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Resveratrol Induces Cell Death through ROS-dependent MAPK Activation in A172 Human Glioma Cells

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Glioblastoma multiforme is the most common and most aggressive type of primary brain tumor in humans. Despite intensive treatment, including surgery, radiation, and chemotherapy, most patients die of the disease. Although the anti-cancer activity of resveratrol has been demonstrated in various cancer cell types, its underlying mechanism in glioma cells is not fully elucidated. The present study was undertaken to investigate the effect of resveratrol on cell viability and to determine the molecular mechanism in A172 human glioma cells. Resveratrol caused the generation of reactive oxygen species (ROS), and resveratrol-induced cell death was prevented by antioxidants (*N*-acetylcysteine and catalase), suggesting that an oxidative mechanism is responsible for resveratrol-induced cell death. Resveratrol-induced phosphorylation of extracellular signal-regulated kinase (ERK), p38 kinase, and c-Jun N-terminal kinase (JNK), and resveratrol-induced cell death were prevented by inhibitors of these kinases. Resveratrol-induced activation of caspase-3 and cell death were prevented by the caspase inhibitors. ERK activation and caspase-3 activation induced by resveratrol was blocked by *N*-acetylcysteine. Taken together, these results suggest that resveratrol causes a caspase-dependent cell death via activation of ERK, p38, and JNK, mediated by ROS generation, in human glioma cells.

Key words : Cell viability, glioma cells, MAPK, resveratrol, reactive oxygen species

Introduction

Glioblastoma multiforme (GBM) is the most common and most aggressive type of primary brain tumor in human. These malignant astrocytic tumors exhibit a high proliferation rate and an aggressive growth pattern and acquire resistance against many therapeutic interventions [19]. Despite aggressive treatment including surgery, radiation, and chemotherapy, most patients die of the disease, with median survival of 1 year [6].

Polyphenols found in various plants reveal anti-oxidant and anti-inflammatory effects, and anti-tumor effects in various cell types [7, 11, 20]. Resveratrol (trans-3,5,4V-trihydroxystilbene) is a polyphenolic phytoalexin which is abundant in grapes, peanuts, red wine, and a variety of food sources. Resveratrol has been reported to induce apoptosis and inhibit growth of various cancer cells [2, 10, 17, 22, 24] and enhances radiosensitivity of human cancer cells [12, 15]. However, the mechanisms underlying the reveratrol-mediated apoptotic signaling pathways are not fully understood.

Mitogen-activated protein kinases (MAPKs) are serine/threonine kinases involved in the regulation of various cellular responses, such as cell proliferation, differentiation, and apoptosis [4]. It has been demonstrated that reactive oxygen species (ROS) can induce the activation of MAPKs to mediate cell responses [18]. We have previously demonstrated that ROS plays a pivotal role in flavonoid-induced glioma cell death. It has also been suggested that MAPKs are involved in cell death mechanism [8, 13, 14].

The present study was undertaken to examine the effect of resveratrol on glioma cell viability and to determine its molecular mechanism in A172 human glioma cells. Our data demonstrated that resveratrol induces human glioma cell death via a caspase-dependent apoptotic signaling cascades involving a ROS/MAPK pathway.

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Materials and Methods

Cell culture

A172 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA,

USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Invitrogen, Carsbad,CA, USA) containing 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved. Unless otherwise stated, cells were treated with resveratrol in serum-free medium.

Reagents

Resveratrol, N-acetylcysteine (NAC), catalase, Hoechst 33258, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). U0126, SB203580, SP600125, z-DEVD-FMK, and DEVD-CHO were purchased from Calbiochem (CA, USA). 2′,7′-dichlorofluorescein diacetate (DCFH-DA) is obtained from Molecular Probes (Eugene, OR, USA). Antibodies were obtained from Cell Signaling Technology Inc. (Beverly, Massachusetts, USA). All other chemicals were of the highest commercial grade available.

Measurement of cell viability, proliferation, and cell death

Cell viability was evaluated using MTT assay. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. Cells were incubated for 2 hr at 37°C, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 0.11 ml of dimethyl sulfoxide. A 0.1-ml aliquot of each sample was then transferred to 96-well plates and the absorbance was measured at 550 nm with ELISA Reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany). Data were expressed as a percentage of control measured in the absence of resveratrol. Test reagents were added to the medium 30 min before resveratrol exposure.

For cell proliferation and cell death, cells were harvested using 0.025% trypsin and incubated with 4% trypan blue solution. The number of viable and nonviable cells was counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable.

