Oxidative stress inhibits growth and induces apoptotic cell death in human U251 glioma cells via the caspase-3-dependent pathway

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Abstract. – OBJECTIVE: To investigate the possible pathway involved in hydrogen peroxide (H_2O_2) induced apoptosis in U251 glioma cells.

MATERIALS AND METHODS: The cultured U251 glioma cells were used in this study. The cells were divided into three groups: control group (untreated glioma cells), H_2O_2 group (treated with 100, 300 and 500 μM H_2O_2) and CI group (treated with 300 μM H_2O_2 and 15 μM caspase inhibitor, CI). The cellular viability was determined by MTT [3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide] assay. A flow cytometer was used for measuring the cell cycles. The mode of cell death was assessed by Annexin-V/PI-flow cytometry analysis. Fluorescence dihydroethidium (DHE) method was conducted to detect the oxygen species (O_2^-). Western blot analysis was performed to confirm the pro-caspase-3, caspase-3 and PARP (poly-ADP-ribose polymerase) protein expression.

RESULTS: The oxidative stress to U251 glioma cells exhibited in a dose-dependent manner with H_2O_2 concentrations increasing. The cell viability was considerably decreased and apoptosis occurred in H_2O_2 treated cells. A G1 cell cycle arrest and O_2^- level increase were found in H_2O_2 group. Western blot analysis showed a decrease of pro-caspase-3 protein level and an increase of caspase-3 and PARP level in 300 μM H_2O_2 treated cells. The H_2O_2 induced apoptosis depicted above was significantly restrained by CI.

CONCLUSIONS: Oxidative stress inhibits growth and induces apoptotic cell death in human U251 glioma cells via the caspase-3-dependent pathway. Mitochondrial pathway might involve in this signaling conduction. These findings are favorable for understanding the mechanisms of oxidative stress-induced apoptosis in U251 glioma cells.

Key Words:
  U251 glioma cells, H_2O_2, Oxidative stress, Caspase, Mitochondrial pathway.

Introduction

Gliomas, the most common type of primary tumor in brain, are characterized by high resistance to apoptosis and invasion pattern[1,2]. Despite treatment with surgery, chemotherapy and radiotherapy, the disease seems intractable to cure completely[3]. Therefore, the inhibition of growth of glioma cells seems necessary for this cancer treatment.

The mitochondrial signaling pathways for regulating apoptosis are complex and changeable derived from extracellular and intracellular stresses[4]. Oxidative stress is one of the most important intracellular stimuli caused by oxygen species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-)[5,6]. The effect of oxidative stress in cell apoptosis has been reported[7,8]. Many research efforts have been taken to explore the molecular mechanism of oxidative stress to glioma cell[9-11]. Zhang et al[12] proved that H_2O_2 induced glioma cell autophagy by interfering with Akt/mTOR and Beclin 1 signaling pathways. Another study[10] suggested that the p53, activated by NF-κB was essential for H_2O_2 induced apoptosis in glioma cells. These results demonstrated that the intracellular apoptotic pathway could be pivotal in induction of apoptosis by oxidative stress.

Caspase activation was proved to execute the apoptosis by induction, amplification and transduction of the intracellular apoptotic signals[13,14]. Caspase-dependent apoptosis is the most common cell death pathway that involves caspase-3, caspase-7, caspase-9 and caspase-12 pathways[15]. During apoptosis, one of the caspase-3 substrates poly ADP-ribose polymerase 1 (PARP-1) plays an important role in repairing the DNA damage[16]. Once activated, it could mediate cell death in reactive oxygen species (ROS)-induced injury[17]. To our knowledge, there are few published data on the defense mechanism of cas-

pase-3 inhibitor (CI) to apoptosis in human U251 glioma cells. Though previous results of Zhang et al[12] suggested a pan-caspase inhibitor Z-VAD-FMK protected the U251 glioma cells from apoptosis, they failed to explain the potential protection mechanism in glioma cells.

In this report, we evaluate the anti-apoptotic effects of the CI (Ac-DEVD-CHO) in U251 glioma cells and try to understand the possible signaling pathway and defense mechanism of CI to oxidative stress in U251 glioma cells.

