canolol lowered intracellular ROS production in t-BH treated ARPE-19 cells (Figure 2). This antioxidative and cytoprotective effect of canolol on t-BH-induced cell damage was much more significant than the conventional antioxidant NAC (Figure 2, Figure 3). Moreover, flow cytometry analysis showed that t-BH-induced cell apoptosis in ARPE-19 cells was significantly reduced by canolol pretreatment (Figure 4). These results indicated that canolol prevented ARPE-19 cells from apoptosis caused by t-BH by reducing intracellular ROS production, and thus conferring a protective effect against t-BH-induced cell damage. Interestingly, we found that canolol more effectively protected ARPE-19 cells from death induced by t-BH than other oxidative stress models (data not shown). The findings indicated that canolol offers distinct protection against oxidative stress.

Several in vitro and in vivo studies have shown that canolol acts as an antioxidant and an anti-inflammatory agent [10-12]. However, the actual mechanisms of its action as an antioxidant are not fully understood. Like many flavonoids and other phenolic compounds from a range of plants, canolol can directly scavenge ROS [10]. In this study, we identified canolol’s antioxidative role through its induction of various antioxidative enzymes such as HO-1, catalase, and GST-pi.

Under physiologic conditions and to overcome the potential toxicity of ROS, cells have different endogenous antioxidant defense systems, including intracellular catalase, and HO-1, as well as antioxidative compounds (e.g., glutathione) [20]. The cellular defenses are considered to be important in protecting the RPE cells from oxidative stress and in reducing the progression of AMD [21]. Among these antioxidative enzymes, catalase appears to be crucial. Other antioxidative enzymes, such as HO-1 and GST-pi, are also known to play essential roles in many ROS-related diseases [22,23]. These enzymes and molecules provide cells with the ability to mount a prolonged and sustained defense against the deleterious effect of oxidants. Many flavonoids and other phenolic compounds from different plants were found to protect cells by stimulating oxidative stress-related genes [24-26]. In this study, we found that there was an increase in the expression of HO-1, catalase, and GST-pi upon canolol treatment (Figure 5), which was positively correlated with the protective effects of canolol. In addition, Nrf-2 is a key molecule for the basal and inducible expression of genes regulating the redox state of a cell [27]. By binding to the antioxidant response element (ARE), Nrf-2 induces the expression of antioxidative genes [28,29]. In keeping with this, we observed the upregulation of Nrf-2 in ARPE-19 cells with canolol treatment, which support the increased expression of HO-1, catalase, and GST-pi (Figure 5). These findings thus indicated that the induction of catalase, HO-1, and GST-Pi is involved in the mechanism of canolol’s protective effects.

The ERK cascade is one of the major mitogen-activated protein kinase (MAPK) pathways and its activation is generally thought to mediate cell survival. ERK was shown to protect against oxidative stress-induced cell injury and death [30]. It has been shown that overexpression of ERK 

\[ \frac{1}{2} \] confers resistance against RPE cell death due to serum-depletion–mediated oxidative stress [31], which suggested that ERK activation may be a general signaling defense against oxidative stress damage. Thus, we detected whether canolol could regulate the ERK signaling pathway. ROS itself could lead to transient and rapid activation of ERK [32], which was also seen in our experiments (Figure 6A). More importantly, canolol could significantly induce ERK activation and this effect was almost fully abolished when cells were pretreated with the ERK inhibitor, U0126. These results suggested that the ERK signaling pathway may lie upstream of the expression of antioxidative enzymes induced by canolol, which certainly warrants further investigation.

ERK phosphorylation was moderately elevated in the 1 h stimulation of t-BH (Figure 6A), whereas canolol treatment significantly enhanced ERK activation, which was partially inhibited by the ERK inhibitor (Figure 6A). These findings suggest that canolol activates survival signals through the ERK signaling pathway, as determined by a cell viability assay. This notion was further supported by the fact that administration of the ERK inhibitor significantly inhibited the antioxidative/cytoprotective effect of canolol (Figure 6B).

Taken together, our results showed that canolol actually protected ARPE-19 cells from t-BH- induced oxidative damage and the protective mechanism was associated, at least partly, with the upregulation (activation) of antioxidative enzymes, probably through an ERK mediated pathway. This study is the first to show the protective function of canolol in ARPE-19 cells and the various mechanisms involved. This suggests that canolol has a remarkable protective effect against the oxidative damage of RPE cells and that
have a therapeutic effect on AMD and other oxidative-stress-related retinal diseases.

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