Epigallocatechin gallate protects against oxidative stress-induced mitochondria-dependent apoptosis in human lens epithelial cells

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Purpose: Oxidative stress has long been recognized as an important mediator of apoptosis in lens epithelial cells and also plays an important role in the pathogenesis of cataracts. (-)-Epigallocatechin gallate (EGCG), the most abundant component in green tea, has potent antioxidant activity. The goals of this study were to determine the protective effect of EGCG against H2O2-induced apoptotic death and the possible mechanisms involved in human lens epithelial (HLE) cells.

Methods: HLEB-3, a human lens epithelial cell line, was exposed to various concentrations of H2O2 and EGCG and subsequently monitored for cell death by the MTT assay and flow cytometric analysis using Annexin V and PI. The effect of EGCG in protecting HLE cells from cell death was determined by various assays after the cells were exposed to H2O2. The ability of EGCG to block the accumulation of intracellular reactive oxygen species and the loss of mitochondrial membrane potential (∆ψm) induced by H2O2 was examined with dichlorofluorescein (DCF) fluorescence and 5,5′,6′,6′-tetrachloro-1,1′,3,3′-tetrathylbenzimidazol carbocyanine iodide (JC-1). The expression of cytochrome c, caspase-9, caspase-3, and Bcl-2 family proteins was measured by western blotting. The changed expression of the mitogen activated protein kinase (MAPK) and Akt pathways was also detected by western blot.

Results: In the present study, EGCG protected against cell death caused by H2O2 in HLEB-3 cells. EGCG reduced the H2O2-induced generation of reactive oxygen species (ROS), the loss of mitochondrial membrane potential (∆ψm), and the release of cytochrome c from the mitochondria into the cytosol. EGCG inhibited the H2O2-stimulated increase of caspase-9 and caspase-3 expression and the decrease of the Bcl-2/Bax ratio. Moreover, EGCG attenuated the reduced activation and expression of ERK, p38 MAPK, and Akt induced by H2O2.

Conclusions: These findings suggest that EGCG protects HLE cells from the mitochondria-mediated apoptosis induced by H2O2 through the modulation of caspases, the Bcl-2 family, and the MAPK and Akt pathways.

Cataract formation, the opacification of the eye lens, is one of the leading causes of human blindness worldwide, accounting for 47.8% of all causes of blindness [1]. Although great advances have been made in surgical treatment, the incidence of cataracts in developing countries is so high that it overwhelms the capacity of surgical programs. Oxidative stress has long been recognized as an important mediator of apoptosis in lens epithelial cells and also plays an important role in the pathogenesis of cataracts [2–4]. The lens exists in an environment that is rich in endogenous sources of reactive oxygen species (ROS), which are produced by the high local oxygen concentration, the chronic exposure to light, and the pathogenic activities of lens epithelial cells [5]. Although multiple physiologic defenses exist to protect the lens from the toxic effects of light and oxidative damage, mounting evidence suggests that chronic exposure to oxidative stress over the longterm may damage the lens and predispose it to cataract development.

Apoptosis is a physiologic process of cell death that plays a critical role in a variety of biologic systems, which has been identified as providing an important molecular basis for both the initiation and progression of cataracts [6,7]. There are distinct mechanisms that execute apoptosis according to various different apoptotic stimuli, and these are classified into the mitochondria-dependent pathway (intrinsic pathway) and the death receptor-dependent pathway (extrinsic pathway). Previous studies have demonstrated the capacity of antioxidant protection of the mitochondria-dependent pathway associated with lens opacification in cultured lenses [8–11]. Mitochondrial damage results in the release of cytochrome c from the impaired mitochondria into the cytoplasm, which contributes to programmed cell death [12].

