The Protective Effect of Quercetin against Oxidative Stress in the Human RPE In Vitro

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Purpose. To investigate the possible protective effect of the dietary antioxidant quercetin on retinal pigment epithelial (RPE) cell dysfunction and cellular senescence occurring in age-related macular degeneration (AMD). The major flavonoid quercetin was studied on RPE cells in vitro.

Methods. Cultured human RPE cells were incubated with different concentrations of quercetin for 24 hours. Cells were then treated with 150 to 300 μM hydrogen peroxide for 2 hours. Mitochondrial function was measured by using MTT assay and cell vitality by live-dead staining assay. Intracellular levels of glutathione were determined by using a glutathione assay kit. Apoptosis was quantified by a caspase-3 assay, and cellular senescence was quantified by β-galactosidase staining. Expression of the senescence-associated transmembrane protein caveolin-1 was investigated by Northern and Western blot analyses.

Results. Hydrogen peroxide treatment caused significant decreases in mitochondrial function (52%) and in cell vitality (71%), whereas preincubation with 50 μM quercetin diminished this decrease in a dose-dependent manner. Quercetin treatment did not show any notable effect on intracellular levels of glutathione in either used concentration of quercetin. Hydrogen peroxide-induced activation of caspase-3 was reduced by 50 μM quercetin, from 1.9- to 1.4-fold, compared with untreated control ($P < 0.001$). Hydrogen peroxide caused a large (>90%) dose-dependent increase in β-galactosidase–positive cells, whereas in the untreated control only single cells expressed this enzyme (<5%). This increase in cellular senescence was significantly attenuated by quercetin in a dose-dependent manner. The highest attenuation was reached at 50 μM quercetin. Quercetin caused a significant dose-dependent reduction of caveolin-1 mRNA 48 hours after treatment with hydrogen peroxide. After 96 hours of incubation, caveolin-1 protein levels were also reduced.

Conclusions. The data demonstrate that quercetin is able to protect RPE cells from oxidative damage and cellular senescence in vitro in a dose-dependent manner. The authors suggest that this increase in antioxidative capacity is—among other mechanisms, such as the intracellular redox state—also mediated by inhibiting the upregulation of caveolin-1. Downregulation of caveolin-1 may be important for the retinal pigment epithelium to prevent apoptotic cell death in response to cellular stress, a condition implicated in the early pathogenesis of AMD. Therefore, the authors believe that the use of antioxidative dietary flavonoids such as quercetin is a promising approach in the prevention of early AMD. (*Invest Ophthalmol Vis Sci.* 2008;49:1712–1720) DOI:10.1167/iovs.07-0477

Age-related macular degeneration (AMD) is the leading cause of severe vision loss in the elderly in the developed world.[1–3] Despite the high prevalence of AMD, the complex pathogenesis of the disease is poorly understood. At present, there is no available efficient treatment for the nonexudative form of AMD that occurs early in disease progression. The clinical hallmark of this dry form of AMD is characterized by changes in the pigmentation of the retinal pigment epithelium and an accumulation of extracellular deposits between retinal pigment epithelial (RPE) cells and Bruch's membrane.[4,5] These alterations lead to RPE cell loss, subsequent death of photoreceptors, and, consequently, central vision impairment.

Evidence from a variety of studies supports an essential role for oxidative stress in the development of age-related RPE cell dysfunction. Because of high partial oxygen pressure from the underlying choriocapillaris, intense light exposure, and high concentrations of polyunsaturated fatty acids in photoreceptor outer segments, RPE cells are susceptible to damage by reactive oxygen intermediates.[6] Furthermore, the phagocytic function of the retinal pigment epithelium provides an additional oxidative burden.[7]

Increased dietary intake and serum levels of specific antioxidant nutrients may reduce the risk for AMD. Several epidemiologic studies have found correlations between the intake of foods high in antioxidants and the decreased risk for AMD.[8–10] Based on the results of the clinical Age-Related Eye Disease Study (AREDS) and the Lutein Antioxidant Supplementation Trial (LAST), the currently recommended supplementary antioxidants include vitamin C, vitamin E, beta carotene, and lutein.[9,10]

Another class of antioxidants that may play an important protective role includes the large group of polyphenolic compounds that provide much of the color and flavor of plant foods, bioflavonoids. The interest in bioflavonoids within the scientific community has been spurred by the dietary anomaly referred to as the French paradox, the correlation of a high-fat, but flavonoid-rich, diet with a lower incidence of coronary heart disease found in Mediterranean cultures.[11] Clinically relevant functions ascribed to flavonoids include antihypertensive activity, anti-inflammatory properties, hypocholesterolemic activity, platelet stabilization, and antiangiogenic effects. The most frequently studied flavonoid, quercetin, exhibits—in addition to its antithrombotic, anticarcinogenic, anti-inflammatory, antiallergic, and antiviral effects—in particular antioxidant and free radical-scavenging activities.[12–15] Intracellular dose-dependent uptake of quercetin and other flavonoids has been demonstrated in various cell types in vitro and is believed to be even higher in vivo than under normal culture conditions.[16,17] The major purpose of the present study was to investigate a possible protective effect of quercetin on cultured RPE monolayer cells treated with oxidative stress. Focusing on

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the possible mechanisms of the cytoprotective activity of quercetin, we investigated the viability of RPE cells treated with oxidative stress mediated by hydrogen peroxide (H_2O_2).

To evaluate the impact of oxidative damage on stress-induced premature senescence (SIPS), we used a histochemical staining procedure for beta galactosidase that had previously been developed to identify senescent cells.[18]

In evaluating the limiting effect of quercetin on SIPS, we investigated the expression of the plasma membrane-bound signal transduction protein caveolin. In other cell types, caveolin-1 has been shown to be upregulated by subcytotoxic levels of H_2O_2.[19]

In this study, we demonstrate that quercetin protects RPE cells from oxidative stress–induced cell death by the inhibition of caspase-3 activity. Subcytotoxic levels of H_2O_2 induce premature senescence in RPE cells and upregulate endogenous caveolin-1 expression. Cotreatment with H_2O_2 and quercetin does not promote premature senescence and does not upregulate caveolin-1 protein expression. Taken together, these results support the protective effect of quercetin in stress-induced changes of the RPE.

Materials and Methods

Isolation of Human RPE Cells

The eyes of five human donors were obtained from the Munich University Hospital Eye Bank and processed within 4 to 16 hours of death. Donor ages ranged from 16 to 76 years. None of the donors had any known history of eye disease. Methods for securing human tissue were humane, included proper consent and approval, were in compliance with the Declaration of Helsinki, and were approved by the local ethics committee. Human RPE cells were harvested by a previously described procedure.[20] Whole eyes were thoroughly cleansed in 0.9% NaCl solution, immersed in 5% poly(1-vinyl-2-pyrrolidone)-iodine (Jodobac; Bode-Chemie, Hamburg, Germany), and rinsed again in NaCl solution. The anterior segment from each eye was removed, and the posterior pole was examined with the aid of a binocular stereomicroscope to confirm the absence of gross retinal disease. The neural retina was carefully peeled away from the RPE-choroid-sclera with the use of fine forceps. The eyecup was rinsed with Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution and filled with 0.25% trypsin (Gibco BRL, Karlsruhe, Germany) for 30 minutes at 37°C. Trypsin was carefully aspirated and replaced with Dulbecco modified Eagle medium (DMEM; Biochrom, Berlin, Germany) supplemented with 20% fetal calf serum (FCS; Biochrom). With the use of a pipette, the medium was gently agitated, releasing the RPE into the medium and avoiding damage to Bruch membrane.