Materials and Methods

Glioma U125 Cell Culture

The human glioma U125 cells (Obio Technology Co., Ltd, Shanghai, China) were cultured in 10% Dulbecco's modified Eagle medium (DMEM) at 37°C and cryopreserved at 4°C with 90% fetal bovine serum (Invitrogen Co., Ltd., Shanghai, China) and 10% DMSO (dimethylsulfoxide, Sigma-Aldrich Co. St. Louis, MO, USA). They were maintained in 37°C 5% CO_2 incubator and allowed to grow to confluence.

Treatment

The experiments were implemented 24 h after the cultured cells were seeded in 96-well plates at densities of 5×10^3 cell/well in 100 μL medium per well. H_2O_2 (Qcbio Science & Technologies co., Ltd, Shanghai, China) was freshly prepared with 100 μM, 300 μM and 500 μM to produce oxidative stress. To examine the inhibitory effects of the CI, cells were divided into three groups: control group (treated with culture medium), H_2O_2-treatment group (treated with 100 μM, 300 μM, 500 μM H_2O_2, respectively) and CI-treatment group (treated with 300 μM H_2O_2 and 15 μM CI, the concentration was chose according to our pre-experiments). All the treatments lasted for 24 h. Each set of experiments were repeated for six times.

Cell Viability Assays

This method depends on the reduction of colorless MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to blue formazan by mitochondrial dehydrogenases in live cells[18]. Viability of the treated U125 cells for 24 h was determined by a MTT-based Cell Proliferation and Cytotoxicity Assay Kit (Sangon Biotech Co., Ltd, Shanghai, China), at densities of 5 mg/mL for 10 μL per well. After 4 h of incubation at 37°C, the supernatants were removed, and 100 μL DM-SO was added to each well. After shaking for 10 min with low speed, the optical density was measured at 570 nm. Cell viability was expressed as the mean percent viable cells *vs*. control.

Cell Cycle Detection

The U251 cells after treatment were treated with 0.25% pancreatin-EDTA. After that, the U251 cells were pelleted via centrifugation at 1500 rpm for 6 min, and the supernatant was removed. Cells were washed once with phosphate buffer solution (PBS), then suspended in 0.5 mL PBS, and resuspended in 5 mL of cold 70% ethanol, incubated overnight at 4°C. The cells were harvested and washed with 6 mL PBS, followed by resuspention in 0.3 mL PBS. Then, they were transferred to a 1.5 mL culture tube. RNase A and propidium iodide (PI) were added subsequently, and incubated in the dark at 4°C for 15 min. The cell cycles were measured using a flow cytometer by standard procedures.

Cell Apoptosis Detection

Different stages of apoptosis were distinguished with a FITC-Annexin V kit (BD Biosciences, Franklin Lakes, NJ, USA) and PI. Briefly, cells (3.5×10^5 cells/well) were seeded in 96-well plates and cultured with H_2O_2 and CI at concentrations (depicted above) for 24 h. After washed once with PBS, and suspended, the cell suspension was moved into the flow tube. FITC-Annexin V and PI were added to these cells. They were incubated in the dark at 25°C for 15 min. Afterward, the apoptotic cells were evaluated by flow cytometry. Necrosis, early apoptosis and late apoptosis were defined by Annexin V^-/PI^+, Annexin V^+/PI^- and Annexin V^+/PI^+, respectively.

Western Blotting Analysis

Cells were washed with PBS and were lysed in RIPA buffer (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). Protein concentrations were evaluated with the bicinchonic acid (BCA) method. The protein samples were then boiled for 5 min before application to electrophoresis.

Proteins of untreated and H_2O_2 treated cells processed as described above were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Shanghai, China) and blocked with 5% skim milk, followed by shaking for 1 h. Then the blocked PVDF membrane was submerged in 1 × phosphate buffered saline with Tween (PBST). The membrane was incubated

with caspase-3 primary antibody (1: 200, BOSTER, Wuhan, Hubei, China) and β-actin antibody (1:10000, Proteintech Biotech Co., Ltd, Wuhan, Hubei, China) at 4°C overnight. After washing for 3 times in PBST, the membranes were incubated with secondary antibodies, goat anti-rabbit IgG (H+L)-HRP and Goat anti-mouse IgG (H+L)-HRP (1:5000). Then the antibody complexes were detected by electrochemiluminescence (ECL) method. The PVDF membrane was washed with $1 \times$ PBST 5 times (5×10 min), reacted with enhanced chemiluminescence ECL reagent (Millipore, Shanghai, China) and exposed to X-ray film for signal detection.