Epidemiologic data have shown that special dietary additives may provide effective defenses against oxidative stress and thus have potential as treatments for a variety of diseases. Recently, extensive studies have suggested a positive correlation between the consumption of green tea (Camellia sinensis) and beneficial antioxidant, anti-inflammatory, and anti-carcinogenic effects [13]. The green tea extracts contain (-)-epigallocatechin gallate (EGCG), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG), (-)-epicatechin (EC), and catechin [14]. EGCG, the most abundant component in green tea, has a potent antioxidant property because of two triphenolic groups in its molecular structure. Several studies have demonstrated that EGCG can protect the heart, brain, and kidney from oxidative injury [15–17]. EGCG provide both short and long-term protection against oxidative stress through a vari-
ety of mechanisms [18-20]. Although the protective effect of EGCG has been reported in various models, there are few studies about the protection of EGCG against apoptosis-related cataracts and the precise mechanism of signal transduction in this pathological condition.

Based on these observations, we hypothesize that EGCG can protect lens epithelial cells from oxidative stress-induced apoptosis and may have benefits in the treatment of cataracts associated with oxidative stress. In the work presented, we used H₂O₂-treated human lens epithelial (HLE) cells as a model to study lens epithelial cell exposure to oxidative stress. This study is designed to investigate the protective effect of EGCG against H₂O₂-induced apoptosis and the possible mechanisms involved in HLE cells.

**METHODS**

**Materials:** HLEB-3 cells (human lens epithelial cells) were obtained from ATCC (Rockville, MD). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from Gibco (Grand Island, NY). Propidium iodide (PI), Annexin-V, EGCG, 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluoresceine diacetate (DCFH-DA), 5,5',6,6'-tetrachloro-1,1',3,3'-tetrathylbenzimidazol carbocyanine iodide (JC-1), and a cocktail of protease inhibitors were from Sigma Chemical Co. (St. Louis, MO). The bicinchoninic acid (BCA) protein assay kit was from Pierce (Lockford, IL). Anti-cytochrome c, anti-Bax, anti-Bcl-2, anti-actin, anti-mouse, and anti-rabbit IgG horseradish peroxidase (HRP) antibodies were from Santa Cruz (Santa Cruz, CA). Anti-p-Akt (Ser473), anti-Akt, anti-p-JNK (Thr183/Tyr185), anti-JNK, anti-p-ERK1/2, anti-ERK1/2, anti-p-p38 (Thr180/Tyr182), anti-p38, anti-pro-caspase-9, and anti-cleaved-caspase 3 antibodies were purchased from Cell Signal Technology (Beverly, MA). The chemiluminescence (ECL) detection kit was acquired from Amersham Pharmacia (Arlington Heights, IL).

**Cell culture:** HLEB-3 cells were cultured in DMEM supplemented with heat-inactivated (56 °C, 0.5 h) 10% FBS at 37 °C in a humidified atmosphere of 5% CO₂. The cells were seeded in a 60 mm culture dish (Falcon; Becton-Dickinson, Oxnard, CA). When grown to 75%-80% confluence, the cells were treated with the indicated concentration of H₂O₂ for the required time or pretreated with EGCG for 1 h before H₂O₂ treatment. At the indicated time points, the cells were collected for the different assays.

**Cell viability assay:** HLEB-3 cells were seeded in 96 well tissue culture plates at an initial concentration of 1x10⁵ cells/ml and incubated with 50, 100, and 200 µM H₂O₂ alone or pretreated with different concentrations of EGCG (10, 25, 50, 75, 100, and 150 µM) for 1 h. After incubation for the indicated time, cells were treated by the addition of 20 µl MTT dye to each well. After an additional 4 h incubation, the growth medium was removed and the formazan crystals, formed by oxidation of the MTT dye, were dissolved with 150 µl DMSO in isopropanol. The absorbance was measured at 490 nm and the cell survival ratio was expressed as a percentage of the control.