Measurement of apoptosis

To cell cycle analysis, cells were grown in 6-well plates and were treated as indicated. Then, attached and floating cells were pooled, pelleted by centrifugation, washed in PBS, and fixed with cold 70% ethanol containing 0.5% Tween 20 at 4°C overnight. Cells were washed and resuspended in 1.0 ml of propidium iodide solution containing 100 μg of RNase A/ml and 50 μg propidium iodide/ml and incubated for 30 min at 37°C. Apoptotic cells were assayed using FACSort Becton Dickinson Flow Cytometer at 488 nm and the data were analyzed with CELLQuest Software. Cells with sub-G_1 propidium iodide incorporation were considered apoptotic. The percentage of apoptotic cells was calculated as the ratio of events on sub-G_1 to events from the whole population.

To detect apoptosis morphologically, cells were treated with resveratrol for 48 hr, fixed them with 4% paraformaldehyde, and stained them with Hoechst 33258 (0.5 μg/ml) for 15 min.

Measurement of ROS

The intracellular generation of ROS was measured using DCFH-DA. The nonfluorescent ester penetrates into the cells and is hydrolyzed to DCFH by the cellular esterases. DCFH is rapidly oxidized to the highly fluorescent compound 20,70-dichlorofluorescein (DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells cultured in 6-well plate were preincubated in the culture medium with 30 μM DCFH-DA for 1 hr at 37°C. After the preincubation, the cells were exposed to 100 μM resveratrol for various times. Data were acquired and analyzed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) with appropriate software.

Western blot analysis

Cells were harvested at various times after resveratrol treatment and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris–HCl, pH 7.4). Cell debris was removed by centrifugation at 10,000g for 10 min at 4°C. The resulting supernatants were resolved on a 10% SDS–PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with different primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Measurement of caspase-3 activity

Caspase-3 activity was measured by the NucViewTM 488 caspase-3 assay kit for live cells (Biotium, Inc. CA. USA) according to the manufacturer's instructions. The substrate consists of a fluorogenic DNA dye and a DEVD substrate moiety specific for activated caspase-3. The substrate, which is both nonfluorescent and nonfunctional as a DNA dye, rapidly crosses cell membrane to enter the cell cytoplasm, where it is cleaved by activated caspase-3 to form a high-affinity DNA dye. The released DNA dye migrates into the nucleus to stain the nucleus bright green. Cells were grown in 6-well plates and were treated as indicated. Detached cells from the culture plates by trypsin were pelleted by centrifugation and washed with phosphatebuffered saline (PBS). A 2 μl caspase-3 substrate stock solution was added directly to 0.3 ml of cells suspension in PBS. Cells were incubated at room temperature for 30 min and changes in caspase-3 activity was acquired and analyzed using a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA) with appropriate software.

Statistical analysis

The data are expressed as means ± SEM and the difference between two groups was evaluated by unpaired Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test using SPSS v10.1 (SPSS Inc., Chicago, Illinois, USA). A probability level of 0.05 was used to establish significance.

Results

Effect of resveratrol on cell viability, proliferation, and cell death

To investigate the effect of resveratrol on A 172 human glioma cell viability, the cells were exposed to various concentrations (0-200 μM) of resveratrol for 48 hr (A) and to 100 μM for various times (B). Resveratrol decreased the cell viability in a dose- and time-dependent manner (Fig. 1A and B). To ascertain if a decrease in cell viability by reveratrol was attributed to changes in cell proliferation, the effect of reveratrol on cell proliferation was examined. As shown in Fig. 1C, resveratrol induced inhibition of proliferation in a dose- and time-dependent manner. We next examined whether a decrease in cell viability and proliferation induced by resveratrol was due to cell death, the cells were exposed to various concentrations of resveratrol for 24 and 48 hr and