Intracellular O_2^- Concentration Detection

The intracellular general ROS H_2O_2 was detected by the oxidation-sensitive fluorescent probe dye. For its highly selective of O_2^- in mitochondrial, dihydroethidium (DHE, Invitrogen Co., Ltd., Shanghai, China) was used as a fluorogenic probe to assess concentration of O_2^- in cells exposed to different concentrations of H_2O_2. In brief, cells were digested to single cell suspension by pancreatin, and washed with 1 mL PBS on ice. For detection of O_2^-, cells were stained with DHE (0.5-5 μM) at 37°C in dark for 30 min and examined by a fluorescence activated cell sorter (FACS) with excitation length at 480-535 nm and emission at length 590-610 nm. ROS positive cells were detected by exhibiting a red fluorescence.

Statistical Analysis

All the statistical analyses were performed using one-way analysis of variance (ANOVA) by SPSS 18.0 software (SPSS, Chicago, IL, USA). All the results were expressed as means ± SEM in the different experiments. $p < 0.05$ was defined as statistical significance.

Results

Effect of H_2O_2 on Cellular Viability

MTT analysis showed that H_2O_2 treatments (100, 300 and 500 μM) caused significant dose-dependent growth inhibition in U251 cells compared to untreated cells ($p < 0.05$, Figure 1).

Effect of CI on H_2O_2 Induced Intracellular Superoxide (O_2^-) Level

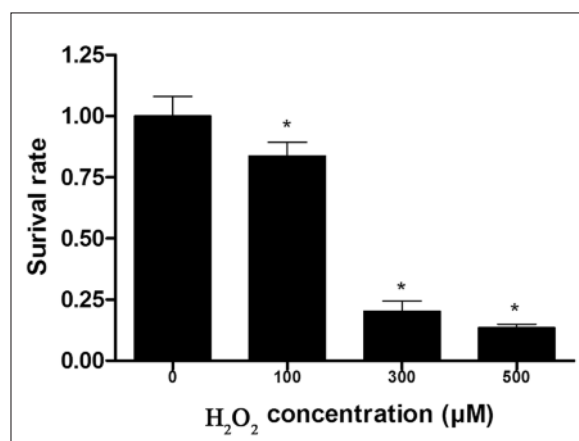To evaluate if the H_2O_2 exposure successfully led to oxidative stress, the quantified O_2^- level in

Figure 1. H_2O_2 induced cell viability assessed by MTT assay.

U251 cell cultures exposed in 100, 300 and 500 μM H_2O_2 were detected using fluorescence DHE (Figure 2). The O_2^- level reflected by red fluorescence was significantly raised with the increasing concentrations of H_2O_2 ($p < 0.05$). While, O_2^- level in CI treated cells were much more suppressed ($p < 0.05$) compared with the 300 μM H_2O_2 treated cells.
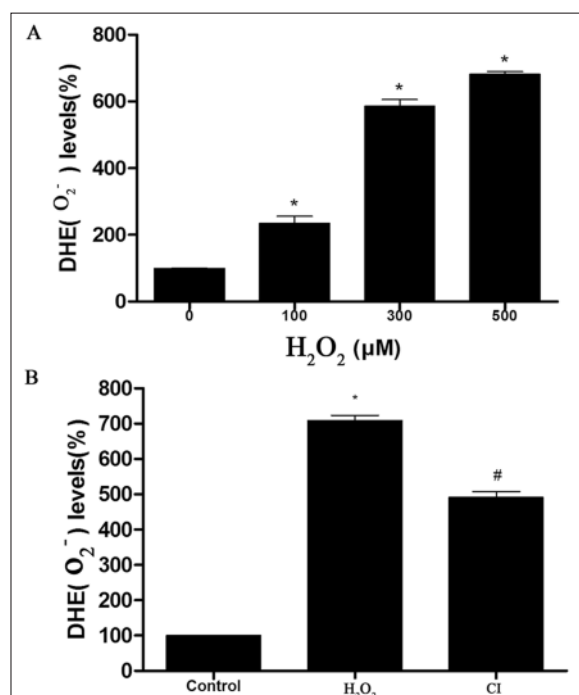
Figure 2. Mitochondrial O_2^- level detected using fluorescence DHE (dihydroethidium) method. **A,** O_2^- level in different H_2O_2 concentrations of glioma cells. **B,** O_2^- level in control, 300 μM H_2O_2 and CI treated cells.