Human RPE Cell Culture

The RPE cell suspension was transferred to a 50-mL flask (Falcon, Wiesbaden, Germany) containing 20 mL DMEM supplemented with 20% FCS and maintained at 37°C in 5% CO_2. Epithelial origin was confirmed by immunohistochemical staining for cytokeratin with a pancytokeratin antibody (Sigma, Deisenhofen, Germany).[21] The cells were tested and found free of contaminating macrophages (anti-CD 11; Sigma) and endothelial cells (anti-von Willebrand factor [Sigma]; data not shown). Primary RPE cells were subcultured and maintained in DMEM supplemented with 10% FCS at 37°C in 5% CO_2. After 2 to 6 weeks, the cells reached confluence. All cells reached the proliferative state, but cultures from older donors took longer to reach the active growth phase than those from younger donors. Growth characteristics of our cultures were in line with previously published data.[22] We found no morphologic differences among the cells of donors of varying ages. To obtain sufficient cells for repeated experiments, cells were split 1:2 and confluent primary RPE cells of passages 4 to 6 were used. Whereas cells of earlier passages exhibited differences in their melanin granule content within the culture plate, cells gradually lost their melanin content during culture passaging. RPE cells of passages 4 to 6 showed diminished but homogenous pigmentation. Because of the antioxidative impact of melanin, recently demonstrated by Wang et al.,[23] only cell cultures with homogenous pigmentation were used for experiments. Before treatment with oxidative stress, cell morphology was assessed with a phase-contrast microscopy system. The cell medium was then changed, and cells were preincubated with different concentrations (1–200 μM) of quercetin dehydrate (Sigma-Aldrich). To simulate the in vivo situation with quercetin showing a half-life of 11 to 28 hours in human plasma, we chose a preincubation period of 24 hours in vitro.[17,24,25] Then phase-contrast microscopy was performed again.

In a first approach to evaluate cytotoxicity of quercetin as a baseline, live-dead assay was performed after incubation with quercetin in different concentrations. To induce oxidative stress, cells were washed three times with serum-free and phenol-free medium (Gibco BRL) and were incubated with 150 to 300 μM H_2O_2 in this medium for 2 hours. To reduce direct interactions with hydrogen peroxide and other reactive oxygen species, quercetin and FCS (containing potential antioxidative agents and containing albumin with a high affinity for quercetin) were not added to the culture medium during exposure to oxidative stress. The medium was subsequently changed and replaced by regular medium containing FCS.

Experiments investigating the effect of quercetin on caveolin-1 expression were performed with and without the addition of the iron chelator desferrioxamine (DFO; Sigma-Aldrich) in a concentration of 0.5 mM before hydrogen peroxide exposure.

Cell Viability Assay

Cell viability was quantified based on a two-color fluorescence assay. In this assay, the nuclei of nonviable cells were stained red by the membrane-impermeable dye propidium iodide (Sigma-Aldrich), whereas the nuclei of all cells were stained with the membrane-permeable dye Hoechst 33342 (Intergen, Purchase, NY). Confluent cultures of RPE cells growing on coverslips in four-well tissue culture plates were exposed to 300 μM H_2O_2 for 2 hours. For the evaluation of cell viability, cells were washed in PBS and incubated with 2 μg/mL propidium iodide and 1.0 μg/mL Hoechst 33342 for 20 minutes at 37°C. Subsequently, cells were analyzed with an epifluorescence microscope (Leica DMR; Bensheim, Germany). Representative areas were documented on film (Fujichrome 400; Fuji Film, Tokyo, Japan). Labeled nuclei were then counted in fluorescence photomicrographs, and dead cells were expressed as a percentage of the total nuclei in the field. Data were based on counts from three experiments performed in duplicate wells, with three to five documented representative fields per well.

MTT Assay

To determine cell viability after oxidative stress, cells were washed with PBS, and MTT solution (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) was added to RPE cell culture wells for 30 minutes at 37°C. After three washes of the cells with PBS (pH 7.4), the insoluble formazan product was dissolved in dimethyl sulfoxide. The optical density of each culture well was then measured using a microplate reader (Molecular Probes, Garching, Germany) at 550 nm. The optical density of formazan formed in control cells was taken as 100% of viability.

Glutathione Assay

RPE cells were rinsed three times with PBS and harvested. According to the protocol for the Calbiochem glutathione (GSH) assay kit (Merck KGaA, Darmstadt, Germany), cells were centrifuged at 2500*g* at 4°C for 5 minutes. Then 4 vol mercaptophosphoric acid solution was added. After vortexing, the solution was centrifuged at 3000*g* at 4°C for 10 minutes, and the clear supernatant was placed on ice for assay. After adding buffer, 4-chloro-1-methyl-7-trifluromethyl-quinolinium methyl sulfate, and 30% NaOH, sample volume was incubated at 4°C for 10 minutes in the dark, and final absorbance was determined by measuring optical density at 400 nm.

Caspase-3 Assay

Caspase-3 activity was determined with a colorimetric assay (ApoAlert; Clontech, Heidelberg, Germany) according to the manufacturer's protocol. In this assay, the capacity of the cellular caspase-3 to cleave the labeled substrate DEVD-p-nitroaniline (DEVD-pNA) was measured spectrophotometrically. In brief, apoptosis was induced by incubation with 300 μM H_2O_2 for 2 hours, as described. The cells were then harvested, and aliquots of 2.5×10^6 cells were used for each reaction. Cell lysates were incubated in the presence or absence of 5 μL caspase-3-substrate (DEVD-pNA) for 1 hour at 37°C. Absorbance was measured at 405 nm in a microplate reader (Versa-Max; Molecular Devices, Sunnyvale, CA). Uninduced and induced cells without substrate served as the background control. Induced cells were incubated with DEVD-CHO, an inhibitor of caspase-3 as a negative control.

β-Galactosidase Assay

Normal β-galactosidase histochemistry identifies the lysosomal form of the enzyme at pH 4.0, whereas senescence-associated beta galactosidase activity is observed at pH 6 in the cytoplasm. RPE cells were subjected to acid β-galactosidase staining using the senescence β-galactosidase protocol.[18] Cells were washed twice with phosphate-buffered saline (PBS) and fixed with fixative solution (2% formaldehyde/ 0.2% glutaraldehyde in PBS, pH 6) at room temperature for 4 minutes Cells were then washed twice with PBS and incubated under light protection for 8 hours at 37°C with fresh senescence-associated β-galactosidase staining solution (1 mg/mL 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside [X-gal], 40 mM citric acid/sodium phosphate, pH 6, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM $NaCl_2$, 2 mM $MgCl_2$). Cells were then examined for the development of blue color and photographed at low magnification (200×) with the use of a light microscope.