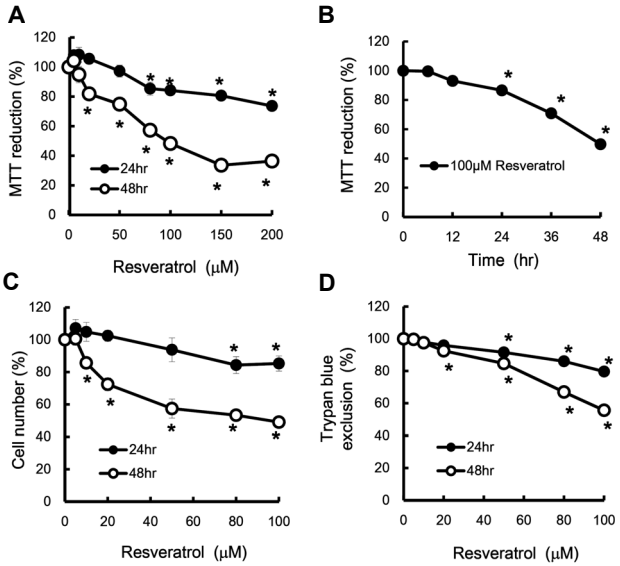

Fig. 1. Effect of resveratrol on cell viability, proliferation, and cell death. Cells were treated with various concentrations of resveratrol for 24 and 48 hr (A) and 100 μM resveratrol for various times (B). Cell viability was determined by MTT assay. Data are mean ± SEM of four independent experiments performed in duplicate. *p< 0.05 compared with control without resveratrol. Cells were treated with concentrations of resveratrol for 24 and 48 hr. Cell number was counted using a hemocytometer under light microscopy (C) and Cell death was estimated by trypan blue exclusion assay (D). Data are mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with control without resveratrol.

cell death was estimated by trypan blue exclusion assay. Resveratrol induced cell death with patterns similar to loss of cell viability and inhibition of cell proliferation (Fig. 1D). These results suggest that resveratrol-induced decrease in glioma cell viability was largely resulted from cell death.

Effect of resveratrol on apoptosis

To delineate whether the resveratrol-induced cell death was due to the induction of apoptosis, changes in nuclear morphology and cell cycle were investigated. Cells treated with resveratrol displayed condensation and fragmentation of nuclei, typical features of apoptosis, as revealed by Hoechst 33258 staining (Fig. 2A). The DNA content analysis by flow cytometry also showed that the proportion of the cells in the sub- G1 phase (apoptotic cells) was increased from 1.72% of control to 24.95% by 100 μM resveratrol (Fig. 2B). These results suggest that resveratrol-induced cell death is largely attributed to induction of apoptosis.

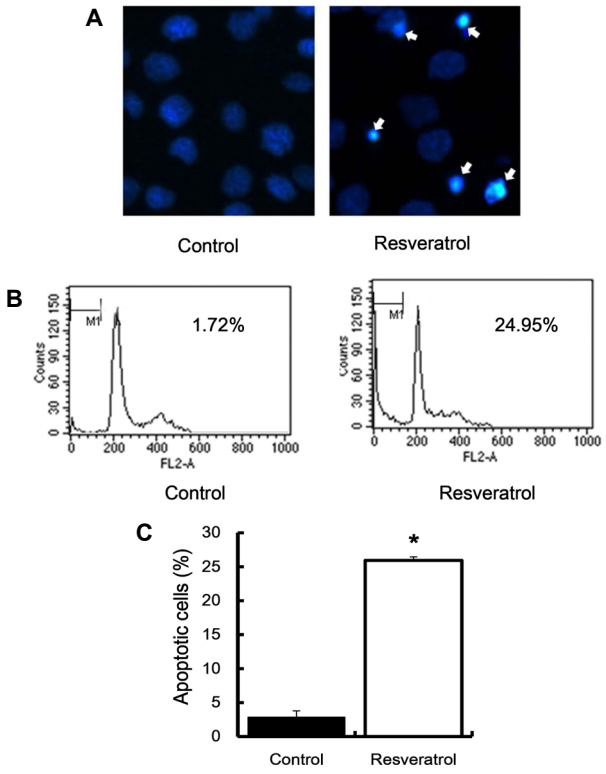

Fig. 2. Effect of resveratrol on apoptosis. Cells were treated with 100 μM resveratrol for 48 hr. Apoptotic cells were evaluated by Hoechst 33258 staining (A) and flow cytometric analysis (B). Arrows in (A) indicate the condensation and fragmentation of nuclei. The percentages within box in (B) indicate the extent of sub-G1phase. Data in (C) obtained from flow cytometric analysis are mean ± SEM of three independent experiments performed in duplicate. * p<0.05 compared with control.

Role of ROS generation in resveratrol-induced cell death

To evaluate whether resveratrol induces ROS generation in A172 cells, the cells were exposed to resveratrol and changes in DCF fluorescence were measured using flow cytometry. Resveratrol caused an increase in ROS generation as evidenced by an increase in the proportion of cells with higher fluorescence intensity (Fig. 3A). A significant change in ROS generation by resveratrol was observed at 1 hr after treatment (Fig. 3B).

To determine whether ROS generation is involved in the resveratrol-induced cell death, the effect of antioxidants NAC and catalase on the cell viability was evaluated. The resveratrol-induced cell death was prevented by these antioxidants (Fig. 3C), indicating that the resveratrol-induced cell death is associated with ROS generation in A172 cells.