**Flow cytometric analysis using annexin V and PI:** Cells were grown on a six-well plate at 1x10⁶ cells per plate and treated with 100 µM H₂O₂ for 24 h. For 50 µM EGCG, cells were pretreated for 1 h before treatment. Cells were centrifuged to remove the medium, washed with PBS, and stained with annexin V-FITC and PI in binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM CaCl₂). Ten thousand events were collected for each sample. Stained cells were analyzed using a FACS calibur (Becton Dickinson, Mountain View, CA) in the FL1-H and FL2-H channels.

**Detection of reactive oxygen species:** The production of reactive oxygen species (ROS) was monitored using flow...
cytometry. Cells were plated on a six-well plate and pretreated with 50 μM EGCG for 1 h followed by treatment with 100 μM H₂O₂ for 2 h. DCFH-DA (10 μM) was added into the medium for 15 min at 37 °C. The intracellular production of ROS was determined with excitation at 480 nm and with emission at 530 nm.

Measurement of mitochondrial membrane potential (Δψₘ):
The cells were incubated with 100 μM H₂O₂ for 6 h or pretreated with 50 μM EGCG for 1 h in the presence of H₂O₂. The Δψₘ of cells was measured by staining with JC-1. When mitochondria were polarized, JC-1 formed aggregates and emitted red fluorescence with 486 nm excitation. The red and green fluorescence were measured simultaneously by FACS caliber.

Immunoblot analysis:
After treatment, cell cultures were washed once in cold PBS and then lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 mM NaF, 1 μg/mL leupeptin, and 1 mM PMSF. The protein extracts were quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce). The samples containing 20-40 μg proteins were boiled in Laemmli sample buffer, electrophoresed in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Milford, MA) in trans-blot transfer medium (20 mM Tris-HCl, 190 mM Glycine, 20% methanol, pH 8.3). All membranes were blocked in TBS buffer (10 mM Tris-HCl, 50 mM NaCl, and 0.05% Tween 20) containing 5% BSA for 1 h at room temperature, and all subsequent antibody dilutions and washings were performed with TBS buffer. Membranes were incubated with the following antibodies overnight at 4 °C: anti-cytochrome c, anti-Bax, anti-Bcl-2, anti-pro-caspase-9, anti-cleaved-caspase-3, anti-p-Akt, anti-Akt, anti-p-p38 MAPK, anti-p38 MAPK, anti-p-ERK, anti-ERK, anti-p-JNK, anti-JNK, and anti-actin. Membranes were washed three times in TBS buffer, incubated with HRP-conjugated secondary antibodies for 1 h at room temperature, then washed and visualized by chemiluminescence.

Statistical analysis:
Data are expressed as the mean±SD. Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was made using one-way ANOVA followed by the Student-Newman-Keuls test. A p-value of less than 0.05 was considered significant.

RESULTS
(-)-epigallocatechin gallate protects against H₂O₂-induced cell death in HLEB-3 cells:
The oxidant H₂O₂ has been used in the design of models of classical oxidative stress in HLEB-3 cells because of its rapid membrane permeability and depolarizing effects on the mitochondrial membrane potential. For these reasons, we selected it for our studies and performed a series of dose- and time-response assays to determine the working concentration and time that led to a consistent degree of cytotoxicity. The cells were exposed to different concentrations of EGCG (10, 25, 50, 75, 100, and 150 μM) for 24 h, and the MTT assay showed no loss of cell viability (data not shown).