RNA Isolation and Northern Blot Analysis of Caveolin-1

After a 2-hour treatment period with H_2O_2, RNA isolation from cultured RPE cells was performed after an interval of 48 hours to allow RNA generation with respect to published data.[26] The concentration of hydrogen peroxide was reduced to 150 μM to diminish lethal oxidative stress to RPE cells. Total RNA was isolated by the guanidium thiocyanate-phenol-chloroform extraction method (Stratagene, Heidelberg, Germany). Structural integrity of the total RNA samples was confirmed by electrophoresis on 1% agarose gels. Total RNA (2 μg) was denatured and size fractionated by gel electrophoresis in 1% agarose gels containing 2.2 M formaldehyde. The RNA was then vacuum-blotted onto a nylon membrane (Roche, Indianapolis, IN) and cross-linked (1600 μJ, Stratalinker; Stratagene). To assess the amount and quality of RNA, the membrane was stained with methylene blue, and images were obtained (LAS-1000; RayTest, Pforzheim, Germany). Prehybridization was performed at 68°C for 1 hour. Hybridizations were performed at 68°C overnight in prehybridization solution (Dig Easy Hyb; Roche) containing 50 ng/mL digoxygenin-labeled, caveolin-1–specific sense 5′-GAGCTGAGCGAGAAGCAAGT-3′ and antisense 5′-ACAGCAAGCGGTAAAACCAG-3′ riboprobe. Riboprobes were synthesized as previously described.[27] After hybridization, the membrane was washed twice with 2 × SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature (RT), followed by two washes in 0.1 × SSC, 0.1% SDS, for 15 minutes at 68°C. After hybridization and posthybridization washes, the membrane was washed for 5 minutes in washing buffer (100 mM maleic acid [pH 7.5], and 150 mM NaCl, 0.3% Tween-20) and incubated for 60 minutes in blocking solution. The blocking solution contained 100 mM maleic acid (pH 7.5), 150 mM NaCl, and 1% blocking reagent (Roche). Anti-digoxigenin alkaline phosphatase (Roche) was diluted 1:10,000 in blocking solution, and the membrane was incubated for 30 minutes. After four additional washes in washing buffer (15 minutes each), the membrane was equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl [pH 9.5]) for 5 minutes. For fluorescence detection, a chemiluminescence substrate (CDP-Star; Roche) was diluted 1:100 with detection buffer, and the filter was incubated for 5 minutes at RT. After air drying, the semidry membrane was sealed in a plastic bag. Chemiluminescence was detected with the imager (LAS-1000; RayTest). Exposure times ranged between 5 and 40 minutes. Quantification of the chemiluminescence signal was performed on computer (AIDA software; RayTest).

Protein Isolation and Western Blot Analysis of Caveolin-1

Ninety-six hours after treatment with 150 μM H_2O_2, RPE cells grown on tissue culture dishes were washed twice with PBS (pH 7.2), collected, and lysed in SDS sample buffer for gel analysis.[28] Samples for gel analysis were boiled for 5 minutes, and their protein content was measured using BCA protein assay reagent (Pierce, Rockford, IL). For protein analysis (2 μg), 5% SDS-PAGE as stacking gel and 12% SDS-PAGE as separating gel were used. After electrophoresis, the proteins were transferred with semidry blotting onto a polyvinyl difluoride membrane (Roche). The membrane was incubated with PBS containing 0.1% Tween-20 (PBST, pH 7.2) and 5% bovine serum albumin for 1 hour. The primary antibody (caveolin-1 diluted 1:1000; BD Biosciences, Heidelberg, Germany) was then added and allowed to react overnight at room temperature. After three washes in PBST, an alkaline phosphatase-conjugated goat–anti-mouse antibody (diluted 1:10,000) was added for 30 minutes. Visualization of the alkaline phosphatase was achieved using chemiluminescence. CDP-star was diluted 1:100 in detection buffer, and the filter was incubated for 5 minutes at room temperature. After drying, the semidry membrane was sealed in a plastic bag. Chemiluminescence was detected with the LAS-1000 workstation. Exposure times ranged between 1 and 5 minutes. Quantification was performed using AIDA software.

Statistical Analysis

All data were collected in a spreadsheet (Excel 2000; Microsoft, Redmond, WA) and were analyzed using SPSS software (SPSS 13.0 for

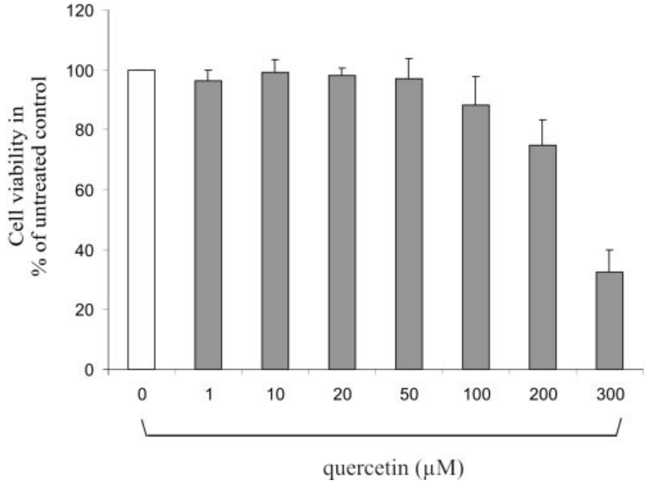

Figure 1. To assess the cytotoxicity of quercetin, RPE cells were incubated with different concentrations of quercetin alone for a period of 24 hours, and cell viability was determined by live-dead assay. Dead cells were stained with propidium iodide. Nuclear staining was conducted with Hoechst 33342. Data represent the average results of three independent experiments. Cell viability is expressed as mean percentage of control (100%). Marked cytotoxicity of quercetin is shown at concentrations 200 μM or greater. Error bars represent the SEM.