Effect of CI on H₂O₂ Induced Cellular Cycle

The U251 cell cycle phase ratio of G1 phase was significantly reduced with the increasing concentration of H_2O_2 ($p < 0.05$, Figure 3A). Conversely, the G2 phase ratio was remarkably increased. While the S phase ratio showed no significant difference between untreated cells and H_2O_2-treated cells. The decrease in the G1 phase ratio was accompanied by much more increase in G2 phase ratio in 300 μM H_2O_2-treated cells.

The flow cytometry cycle diagrams (Figure 3B) showed that the U251 cell cycle ratio in untreated cells was as follows: G1% = 41.15, G2% = 14.19, S% = 44.66; the U251 cell cycle ratio in H_2O_2 group (in 300 μM H_2O_2 treatment) was as follows: G1% = 19.88, G2% = 33.64, S% = 46.48; the U251 cell cycle ratio in CI group was as follows: G1% = 36.05, G2% = 16.91, S% = 47.03. These results showed that CI inhibited the G1 phase reduction and G2 increment (Figure 3C).

Effect of CI on H₂O₂ Induced Cellular Apoptosis

The H_2O_2 induced apotosis in U251 cells was in a dose-dependent manner (Figure 4A). The apoptosis in 300 μM H_2O_2-treated cells was more obvious (Figure 4B), while it was significantly inhibited by addition of CI (Figure 4C). As shown in Figure 4B, the percentage of early apoptotic cells was 11.35%, 17.55% and 8.50% in untreated cells, 300 μM H_2O_2 treated and CI treated cells, respectively.

Effect of H₂O₂ on Caspase-3 and PARP-1 Protein

As seen in Figure 5, the expression of pro-caspase-3 was down-regulated with H_2O_2 concentration increasing. While the caspase-3 and its substrate (PRAP-1) were significantly up-regulated in 300 μM H_2O_2 treated cells.

