Hydrogen peroxide-induced apoptosis of human lens epithelial cells is inhibited by parthenolide

Xing-Chao Shentu¹, Xi-Yuan Ping¹, Ya-Lan Cheng¹, Xin Zhang¹, Ye-Lei Tang², Xia-Jing Tang¹

¹Eye Center, the Second Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou 310009, Zhejiang Province, China
²The Second Affiliated Hospital of Zhejiang University the School of Medicine, Hangzhou 310000, Zhejiang Province, China

Correspondence to: Xia-Jing Tang. Eye Center, Second Affiliated Hospital of Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou 310009, Zhejiang Province, China. xiajingtang@zju.edu.cn

Received: 2017-10-09 Accepted: 2017-12-02

Abstract

- **AIM:** To explore the effect of parthenolide on hydrogen peroxide (H$_2$O$_2$)-induced apoptosis in human lens epithelial (HLE) cells.
- **METHODS:** The morphology and number of apoptotic HLE cells were assessed using light microscopy and flow cytometry. Cell viability was tested by MTS assay. In addition, the expression of related proteins was measured by Western blot assay.
- **RESULTS:** Apoptosis of HLE cells was induced by 200 μmol/L H$_2$O$_2$, and the viability of these cells was similar to the half maximal inhibitory concentration (IC50), as examined by MTS assay. In addition, cells were treated with either different concentrations (6.25, 12.5, 25 and 50 μmol/L) of parthenolide along with 200 μmol/L H$_2$O$_2$ or only 50 μmol/L parthenolide or 200 μmol/L H$_2$O$_2$ for 24h. Following treatment with higher concentrations of parthenolide (50 μmol/L), fewer HLE cells underwent H$_2$O$_2$-induced apoptosis, and cell viability was increased. Further, Western blot assay showed that the parthenolide treatment reduced the expression of caspase-3 and caspase-9, which are considered core apoptotic proteins, and decreased the levels of phosphorylated nuclear factor-κB (NF-κB), ERK1/2 [a member of the mitogen-activated protein kinase (MAPK) family], and Akt proteins in HLE cells.
- **CONCLUSION:** Parthenolide may suppress H$_2$O$_2$-induced apoptosis in HLE cells by interfering with NF-κB, MAPKs, and Akt signaling.
- **KEYWORDS:** parthenolide; apoptosis; human lens epithelial cells; hydrogen peroxide

DOI:10.18240/ijo.2018.01.03
However, the total levels of these proteins did not change.

Parthenolide inhibited apoptosis by decreasing the activating phosphorylation of NF-κB, MAPKs/ERK and Akt.

**MATERIALS AND METHODS**

**Cell Culture** The adherent HLE cell line (SRA 01/04), which was obtained from the Riken cell bank, was cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 units of penicillin, 100 μg of streptomycin, 2 mmol/L glutamine and 1% non-essential amino acids in a 37°C incubator with a 5% CO2 atmosphere. As a model to study cataract formation, an HLE cell line (SRA 01/04) was immortalized by infection with adenovirus 12-SV40, and differentiation was inhibited. For the chemical treatments, cells were uniformly distributed on 6-well or 96-well plates 24h before the experiments and cultured until the cell confluence reached 70%-80%. The culture medium was replaced, and then cells were treated with 50 μmol/L parthenolide or 200 μmol/L H2O2 for an additional 24h or pretreated with different concentrations of parthenolide for 1h before the 200 μmol/L H2O2 treatment.

**Chemicals and Reagents** H2O2, which was purchased from Sigma (Sigma, St. Louis, MO, USA), prepared immediately before use and diluted with PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 2 mmol/L KH2PO4, and pH 7.2-7.4) to 200 μmol/L. Parthenolide (Sigma, St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO) (Shenggong, Shanghai, China) to 100 mmol/L and stored at -20°C before use, parthenolide was diluted to final concentrations of 12.5, 25 and 50 μmol/L.