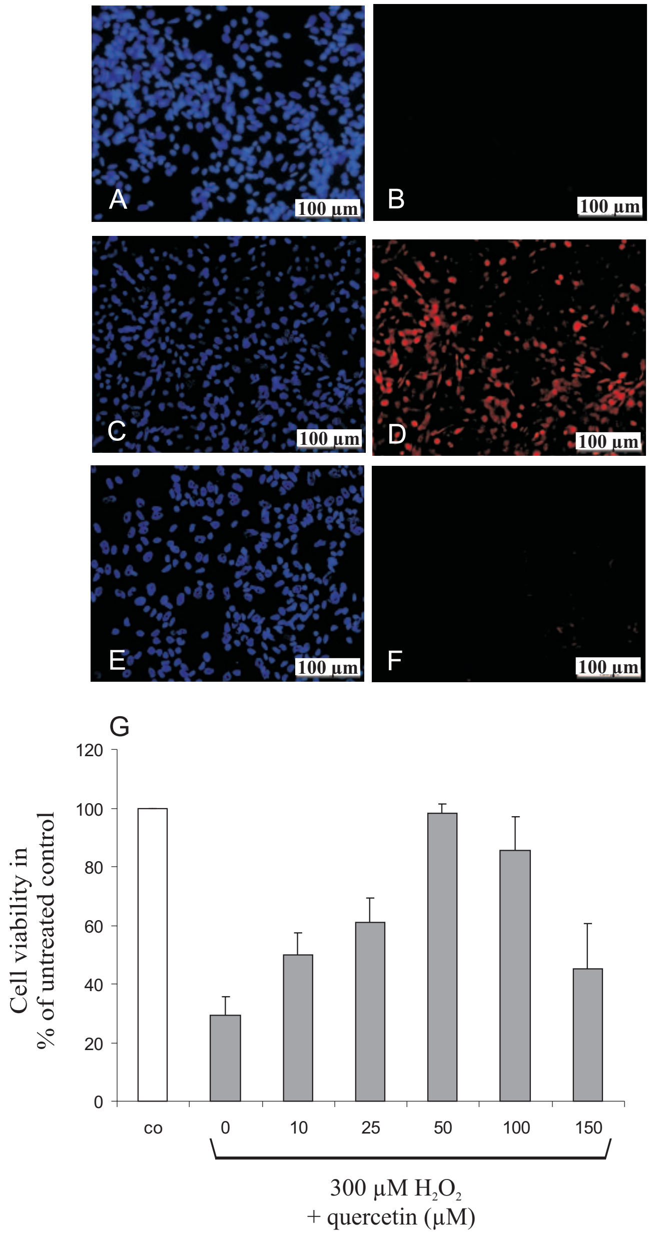

Figure 2. Oxidative stress–induced cell death is inhibited by preincubation with quercetin. RPE cells were treated with 300 μM H_2O_2 for 2 hours. Cell viability was determined 1 hour after exposure to oxidative stress by live-dead assay. **(A)** Representative fluorescence photomicrograph of Hoechst 33342-stained RPE cells. **(B)** Nonviable cells in the corresponding field. Propidium iodide staining is indicative of a loss of membrane integrity. **(C)** Representative fluorescence photomicrograph of Hoechst 33342-stained H_2O_2 treated RPE cells. **(D)** Nonviable cells in the corresponding field. **(E)** Fluorescence photomicrograph of cells pretreated with 50 μM quercetin and exposed to 300 μM H_2O_2 labeled with Hoechst 33342. **(F)** Nonviable cells in the same field as in **(E)**. **(G)** Quantification of the effect of quercetin treatment on the numbers of nonviable cells. The percentage of dead cells was scored by counting at least 700 cells in fluorescence photomicrographs of representative fields. Data (mean ± SEM) are based on the sampling of 6 to 10 photomicrographs per condition in three independent experiments performed in duplicate (*$P < 0.001$).

Windows; SPSS Inc., Chicago, IL). On all statistical tests, $P < 0.05$ was considered significant. Nonparametric tests were used where appropriate and multiple testing was corrected for (e.g., multiple comparisons of pairs were analyzed by the Friedman test method).

Results

Quercetin Protects RPE Cells from Oxidative Stress–Mediated Cell Death

Live-dead assay demonstrated that incubation with quercetin alone as a baseline induced no significant decrease in the number of living cells in concentrations of 0 to 100 μM. However, significant reductions in cell viability were noted in concentrations of 200 μM or greater (Fig. 1).

After exposure to oxidative stress, preincubation with quercetin protected RPE cells in a dose-dependent manner, with the highest protective effect at a concentration of 50 μM and a decreasing protective effect at concentrations of 100 μM or greater. Treatment with 300 μM H_2O_2 decreased the number of living cells to 29.4% (±6.2%; $P < 0.05$) of the untreated control. Preincubation with quercetin diminished this effect in a dose-dependent manner. Fifty percent (±7.4%; $P < 0.05$) of cells were alive after preincubation with 10 μM quercetin, 98.2% (±3.2%; $P < 0.05$) with 50 μM quercetin, 85.7% (±11.2%; $P < 0.05$) with 100 μM quercetin, and 45.2% (±15.3%; $P < 0.05$) with 200 μM quercetin (Figs. 2A–G). No statistically significant difference in cytotoxicity of incubation with quercetin alone or in oxidative stress response was noted for RPE cells of different donor ages (data not shown).

Treatment with 300 μM hydrogen peroxide for 2 hours caused a reduction in mitochondrial activity of 52% (±11%; $P < 0.05$). Preincubation with quercetin diminished this decrease in a dose-dependent manner. Mitochondrial activity after preincubation with 10 μM quercetin was 75.2% (±15.5%; $P < 0.05$) of control cells; 50 μM quercetin showed the highest protective effect, resulting in 84.8% (±12.1%; $P < 0.05$) mitochondrial activity in comparison with the untreated control. Quercetin concentrations higher than 50 μM led to decreased mitochondrial function (71.6% ± 10.9%; $P < 0.05$) at 100 μM, 68.8% (±10.1%; $P < 0.05$) at 150 μM, and 64.7% (±10.3%; $P < 0.05$) at 200 μM (Fig. 3).

Quercetin Does Not Influence Intracellular Glutathione Level in RPE Cells

To determine the cytoprotective effect of quercetin, intracellular GSH levels were measured. Average intracellular extinction of GSH in cultured RPE cells was set to 100%. Treatment with hydrogen peroxide alone did not decrease GSH levels significantly. Preincubation with quercetin at concentrations of

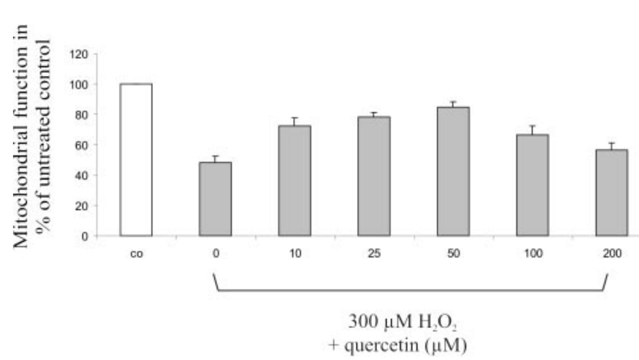

Figure 3. Quercetin prevents the reduction of mitochondrial activity by H_2O_2. After oxidative stress, mitochondrial activity was statistically reduced ($P < 0.05$) compared with untreated control cells. Preincubation with quercetin diminished this decrease in a dose-dependent matter. Results are expressed as the mean percentage of control mitochondrial activity (control OD 0.8 at 550 nm assigned as 100% mitochondrial activity). Bars represent the mean of five experiments, each performed in triplicate. Error bars represent the SEM.

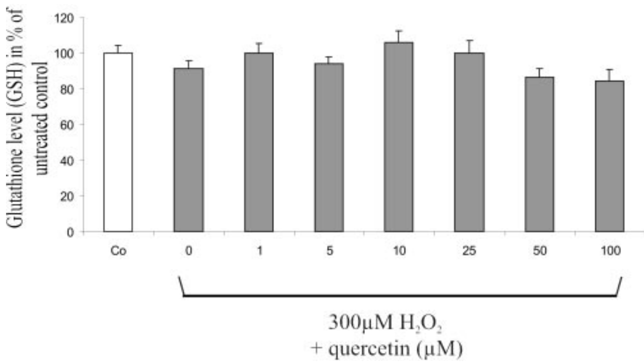

FIGURE 4. GSH concentration of RPE cells is not influenced by quercetin treatment. Cells were treated for 24 hours with quercetin before GSH assay was performed. Bars represent the mean of five experiments, each performed in triplicate. Error bars represent the SEM.