Figure 2. (-)-epigallocatechin gallate reduces the generation of ROS and prevents the loss of Δψₘ and the release of mitochondrial cytochrome c into the cytosol in HLEB-3 cells. A: The cells were pretreated with 50 μM EGCG for 1 h followed by treatment with 100 μM H₂O₂ for 2 h. The production of ROS was examined by measuring the level of ROS production using DCF-DA by flow cytometry. B: The cells were incubated with either 100 μM H₂O₂ for 6 h or pretreated 50 μM EGCG for 1 h in the presence of H₂O₂. The Δψₘ of cells was measured by staining with JC-1 by flow cytometry. C: After treatment for 24 h with 100 μM H₂O₂ or with 25 and 50 μM EGCG in the presence of H₂O₂, the cells were analyzed by western blot analysis. The result is one representative example of three separate experiments.
shown). Treatment with H$_2$O$_2$ at 50, 100, and 200 µM for 24 h caused a dose-dependent loss of viability (50.0%±1.2%, 38.3%±1.7%, and 24.5%±2.6% of control value) whereas the EGCG pretreatment at a concentration from 10-150 µM for 1 h prevented the loss of viability (Figure 1A). The viability of cells pretreated with EGCG at 100 µM for 1 h before exposure to 100 µM H$_2$O$_2$ was up to 64.0%±2.6% whereas the increased concentration of 150 µM did not cause any enhancement of this preventive effect. We analyzed the effective half maximal concentration for protection (EC$_{50}$s) from the dose-response curves and decided to adopt the concentration of 50 µM for our subsequent studies. Moreover, the application of 100 µM H$_2$O$_2$ induced time-dependent cytotoxicity which was prevented by EGCG in a time-dependent manner (Figure 1B).

To examine whether EGCG protects against H$_2$O$_2$-induced apoptosis, the HLEB-3 cells were incubated with 50 µM EGCG for 1 h then treated with 100 µM H$_2$O$_2$ for 24 h. Flow cytometric analysis was used to quantify the rate of cell apoptosis using double staining of Annexin V-FITC and PI. A significant increase of apoptosis was observed in HLEB-3 cells treated with 100 µM H$_2$O$_2$ compared with control cells (20.12% versus 1.2%; Figure 1C). However, EGCG-pretreated HLE cells showed significant resistance to H$_2$O$_2$-induced apoptosis (2.2%).

(-)-epigallocatechin gallate reduces the generation of reactive oxygen species and prevents the loss of ∆ψm and the release of mitochondrial cytochrome c into the cytosol in HLEB-3 cells:

The ROS induced by H$_2$O$_2$ were examined by measuring the level of ROS production using DCF-DA. HLEB-3 cells treated with 100 µM H$_2$O$_2$ for 2 h resulted in the production of ROS with an approximately two-fold increase compared to nontreated cells (Figure 2A). However, pretreatment with EGCG at 50 µM before H$_2$O$_2$ exposure markedly reduced the ROS levels generated by H$_2$O$_2$ in HLEB-3 cells.

Loss of ∆ψm is an early event of apoptosis induced by a variety of stimuli. To examine whether the ∆ψm pathway is involved in H$_2$O$_2$-induced apoptosis or is changed by EGCG in the presence of H$_2$O$_2$, ∆ψm detection was performed using dye JC-1, which was used to assess mitochondrial membrane depolarization. As shown in Figure 2B, cells treated with H$_2$O$_2$ exhibited a substantial increase in mitochondrial depolarization; the percentage of cells testing positive for depolarized mitochondria increased from 4.2% of control cells to 44.5% of cells treated with 100 µM H$_2$O$_2$. Depolarization of mitochondria releases several apoptogenic proteins, most notably cytochrome c, from the mitochondria into the cytosol. Western blot revealed that 100 µM H$_2$O$_2$ led to an accumulation of cytochrome c in the cytosol for 24 h (Figure 2C). The observed cytochrome c in the cytosol was significantly reduced when the cells were pretreated with 50 µM EGCG before H$_2$O$_2$ treatment. These results indicate that H$_2$O$_2$ causes a mitochondrial dysfunction via the mitochondrial pathway and EGCG is protective against such dysfunction.