**Cell Viability Assays** Cell viability was examined using a Promega Cell Titer 96 aqueous cell viability assay (MTS) kit. Cells were plated into 96-well plates, allowed to attach and grow overnight until the cell density reached 70%-80%, and then treated with the chemicals mentioned above for 24h. The assay was performed according to the manufacturer’s instructions. First, 20 μL of an MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt]/phenazine ethosulfate (PES) mixture were added to each well. Then, cells were incubated at 37°C for 1h, and the quantity of formazan crystals produced from the MTS compound was measured by monitoring the absorbance at 490 nm, which is directly proportional to the number of living cells in culture[24]. Background absorbance from the readings from blank controls was subtracted.

**Flow Cytometry Analysis** Twenty-four hours after the H2O2 treatment, the apoptosis of cells treated with or without parthenolide was monitored. Annexin V binding and propidium iodine staining were determined by flow cytometry. Cells were washed with PBS twice, and double stained with the FITC-conjugated Annexin V protein and propidium iodine for 20min. Flow cytometry was performed using a 488 nm laser coupled to a cell sorter (FACSC alibur; BD Biosciences, San Jose, CA, USA). Cells stained with the Annexin V protein alone were considered apoptotic cells.

**Western Blot Analysis** Levels of caspase-3, caspase-9, ERK1/2, p-ERK1/2, p-NF-κB, p-NF-κB, Akt, and p-Akt were detected using Western blotting. Antibodies used in the Western blot analysis included anti-caspase-3 and anti-caspase-9 polyclonal antibodies, as well as anti-ERK1/2, anti-p-ERK1/2, anti-NF-κB, anti-p-NF-κB, anti-Akt from Chemicon (CA, USA), and an anti-p-Akt antibody from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies were purchased from Pierce. The lysis buffer contained 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.5% Triton X-100 and protease inhibitors. Protein samples were mixed with 5×SDS sample buffer, and boiled at 95°C for 5min. Forty microliters of proteins were separated on an 8%-12% polyacrylamide gel and then blotted on a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked in blocking buffer (PBS containing 5% BSA and 0.1% Tween) for 1h at room temperature and incubated with antibodies (1:1000 in blocking buffer) overnight at 4°C. Afterwards, the blots were washed three times with washing buffer (PBS containing 0.1% Tween-20), incubated with secondary antibodies (1:10000 in blocking buffer) for 1h at room temperature, washed three times with washing buffer, and then signals were amplified using the ECL chemiluminescence detection system (Amersham). Experiments were performed at least three times.

**Statistical Analysis** All the experiments were performed more than three times for confirmation. Data are presented as the mean±SEM. One-way analysis of variance was used to analyze statistical significance. P values less than 0.05 were considered statistically significant differences.

**RESULTS**

Parthenolide Inhibited Morphological Changes Characteristic of Apoptotic Human Lens Epithelial Cells HLE cells were treated with different concentrations (50, 100, or 200 μmol/L) of H2O2 to induce apoptosis. Only the 200 μmol/L H2O2 treatment caused a series of morphological changes, such as cytoplasmic condensation and increased intercellular gaps (Figure 1), which are characteristic features of cell apoptosis. Meanwhile, the effects of different concentrations of parthenolide (6.25, 12.5, 25 and 50 μmol/L) or a single 50 μmol/L parthenolide treatment on HLE cells were assessed. However, following treatment with higher concentrations of parthenolide (50 μmol/L) (Figure 1C), fewer HLE cells underwent H2O2-induced apoptosis and cell viability increased, as examined using the MTS assay (Figure 1H). Thus, parthenolide exerts a dose-dependent anti-apoptotic effect.