1 to 100 μM before exposure to oxidative stress showed no significant effect on intracellular GSH level (Fig. 4).

Quercetin Reduces Oxidative Stress–Induced Caspase-3 Activity

In a previous study, we showed that oxidative stress induces caspase-3 activation in cultured RPE cells.[20] In accordance with these results, treatment of RPE cells with 300 μM H_2O_2 for 2 hours caused an elevation of caspase-3 (1.9-fold). Preincubation with 50 μM quercetin inhibited H_2O_2-induced caspase-3-elevation (1.4-fold). The difference in the increase of caspase-3 activity of cells treated with quercetin and H_2O_2 and of cells treated with H_2O_2 alone was statistically significant ($P < 0.001$). Incubation with 50 μM quercetin alone for 24 hours did not show any change in caspase-3 activity (Fig. 5).

Quercetin Inhibits the Increase of Caveolin-1 by Oxidative Stress

Northern blot analysis of caveolin-1 mRNA showed a 2.4-fold increase of caveolin-1 48 hours after treatment with 150 μM H_2O_2 for 2 hours. Preincubation with 10 μM quercetin for 24 hours was able to inhibit this oxidative stress–induced increase in caveolin-1 expression. Higher concentrations of quercetin showed a similar effect in the decrease of caveolin-1 expression, reducing the caveolin-1 level to 50% of accordant control RPE cells (Fig. 6). On the protein level, H_2O_2 exposure caused a 2-fold increase of caveolin-1 96 hours after exposure to hydrogen peroxide incubation. Once again, this increase was prevented by preincubation with 10 to 150 μM quercetin.

Experiments performed with the addition of DFO to the culture medium before treatment with H_2O_2 showed different results. Incubation with DFO alone induced caveolin expression to 1.4-fold compared with the untreated control cells. Incubation with DFO and subsequent treatment with H_2O_2 led to caveolin expression that was even more increased, to 2.3-fold. Preincubation with 10 μM quercetin diminished this oxidative stress–induced increase to 1.5-fold, and preincubation with 50 μM quercetin diminished it to 1.8-fold. Preincubation with 100 μM and 150 μM quercetin showed no protective effect on caveolin expression (2.2-fold and 2.3-fold, respectively).

Quercetin Prevents Oxidative Stress–Induced Cellular Senescence

Treatment of RPE cells with 150 μM H_2O_2 caused a significant increase in β-galactosidase–positive cells, whereas in the untreated control only single cells (4.4% [± 2.1%]; $P < 0.05$) expressed this enzyme. After treatment with hydrogen peroxide for 2 hours, 91.9% (± 9.7%; $P < 0.05$) of RPE cells stained positive for β-galactosidase. This increase in cellular senescence was significantly attenuated by preincubation with quercetin in a dose-dependent manner. Preincubation with 1 μM quercetin resulted in 46.4% (± 7.8%; $P < 0.05$) positively stained cells, 5 μM quercetin in 42.3% (± 7.2%; $P < 0.05$) positively stained cells, 10 μM in 42.4% (± 4.3%; $P < 0.05$) positively stained cells, 20 μM quercetin in 15.5% (± 8.2%; $P =$ 0.052) positively stained cells, and 50 μM quercetin with maximal attenuation to 5.0% (± 2.1%; $P < 0.05$) positively stained cells. However, preincubation with 100 μM quercetin decreased the number of β-galactosidase–positive cells to only 14.3% (± 13.6%; $P < 0.05$), as shown in Figure 7D. In the β-galactosidase assay, no statistically significant difference in oxidative stress–induced staining was noted for cells of different donor ages (data not shown).

DISCUSSION

Our results demonstrate for the first time that quercetin is able to protect human RPE cells in vitro from oxidative damage and cellular senescence in a dose-dependent manner. Quercetin is well known as a powerful free radical scavenger and as a chelating agent that inactivates the metal iron responsible for the generation of reactive oxygen species.[29] In line with these data are our findings that inhibition of the iron-chelating effect diminished the impact of quercetin on oxidative stress–induced caveolin expression. However, the addition of the iron chelator DFO to the culture medium itself increased caveolin

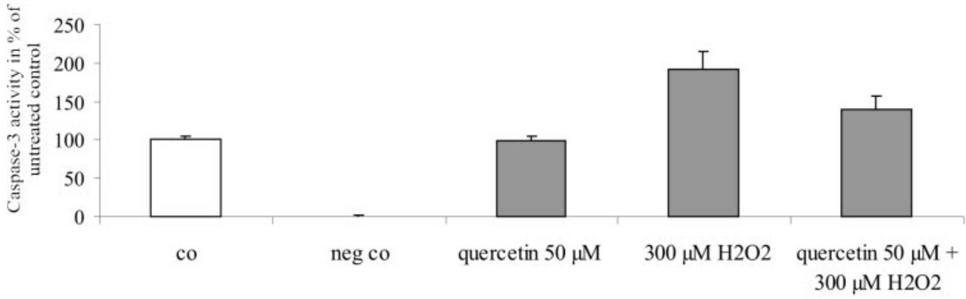

FIGURE 5. Activation of caspase-3 in RPE cells after exposure to 300 μM H_2O_2 for 75 minutes. Caspase-3 protease activity in RPE cells was determined as release of *p*NA from the substrate and monitored colorimetrically at 405 nm. Bars indicate average values ± SEM of three independent experiments performed in duplicate. Values were normalized with respect to untreated cells (control; co = 100%). Negative control (neg co); induced sample incubated with caspase-3 inhibitor before the addition of substrate.