(-)-epigallocatechin gallate inhibits the activities of caspase-9 and caspase-3 and modulates the expression of the Bcl-2 family proteins induced by H$_2$O$_2$ in HLEB-3 cells:

Caspases are known to play important roles in apoptosis. To examine whether caspases are activated in H$_2$O$_2$-induced apoptosis, we examined the expression of caspase-9 and caspase-3 mRNA was detected by RT-PCR. The expression of caspase-9 and caspase-3 mRNA was detected by RT-PCR. The expression of caspase-9 and caspase-3 mRNA was detected by RT-PCR. The expression of caspase-9 and caspase-3 mRNA was detected by RT-PCR.
caspase-3 at the protein level. Western blot analysis revealed a decrease of pro-caspase-9 as a result of caspase-9 activation in H₂O₂-treated cells, which was prevented by pretreatment with EGCG (Figure 3A). The decrease in pro-caspase-9 was associated with increased caspase-3 cleavage with 17 kDa and 19 kDa, a downstream substrate of caspase-9. The effect of H₂O₂-induced caspase-3 cleavage was also rescued by EGCG.

Either the pro-apoptotic or anti-apoptotic Bcl-2 family members can affect the execution of apoptosis. To determine whether EGCG protects against H₂O₂-induced apoptosis in HLEB-3 cells by modulating Bcl-2 family, the protein levels of Bax and Bcl-2 were examined by western blot analysis (Figure 3B). Treatment with 100 µM H₂O₂ decreased the expression of Bcl-2 and increased the expression of Bax while pretreatment with EGCG inhibited downregulation of Bcl-2 and upregulation of Bax. The ratio of Bcl-2 to Bax was significantly reduced by H₂O₂ and attenuated by pretreatment with EGCG (Figure 3C).

(-)-epigallocatechin gallate protects against H₂O₂-induced apoptosis through activation of mitogen activated protein kinases and Akt: To gain further insight into the molecular mechanisms involved, we studied the effect of EGCG on the possible pathways potentially activated during apoptosis. Therefore, we investigated whether the MAPKs and Akt pathways were induced by treatment with or without EGCG. As shown in Figure 4, stimulation with H₂O₂ resulted in a significant decrease in the phosphorylated forms of ERK (p44 and p42) and p38 MAPK and Akt (Ser 473) whereas there was no change in total ERK, p38, or Akt expression. The H₂O₂-induced downregulation of ERK and p38 MAPK and Akt was significantly inhibited by pretreatment with EGCG at concentrations of 25 and 50 µM, respectively. However, JNK was not activated by treatment with H₂O₂ although this kinase was present in the cells.

DISCUSSION

Cellular defenses that protect the lens epithelial cells against oxidative stress have been proposed to be an important way to reduce the progression of various types of cataracts [21]. Previous studies have demonstrated that H₂O₂-induced apoptosis in HLE cells is a useful model for studying cataractogenesis [22,23]. Although EGCG has been reported to protect against oxidative stress in other human cell lines [24,25], little had been demonstrated before this study in terms of its effect in lens epithelial cells. In this study, EGCG remarkably inhibited the cytotoxicity of HLE cells caused by H₂O₂ in a range of 10-100 µM compared to treatment with H₂O₂ alone. However, EGCG at a concentration 150 µM did not cause any increase of cell viability. One possible reason is the cell toxicity due to the accumulation of the two drugs at high concentrations. Moreover, flow cytometry analysis using PI and annexin V showed that H₂O₂ induced cell apoptosis in HLE cells whereas EGCG significantly reduced the apoptosis in H₂O₂-treated cells. This indicates that EGCG at low concentrations exerts a protective effect by inhibiting H₂O₂-induced cell death.

Oxidative stress can disrupt the balance between reactive oxygen radical production and the radical scavenging effect and lead to apoptotic cell death through the mitochondrial apoptosis pathway. Previous studies have shown mitochondrial protection to be important in the process of lens opacification in cultured lenses [8,10,11]. The collapse of the mitochondrial membrane potential potential results in the rapid release of cytochrome c into the cytoplasm [12]. Although the mechanism is still not completely understood, EGCG has recently been recognized to scavenge intracellular ROS and regulate antioxidant enzyme activities [26]. Consistent with these findings, H₂O₂-treated cells showed an increased production of intracellular ROS, a loss of mitochondrial potential, and an increased release of mitochondrial cytochrome c into the cytoplasm. However, pretreatment with EGCG attenuated the increase in ROS level and prevented the loss of mitochondrial potential and the release of cytochrome c. These results indicate H₂O₂ is an oxidative stress which stimulates the produc-