Discussion

There are four pathways related to caspase activation apoptosis as follows: (1) the death receptor pathway, started by binding the ligands of death receptors on cell membrane; (2) the mitochondrial pathway, released the cy-
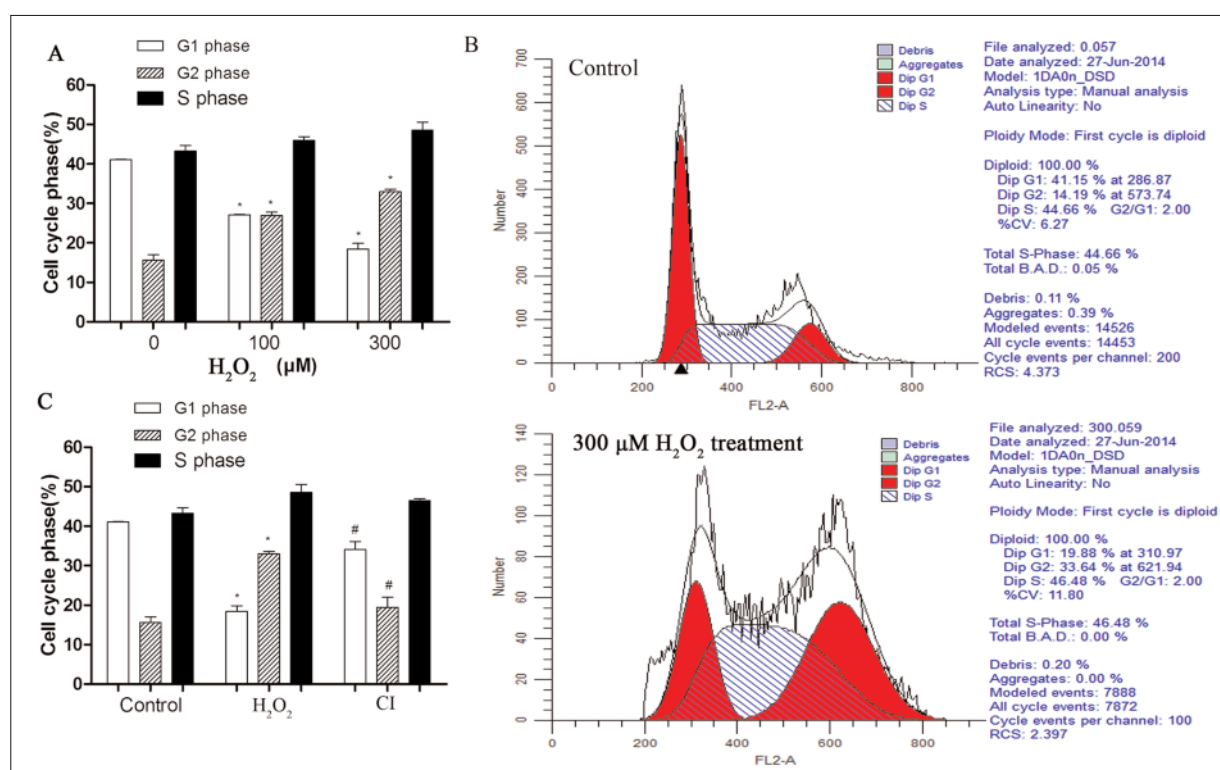

Figure 3. Cell cycle distribution among different treatment groups in G1, G2 and S phase. **A,** Cell cycle distribution in different concentrations of H_2O_2; **B,** and **C,** Cell cycle distribution in H_2O_2 group, 300 μM H_2O_2 **(B)**, and CI (caspase inhibitor) group **(C)**. *$p < 0.05$, when compared with untreated cells; #$p < 0.05$, when compared with H_2O_2 treated cells.
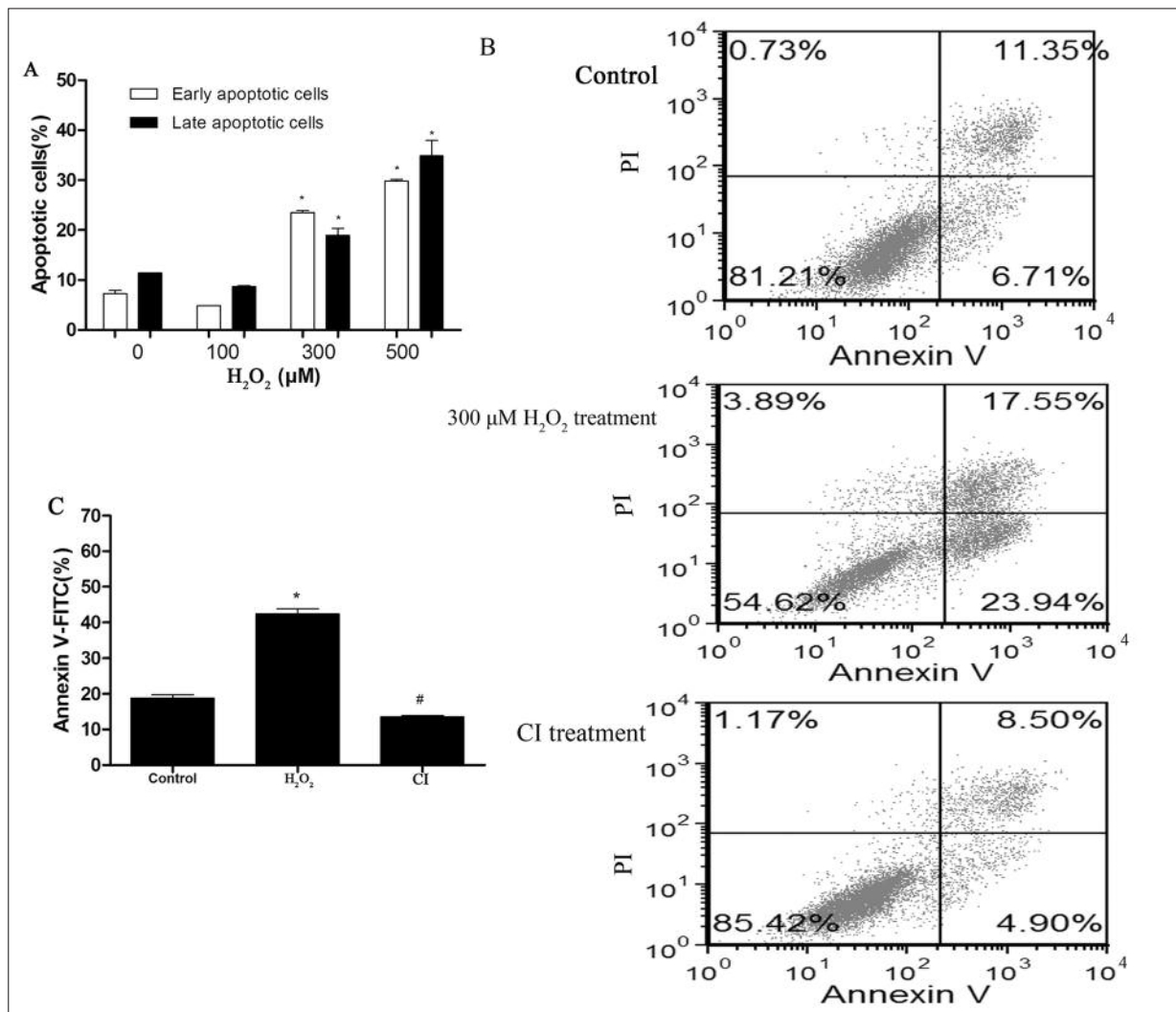
Figure 4. Apoptotic cell death results in U251 glioma cells. *A,* Early apoptotic cells and late apoptotic cells caused by different concentrations of H_2O_2. *B,* and *C,* Annexin V/PI results of apoptosis. The four quadrants in each panel of figure 4B correspond to: necrosis or debris cells (*upper left*), late apoptotic cells (*upper right*), early apoptotic cells (*lower right*) and viable cells (*lower left*), respectively *(B)*. *$p < 0.05$, when compared with untreated cells; #$p < 0.05$, when compared with H_2O_2 treated cells.