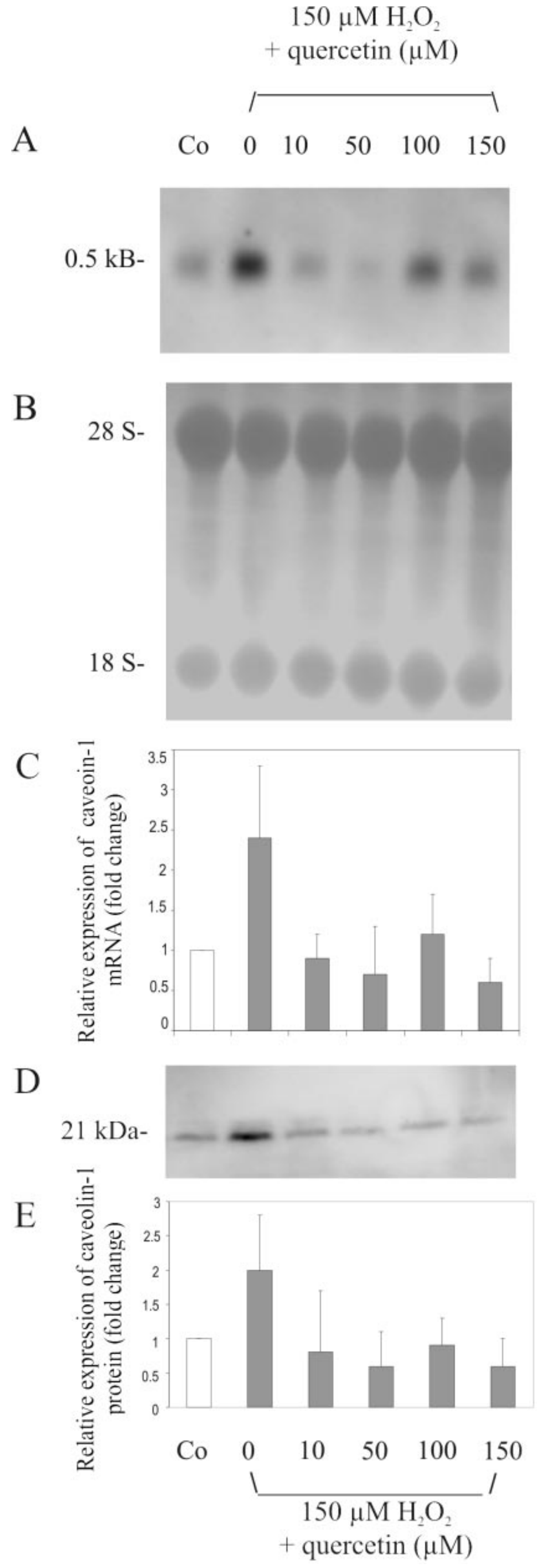

FIGURE 6. Quercetin prevents hydrogen peroxide-mediated increase of caveolin-1 mRNA (**A**) and protein (**D**) in cultured RPE cells. RPE cells were incubated with 150 μM H_2O_2 for 2 hours or were preincubated with 100 μM quercetin and then exposed to oxidative stress. Methylene blue staining of the 28S and 18S rRNA is also shown (**B**), demonstrating the relative integrity and even loading of the RNA. Northern blot analysis of caveolin-1 mRNA in confluent cultured RPE cells (**C**). Western blot analysis of caveolin-1 in confluent cultured RPE cells (**D**). Each lane was loaded with 2 μg protein. The table below depicts the relative chemiluminescence measurement (**E**). RDI, relative densitometric intensity (normalized to 28S rRNA); Co, control; MW, molecular weight.

expression significantly. This effect of DFO probably biased our results. Nevertheless, the reduction of hydrogen peroxide-induced caveolin expression by quercetin preincubation was lower with the addition of DFO than without it, supporting evidence that quercetin acts as a chelating agent. Several in vitro studies have shown that quercetin protects DNA against oxidative damage.[30–32] Other data have indicated that quercetin, at concentrations ranging between 5 and 40 μM, changes the redox state of cells by significantly increasing the level of total GSH.[33,34] In contrast, our results did not show any significant effect of quercetin treatment in either used concentration on GSH levels in RPE. However, our assay only measured the reduced form of GSH and not the ratio GSH/(GSH+GSSG), which might explain the different results obtained in our study (GSSG is the oxidized form of GSH). Nevertheless other mechanisms of the flavonoid quercetin are likely to protect RPE cells from oxidative stress.

In cardiomyoblast cells, quercetin prevents hydrogen peroxide-mediated mitochondrial dysfunction, including disruptions of mitochondrial membrane permeability transition and increases in the expression of apoptotic proteins. Furthermore, quercetin inhibited the activation of caspase-3-activity.[35] In accordance with these data in human RPE cells, caspase-3 activity after oxidative stress was similarly markedly diminished by quercetin.

Some authors have shown that in other cell lines, quercetin pretreatment impaired, rather than increased, the expression of enzymes of the antioxidant-system (e.g., CuZn superoxide dismutase, Mn superoxide dismutase, and GSH peroxidase) but still protected against oxidative stress provoked by H_2O_2 treatment.[36] This indicates that the antioxidant effect of quercetin might be mediated by cellular mechanisms other than the redox state or apoptosis in RPE cells. Recently, quercetin was shown to act on the wnt/β-catenin pathway or its downstream elements. It regulates cell proliferation and plays a role in carcinogenesis.[37] Our findings suggest that this increase in antioxidant capacity is among other given mechanisms mediated by a downregulation of the 21- to 24-kDa integral plasma membrane-bound signal transduction protein caveolin-1. It is known that caveolin-1 decreases expression of the apoptosis protein inhibitor survivin through the wnt pathway.[38] Kim et al.[39] showed that another polyphenol, epigallocatechin, is able to suppress the wnt signaling pathway in human cancer cells in vitro. This inhibition of the wnt signaling pathway might be mediated by caveolin expression. Recently published data demonstrating that caveolin plays a critical role in activating the wnt signaling pathway supports this hypothesis.[40] Inhibition of the upregulation of caveolin-1 via inhibition of the wnt signaling pathway might therefore have an antiapoptotic effect on human RPE cells.

In other cellular systems, caveolin-1 has been induced by oxidative stress. Cotreatment with quercetin prevented this increase.[19] Several studies indicate that caveolin-1 belongs to the group of senescence-associated genes. Another cellular marker for cellular senescence is β-galactosidase activity. β-Galactosidase expression was previously demonstrated to be age dependent in the retinal pigment epithelium of primates. In addition, it was recently shown that mild hyperoxia, another mediator of free radicals, induces the expression of this cellular senescence marker in RPE cells.[41] Consistent with the decreased expression of caveolin-1 after