Parthenolide Protected Apoptotic Human Lens Epithelial Cells from Apoptosis Caused by H2O2 We used the MTS
assay to assess cell viability and explore the effect on parthenolide on oxidative stress in HLE cells in vitro 24, 48, and 72h after treatment with H2O2 and different concentrations of parthenolide (6.25, 12.5, 25 or 50 μmol/L). The H2O2 treatment significantly decreased cell viability, particularly after 72h of treatment (Figure 2A). At 24 h, cell viability was similar to the half maximal inhibitory concentration (IC50) of parthenolide. When cells were incubated with parthenolide alone, 50 µmol/L was the optimal parthenolide concentration, inducing the greatest statistically significant increase in cell viability (Figure 2B). Moreover, cell death was blocked, even by the lowest parthenolide concentration (Figure 2A).

Additionally, Annexin V-FITC and PI staining was used to detect the apoptosis rate in response to the H2O2 treatment. A significant increase in the apoptosis rate was observed in cells treated with 200 µmol/L H2O2 compared to the control (Figure 3). Parthenolide reduced the percentage of apoptotic cells in a dose-dependent manner (Figure 3).

Parthenolide Inhibited the H2O2-induced Activation of ERK1/2, Akt and NF-κB

Based on the evidence that parthenolide protect HLE cells from H2O2-induced apoptosis, we next considered the probable protective pathways. We examined the expression of the initiators and executioners of apoptosis, caspase-9 and caspase-3, during H2O2-induced apoptosis of HLE cells treated with various concentrations of parthenolide (6.25, 12.5, 25 or 50 µmol/L). The total expression of caspase-3 and caspase-9 was significantly increased in cells treated with 200 µmol/L H2O2 compared with the control (Figure 4A, 4B). Moreover, treatment with increasing concentrations of parthenolide decreased caspase-3 and caspase-9 expression in a dose-dependent manner.

As parthenolide inhibited the expression of caspase-3 and caspase-9 during H2O2-induced apoptosis, we performed a further study to examine the potential molecular mechanisms involved in this effect. As the NF-κB, MAPK and Akt pathways are important for cell apoptosis, proliferation and signal transduction, we determined whether those pathways were involved in the inhibitory effects of parthenolide on H2O2-induced apoptosis in HLE cells.

Interestingly, according to the Western blot analysis, neither H2O2 nor the parthenolide treatments changed the levels of the original form of these proteins, but both treatments altered the levels of the active, phosphorylated forms of the proteins (Figure 4C). Levels of phosphorylated ERK1/2, Akt and NF-κB were substantially increased compared to the normal control. The phosphorylation of these proteins represents their activation, indicating that parthenolide blocks the activation of ERK1/2, Akt and NF-κB, thereby reducing apoptosis.

DISCUSSION

Oxidative stress-induced apoptosis of HLE cells is confirmed as one of the most important causes of cataract formation, and it is induced by H2O2 in vitro, which creates a good model to investigate possible therapies for cataract. Meanwhile, based on accumulating evidence, parthenolide has diverse functions in cell apoptosis. Parthenolide inhibits H2O2-induced HLE cell apoptosis. This study was performed to identify
the molecular mechanisms involved in the inhibitory effect of parthenolide on H$_2$O$_2$-induced apoptosis in HLE cells. Parthenolide inhibits apoptosis and protects against oxidative stress in HLE cells. In the present study, cell morphology and cell viability were measured in HLE cells treated with both H$_2$O$_2$ and parthenolide. Cells treated with H$_2$O$_2$ alone showed characteristic apoptotic features, such as cytoplasmic condensation and increased intercellular gaps. Parthenolide was added at concentrations ranging from 6.25-50 μmol/L. Even the lowest concentration of parthenolide, 6.25 μmol/L, increased cell viability compared to the H$_2$O$_2$ group. The morphology and viability of HLE cells treated with 50 μmol/L parthenolide were similar to those of the normal control group. Moreover, this herbal constituent inhibited the expression of the caspase-3 and caspase-9 proteins in HLE cells treated with 200 μmol/L H$_2$O$_2$. Apoptosis is a series of proteolytic cascades in which initiator caspases mediate the activation of effector caspases[26-27] to promote the cleavage of target proteins and the orderly demise of the cell; caspase-9 is an initiator and caspase-3 is an effector[28-29]. These proteins are considered the

Figure 2 Cell viability after treatment with H$_2$O$_2$ and different concentrations of parthenolide  A: Cells were incubated with the indicated concentrations of parthenolide and H$_2$O$_2$ for 24, 48, or 72h, and cell viability was determined using the MTS assay. Cell viability was obviously altered after cells were treated for 24h. B: Cells were incubated with the indicated concentrations of parthenolide for 24 h. Cell viability was also determined using the MTS assay. Data are derived from three independent experiments (mean ±SEM). $^aP<0.05$, $^bP<0.01$ compared with the corresponding normal control.