Figure 4. (-)-epigallocatechin gallate protects against H₂O₂-induced apoptosis through activation of the MAPKs and Akt pathways. A: After the cells were incubated for 24 h with 100 µM H₂O₂ or with 25 and 50 µM EGCG 1 h before H₂O₂ treatment, the expression of ERK, p38 MAPK, JNK, and Akt protein was detected using western blot analysis. B: Densitometric analyses of western blot are presented as the means±SD for three independent experiments performed in triplicate. Data are presented as the fold induction over control cells. The asterisk indicates that p<0.05 compared to the untreated control and the sharp (hash mark) means that p<0.05 compared to the H₂O₂-treated group.
tion of ROS and triggers the mitochondria-mediated apoptosis pathway, and these effects can be suppressed by EGCG.

Caspases play an important role in regulating cell apoptosis. Caspases transduce the apoptotic signal cascade and engage cellular targets leading to programmed cell death [27,28]. As one of the key effectors, caspase-3 is initiated by caspase-9 and involved in the mitochondria-mediated pathway. Previous studies have shown that H$_2$O$_2$ induced the activation of caspase-9 and caspase-3 in lens epithelial cells [23,29]. Consistent with these results, we demonstrated that the induction of apoptosis induced by H$_2$O$_2$ was accompanied by an increase of caspase-9 and caspase-3 activities at the protein level. Furthermore, this effect could be attenuated by a pretreatment with EGCG before H$_2$O$_2$ treatment. Caspase-9 and caspase-3 might be important effector caspases in H$_2$O$_2$-induced apoptosis, and EGCG protects against the apoptosis of HLE cells by blocking the expression of caspase-9 and caspase-3.

The Bcl-2 family, which possesses both anti- and pro-apoptotic members, constitutes a decisive checkpoint within the common portion of the cell death pathway [30]. Bcl-2 can prevent ROS production and regulate the mitochondrial transition pore opening by opposing the effect of Bax thereby blocking cytochrome c release and inhibiting caspase activities [31]. An altered ratio of anti-apoptotic/pro-apoptotic Bcl-2 family proteins is critical in determining whether apoptosis is performed. In this study, western blot analysis revealed that the Bcl-2/Bax ratio was significantly decreased by the treatment with H$_2$O$_2$, and this decrease was inhibited by the pretreatment with EGCG. This result indicates that Bcl-2 family proteins may play a critical role in regulating HLE cell death induced by H$_2$O$_2$, and EGCG is able to protect against H$_2$O$_2$-stimulated apoptosis through a modulation of Bcl-2/Bax expression.

Several investigations have reported that EGCG can modulate growth and survival in many tumor cells through an activation of intracellular signaling cascades such as the mitogen activated kinase pathway (MAPK) and phosphoinositol-3-kinase/Akt (PI3K/Akt) pathway [32,33]. Both of these pathways have roles in anti-apoptotic and growth stimulatory signaling. To detect the pathways, which are involved in the pro-apoptotic caspases, the MAPKs and Akt pathways. Our data showed that H$_2$O$_2$ significantly decreased the activation of ERK, p38 MAPK, and Akt but not the expression of JNK whereas pretreatment with EGCG inhibited the downregulation of these proteins. This implies that EGCG may offer protection against oxidative stress-induced cell death by regulating the MAPKs and Akt pathways.

Taken together, the present study suggests H$_2$O$_2$ to be an oxidative stress which can induce HLE cell apoptosis through mitochondrial pathway, EGCG, a potent antioxidant, protects against H$_2$O$_2$-induced apoptosis by regulating caspases, the Bcl-2 family, and the MAPKs and Akt pathways. It may be exploited as a potentially useful method for cataract prevention.

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