tochrome C from mitochondria for formation of apoptosomes; (3) the endoplasmic reticulum stress induced apotosis pathway, activated caspase-4 and caspase-2 in turn caspase-3; (4) the granzyme B activation pathway, injected natural killer cells to target cells[19]. All these pathways are connected to each other. In the current study, we focused on assessing the defense effects of caspase-3 inhibitor (Ac-DEVD-CHO) in relation to the changes in cellular cycle, cellular apoptosis and O_2^- level in $H_2O_2^-$ treated human U251 glioma cells, to reveal the possible apoptosis pathway in oxidative stress U251 glioma cells. Treatment with H_2O_2 successfully

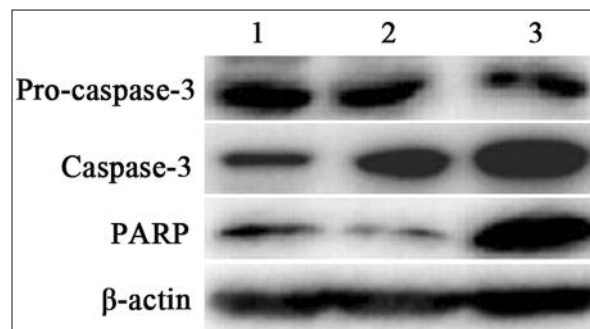**Figure 5.** Western blot analysis of the expression of pro-caspase-3, caspase-3 and PARP (poly-ADP-ribose polymerase) in U251 cells treated by 0 μM H_2O_2 (*band 1*), 100 μM H_2O_2 (*band 2*) and 300 μM H_2O_2 (*band 3*).

led to oxidative stress, increased cell apoptosis and affected the cell cycle and caspase-3 expression. Addtion of CI remarkably elevated the G1 cycle phase cells and reduced the G2 cycle phase cells, prevented the apoptosis in U251 cells and suppressed the mitochondrial O_2^- level. These data imply that the H_2O_2 induced apoptosis in human U251 glioma cells via a mechanism involving a caspase-3-dependent mitochondrial pathway.