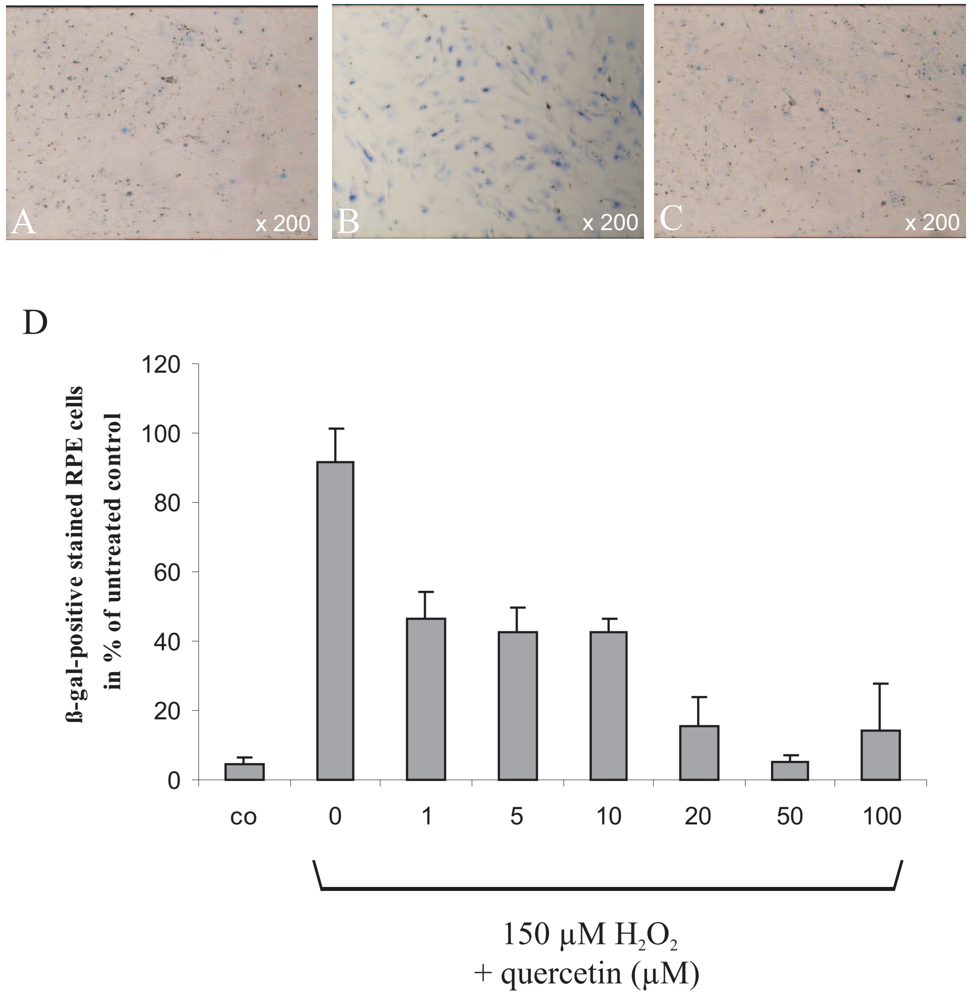

FIGURE 7. Quercetin prevented the upregulation of SA-β-gal activity induced by H_2O_2. **(A)** Morphology and β-gal activity of untreated cultured RPE cells. Single cells are stained *blue*, indicating β-gal activity. **(B)** In contrast, RPE cells of the same passage exposed to 150 μM H_2O_2 for 2 hours showed a marked increase of β-gal activity. **(C)** Pretreatment with 20 μM quercetin prevented H_2O_2-induced increase of β-gal activity. **(D)** Quantification of the effect of quercetin on the number of cells expressing β-gal activity. Percentage of β-gal activity was scored by counting at least 300 cells in phase-contrast photomicrographs of representative fields. Data (mean $\pm$ SEM) are based on the sampling of 6 to 10 photomicrographs per condition in three independent experiments performed in duplicate for the quantification of staining for β-gal (pH 6). Experiments were performed as described in Materials and Methods and repeated three times with five different cell cultures from different donors. Original magnification, (**A**–**D**) ×200.

cotreatment with quercetin and oxidative damage, we found that quercetin prevented the stress-induced premature senescence of RPE cells by oxidative stress. Therefore, we postulate that quercetin is a powerful agent in the prevention of stress-induced premature senescence. Another mark of cellular stress is the flattening and enlargement of cultured cells.[41] In our RPE cell cultures, quercetin prevented these characteristic changes. We can only speculate whether this effect was related to the decreased expression of caveolin. In other cell systems, it has been shown that caveolin knockout senescent cells, achieved by the use of small interfering RNA and antisense oligonucleotide, resulted in morphologic adjustment to the young cell-like shape.[42] Therefore, these authors do believe that the downregulation of caveolin in senescent cells adjusts functional efficiency and restores morphologic appearance.

Above all, we interpret our results as indicating that quercetin is able to inhibit molecular changes associated with stress-induced senescence in human RPE cells in vitro. Our results reveal a dose-dependent cytoprotective effect of quercetin in vitro, presumably with the highest protective effect at concentrations ranging between 10 and 50 μM. These results are in line with the latest published data about the antioxidative effects of a variety of dietary and synthetic flavonoids on human RPE cells.[43] On transferring our in vitro results to the in vivo situation, one might assume that prolonged exposure to quercetin might lower the cytoprotective concentration of quercetin in vivo. Regarding physiological concentrations of quercetin, it was shown that the plasma level of quercetin may be maintained in the range of 0.1 to 1 μM by the daily ingestion of 100 to 200 g onion (containing 200–600 mg quercetin/kg), although plasma levels may be transiently elevated for a few hours after consumption.[44,45] High concentrations of quercetin are also listed in capers, ancho peppers, cranberries, fennel, cocoa, black currants, buckwheat, black tea, spinach, and wild greens.

In relatively high concentrations ($\geq$100 μM), however, quercetin seems to have a cytotoxic rather than an antioxidative effect in vitro. Our data are thereby also consistent with those of several other studies, indicating that relatively high concentrations of quercetin induce chromosomal damage or cytotoxicity.[46–48] Therefore, like many other especially lipid-soluble antioxidants, excessively elevated serum levels of quercetin may cause cellular injury.

In addition to its antiapoptotic and antioxidative effects, quercetin has been shown to have anti-inflammatory properties.[13] Inflammatory processes are thought to be involved in the pathogenesis of AMD. In other cellular systems, quercetin is able to inhibit the expression of monocyte chemoattractant protein-1, which is known to be potentially involved in the pathogenesis of early AMD.[49,50]

In accordance with several clinical studies indicating the antioxidant cytoprotective effect of quercetin and based on our own in vitro findings of quercetin on human RPE cells, quercetin appears to be a candidate as food supplement in the prevention of early pathologic changes in AMD.