Figure 3 Parthenolide inhibited HLE cells from H$_2$O$_2$-induced apoptosis  The apoptotic cells were detected by flow cytometry. A: HLE cells without H$_2$O$_2$ or parthenolide treatment (Normal); B: HLE cells with only 200 μmol/L H$_2$O$_2$ treatment; C-F: HLE cells with 200 μmol/L H$_2$O$_2$ treatment and 6.25, 12.5, 25, 50 μmol/L parthenolide treatment, respectively. Data are representative of three independent experiments with similar results.
core of the apoptotic process. Once both caspases are activated, the inevitable programmed cell death occurs. When cells were incubated with H$_2$O$_2$ for 24h, the number of apoptotic cells was substantially increased, and the total caspase level was concomitantly changed. Thus, H$_2$O$_2$-induced apoptosis were blocked by parthenolide. However, parthenolide, which targets selenocysteine-containing antioxidant enzymes, has been reported to induce reactive oxygen species accumulation and apoptosis of HeLa cells\[14\]. Thus, the mechanism underlying the complicated regulatory functions of parthenolide in HLE cell apoptosis should be investigated in further detail.

As the NF-κB, MAPK and Akt pathways are vital for cell apoptosis and signal transduction, we wondered whether the NF-κB, MAPK and Akt pathways were involved in the inhibitory effects of parthenolide on H$_2$O$_2$-induced apoptosis in HLE cells. Therefore, we examined the levels of total and phosphorylated NF-κB, ERK1/2 and Akt, which correlated with NF-κB, ERK1/2 and Akt activation\[10\]. Surprisingly, the results revealed a dose-dependent change in the levels of those molecules in H$_2$O$_2$-treated HLE cells after exposure to parthenolide. Levels of phosphorylated NF-κB, ERK1/2 and Akt were reduced, but the total levels of these proteins were unaffected. Thus, parthenolide induced a dose-dependent decrease in NF-κB, ERK1/2 and Akt activation.

In conclusion, the herbal constituent parthenolide inhibits H$_2$O$_2$-induced apoptosis in HLE cells, suggesting a protective effect on cataractogenesis. The mechanisms underlying the complicated regulatory effects of parthenolide on HLE cell apoptosis include the inhibition of various cell signaling pathways, including the NF-κB, ERK1/2 and Akt pathways. Therefore, parthenolide could be used as a potential treatment for cataracts in future clinical trials.

ACKNOWLEDGEMENTS

Authors’ contributions: Shentu XC analyzed data and wrote the manuscript; Ping XY performed the experiments; Cheng YL performed the experiments; Zhang X performed the experiments; Tang YL performed the experiments; Tang XJ analyzed data and wrote the manuscript.

Foundations: Supported by the National Natural Science Foundation of China (No.81371000; No.81670834); the Natural Science Foundation of Zhejiang Province (No. LY17H090004); the Zhejiang Traditional Chinese Medicine Project (No.2013ZA080); the Fundamental Research Funds for the Central Universities (No.2017FZA7002).

Conflicts of Interest: Shentu XC, None; Ping XY, None; Cheng YL, None; Zhang X, None; Tang YL, None; Tang XJ, None.

REFERENCES


7 Li L, Duker JS, Yoshiida Y, Niki E, Rasmussen H, Russell RM, Yeum KJ. Oxidative stress and antioxidant status in older adults with early cataract. Eye (Lond) 2009;23(6):1464-1468.