The choice of H_2O_2 concentration should be discreet in this study, for different levels responding to different results. A higher degree of oxidative stress triggers cell necrosis, while lower level cause apoptosis[20,21]. A recent research[22] has been reported that 300 μM H_2O_2 triggered apoptosis in T98 glioma cells. In the present study, we have proved the H_2O_2 inducing dose-dependent apoptosis as well. According to these results, 300 μM H_2O_2 was chosen in the analysis of U251 glioma cells apoptotic pathways.

Although the oxidative stress dose not act directly in the death receptors, it influences the mitochondrial pathway by making the environment conducive for receptors and ligands matching or downstream apoptosis execution[23]. In fact, oxidative stress has several effects on cells, such as the DNA damage, mitochondrial dysfunction and signaling pathway activation[24]. We found in the present study that the proliferation of normally grown U251 glioma cells was significantly suppressed after exposure to H_2O_2 in a dose-dependent manner. Moreover, flow cytometry indicated apoptosis induced by H_2O_2 seems to be a consequence of cycle arrest in G1 phases of U251 glioma cells, indicating a DNA damage checkpoints occurs in this phase. The damaged DNA can not encode some critical proteins in mitochondrial electron transport chain and, then, result in mitochondrial dislocation. Activated caspase-3, after DNA damage by oxidative stress, might either triger the initiation of DNA repair, resulting in the completion of the cell cycle or stop the cell cycle to apoptosis.

Caspases are a series of vital downstream executoners. The initiation phases of apoptosis includes activation of caspase-3, in which the zymogen pro-caspase was splitting into active units by proteolytic cleavage[25]. The activated caspase-3, then, decomposes the critical substrate PARP-1 and finally leads to apoptosis[26,27]. Therefore, the interaction of caspase-3 and its substrate PARP-1 are regarded as key executioners of apoptosis[28]. The present study showed that the protein level of caspase-3 was increased accompanied with decrease of pro-caspase-3 level during the apoptotic process, especially in the 300 μM H_2O_2-treated cells. In addition, PARP-1 exhibited a dominant upregulation in glioma cells when exposed to 300 μM H_2O_2 in our study. The results could be explained by decomposition of PARP-1 from caspase-3 during apoptosis[29,30].

The treatment results of H_2O_2-induced cells co-treated withCI suggested that Ac-DEVD-CHO restrained the apoptosis by H_2O_2 via reducing the cell cycles, suppressing the cycle arrest in G1 phase. It has been shown that the tumor suppressor p73 induced apoptosis by mediating the PUMA (p53 up-regulated modulator of apoptosis) protein, then in turn activated Bax eventually induced the mitochondrial dislocation and cytochrome C release in cancer cells[31]. The function of cytochrome C release[32] has been shown to be modulated by O_2^-, which was assessed in our study. Interestingly, our study also demonstrated that the O_2^- level induced by inhibitor was significantly refrained by inhibitor. Recently, the ROS burst was usually observed involving in the mitochondrial pathway apoptosis in cancer cells[33,34]. Based on these studies, we confirmed our hypothesis that caspase-3/PARP involved in the mitochondrial pathway in oxidative stress induced apoptosis in U251 glioma cells. However, more researches should be focused on the mediation in the future.

Conclusions

We suggest the slight concentration (300 μM) of H_2O_2 lead to apoptosis in U251 glioma cells based on the evidence such as a significant decrease in the cell viability, a G1 cell cycle arrest and a remarkably increase in the O_2^- level. These changes are, then, significantly suppressed by caspase-3 inhibitor, supporting the caspase-3-dependent pathway involving in apoptosis induced by oxidative stress response. We also suggest the apoptosis was triggered by O_2^--mediation. These findings are favorable for understanding the mechanism of oxidative stress induced apoptosis in U251 glioma cells.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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