References

1. Klein R, Klein BE, Linton KLP. Prevalence of age-related maculopathy: the Beaver Dam Eye Study. *Ophthalmology*. 1992;99:933–943.
2. Vingerling JR, Dielemans I, Hofmann A, et al. The prevalence of age-related maculopathy in the Rotterdam Study. *Ophthalmology*. 1995;102:205–210.
3. Hawkins BS, Bird A, Klein R, West SK. Epidemiology of age-related macular degeneration. *Mol Vis*. 1999;5:26.
4. Coffey AHJ, Brownstein S. The prevalence of macular drusen in postmortem eyes. *Am J Ophthalmol*. 1986;102:164–171.
5. Spraul CW, Lang GE, Grossniklaus HE. Morphometric analysis of the choroid, Bruch's membrane, and retinal pigment epithelium in eyes with age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 1996;37:2724–2735.
6. Young RW. Solar radiation and age related macular degeneration. *Surv Ophthalmol*. 1988;32:252–269.
7. Kennedy CJ, Rakoczy PE, Constable IJ. Lipofuscin of the retinal pigment epithelium: a review. *Eye*. 1995;9:763–771.
8. Mares-Perlman JA, Klein R, Klein BE, et al. Association of zinc and antioxidant nutrients with age-related maculopathy. *Arch Ophthalmol*. 1996;114:991–997.
9. AREDS Group. A randomized, placebo-controlled, clinical trial of high-dose supplementation with vitamins C and E, beta carotene, and zinc for age-related macular degeneration and vision loss: AREDS report no. 8. *Arch Ophthalmol*. 2001;119:1417–1436.
10. Richer S, Stiles W, Statkute L, et al. Double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation in the intervention of atrophic age-related macular degeneration: the Veterans LAST study (Lutein Antioxidant Supplementation Trial). *Optometry*. 2004;75:216–230.
11. Dolnick E. Le paradoxe François. *Health*. 1990;41–47.
12. Swain T. The evolution of flavonoids. *Prog Clin Biol Res*. 1986;213:1–14.
13. Formica JV, Regelson W. Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol*. 1995;33:1061–1080.
14. Hollman P, Katen MB. Bioavailability and health effects of dietary flavonoids in man. *Arch Toxicol (suppl)*. 1998;20:237–248.
15. Ross JA, Kasum CM. Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr*. 2002;22:19–34.
16. Spencer JPE, El Mohsen MMA, Rice-Evans C. Cellular uptake and metabolism of flavonoids and their metabolites: implications for their bioactivity. *Arch Biochem Biophys*. 2004;423:148–161.
17. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr*. 2004;79:727–747.
18. Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*. 1995;26:92:9363–9367.
19. Volonte D, Zhang K, Lisanti MP, Galbiati F. Stress-induced premature senescence upregulates the expression of endogenous caveolin-1. *Mol Biol Cell*. 2002;13:2502–2517.
20. Alge CS, Priglinger SG, Neubauer AS, et al. Retinal pigment epithelium is protected against apoptosis by αB-crystallin. *Invest Ophthalmol Vis Sci*. 2002;43:3575–3582.
21. Leschey KH, Hackett SF, Singer JH, Campochiaro PA. Growth factor responsiveness of human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 1990;31:839–846.
22. Flood MT, Gouras P, Kjeldbye H. Growth characteristics and ultrastructure of human retinal pigment epithelium in vitro. *Invest Ophthalmol Vis Sci*. 1980;19:1309–1320.
23. Wang Z, Dillon J, Gaillard ER. Antioxidant properties of melanin in retinal pigment epithelial cells. *Photochem Photobiol*. 2006;82:474–479.
24. Graefe EU, Wittig J, Mueller S, et al. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J Clin Pharmacol*. 2001;41:492–499.
25. Hollmann PCH, van Trijp JMP, Buysman MNCP, et al. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett*. 1997;418:152–156.
26. Jayachandran M, Hayashi T, Sumi D, Iguchi A, Miller VM. Temporal effects of 17 beta-estradiol on caveolin-1 mRNA and protein in bovine aortic endothelial cells. *Am J Physiol Heart Circ Physiol*. 2001;281:H1327–H1333.
27. Welge-Luessen U, May CA, Eichhorn M, Bloemendal H, Lütjen-Drecoll E. αB-crystallin in the trabecular meshwork is inducible by transforming growth factor-beta. *Invest Ophthalmol Vis Sci*. 1999;40:2235–2241.
28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970;227:680–685.
29. Kaeko M, Yuki M, Mami I, Tojiro T, Sayuri M, Junji T. Quercetin-4′-glucoside is more potent than quercetin-3-glucoside in protection of rat intestinal mucosa homogenates against iron-induced lipid peroxidation. *J Agric Food Chem*. 2004;52:1907–1912.
30. Szeto YT, Benzie IF. Effects of dietary antioxidants on human DNA ex vivo. *Free Radical Res*. 2002;36:113–118.
31. Duthie SJ, Collins AR, Duthie GG, Dobson VL. Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. *Mutat Res*. 1997;393:223–231.
32. Noroozi M, Angerson WJ, Lean ME. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am J Clin Nutr*. 1998;67:1210–1218.
33. Cipak L, Berczeliova E, Paulikova H. Effects of flavonoids on glutathione and glutathione-related enzymes in cisplatin-treated L1210 leukemia cells. *Neoplasma*. 2003;50:443–446.
34. Boadi WY, Iyere PA, Adunyah SE. In vitro exposure to quercetin and genistein alters lipid peroxides and prevents the loss of glutathione in human progenitor mononuclear (U937) cells. *J Appl Toxicol*. 2005;25:82–88.
35. Park C, So HS, Shin CH, et al. Quercetin protects the hydrogen peroxide-induced apoptosis via inhibition of mitochondrial dysfunction in H9c2 cardiomyoblast cells. *Biochem Pharmocol*. 2003;66:1287–1295.
36. Röhrdanz E, Bittner A, Tran-Thi QH, Kahl R. The effect of quercetin on the mRNA expression of different antioxidant enzymes in hepatoma cells. *Arch Toxicol*. 2003;77:506–510.
37. Park CH, Chang JY, Hahm ER, Park S, Kim HK, Yang CH. Quercetin, a potent inhibitor against β-catenin/Tcf signaling in SW480 colon cancer cells. *Biochem Biophys Res Commun*. 2005;328:227–234.
38. Torres VA, Tapia JC, Rodriguez DA, et al. Caveolin-1 controls cell proliferation and cell death by suppressing expression of the inhibitor of apoptosis protein surviving. *J Cell Sci*. 2006;119:1812–1823.
39. Kim J, Zhang X, Rieger-Christ KM, et al. Suppression of Wnt signaling by the green tea compound (−)-epigallocatechin 3-gallate (EGCG) in invasive breast cancer cells: requirement of the transcriptional repressor HBP1. *J Biol Chem*. 2006;281:10865–10875.
40. Yamamoto H, Komekado H, Kikuchi A. Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. *Dev Cell*. 2006;11:213–223.
41. Honda S, Hjelmeland LM, Handa JT. Senescence associated beta galactosidase activity in human retinal pigment epithelial cells exposed to mild hyperoxia in vitro. *Br J Ophthalmol*. 2002;86:159–162.
42. Cho KA, Park SC. Caveolin-1 as a prime modulator of aging: a new modality for phenotypic restoration? *Mech Ageing Dev*. 2005;126:105–110.
43. Hanneken A, Lin FF, Johnson J, Maher P. Flavonoids protect human retinal pigment epithelial cells from oxidative-stress–induced death. *Invest Ophthalmol Vis Sci*. 2006;47:3164–3177.
44. Hertog MGL, Hollman PCH, Katan MB. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J Agric Food Chem*. 1992;40:2379–2383.

45. Moon J, Nakata R, Oshima S, Inakuma T, Terao J. Accumulation of quercetin conjugates in blood plasma after the short-term ingestion of onion by women. *Am J Physiol*. 2000;279:461–467.
46. Akiko S, Ayako S, Keizo U, Hiroyuki S. Protective effects of quercetin and its metabolites on H_2O_2-induced chromosomal damage to WIL2-NS cells. *Biosci Biotechnol Biochem*. 2004;68: 271–276.
47. Yen GC, Duh PD, Tsai HL, Huang SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotechnol Biochem*. 2003;67:1215–1222.
48. Cao XG, Li XX, Bao YZ, Xing NZ, Chen Y. Responses of human lens epithelial cells to quercetin and DMSO. *Invest Ophthalmol Vis Sci*. 2007;48:3713–3718.
49. Chen L, Wu W, Dentchev T, et al. Light damage induced changes in mouse retinal gene expression. *Exp Eye Res*. 2004;79:239–247.
50. Kalayoglu MV, Bula D, Arroyo J, Gragoudas ES, D'Amico D, Miller JW. Identification of *Chlamydia pneumoniae* within human choroidal neovascular membranes secondary to age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol*. 2005;243: 1080–1090.