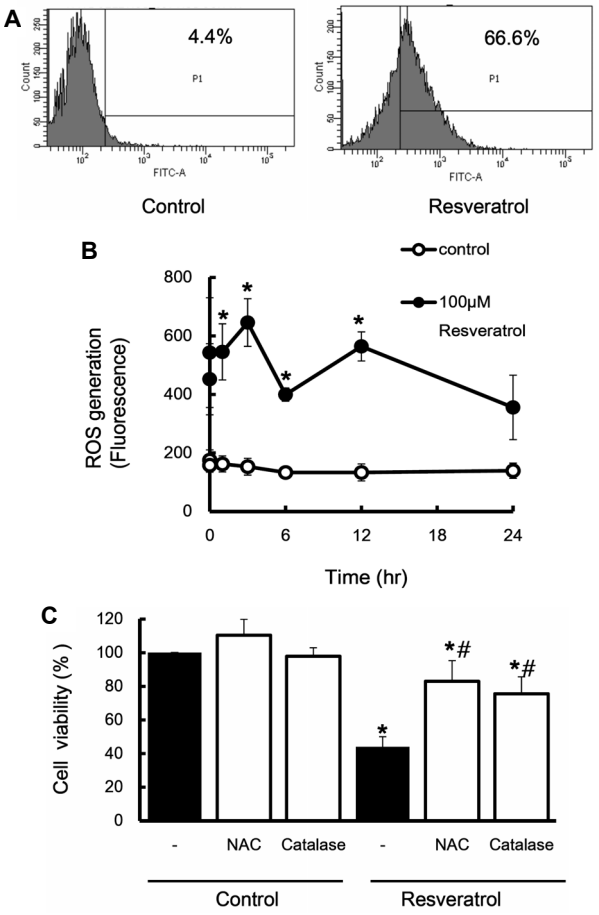

Fig. 3. Role of reactive oxygen species (ROS) generation in resveratrol-induced cell death. Cells were exposed to 100 μM resveratrol for 1 hr (A) or various times (B) and the DCF fluorescence intensity was measured by a flow cytometer. Data in (B) are mean ± SEM of four independent experiments performed in duplicate. * p<0.05 compared with control. Cells were exposed to 100 μM resveratrol for 48 hr in the presence or absence of 5 mM N-acetylcysteine (NAC) and 800 units/ml catalase (C). Cell viability was estimated by MTT reduction assay. Data are mean ± SEM of four independent experiments performed in duplicate. * p<0.05 compared with respective control; # p<0.05 compared with resveratrol alone.

Role of MAPKs in resveratrol-induced cell death

We examined if the resveratrol-induced cell death is associated with activation of these kinases. Cells were exposed to resveratrol for various times and activity of these kinases was evaluated by detecting their phosphorylation forms. Resveratrol induced activation of ERK, p38 kinase, and JNK in a time-dependent manner (Fig. 4A and B). Activation of these kinases was inhibited by the antioxidant NAC (Fig. 4C), suggesting that ROS generation mediates activation of

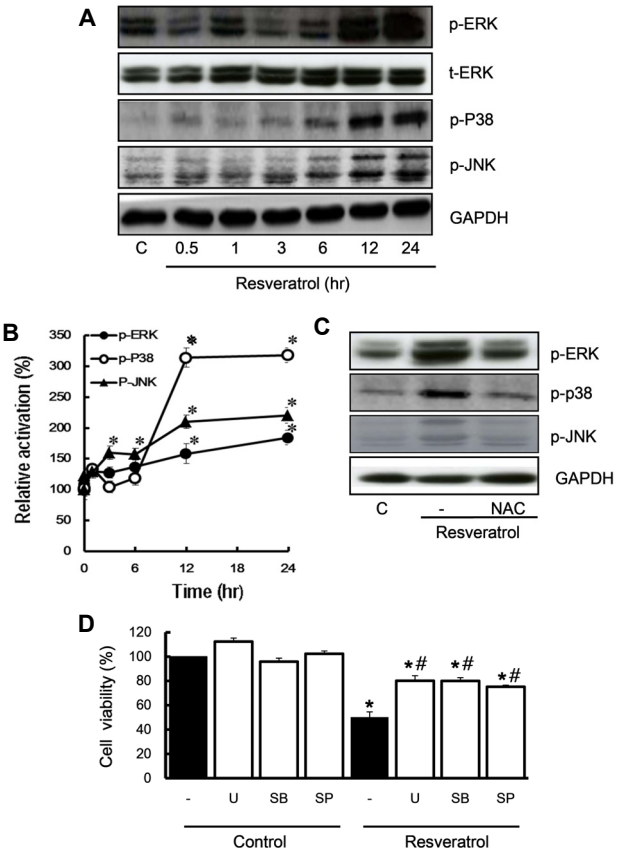

Fig. 4. Role of mitogenactivated protein kinases (MAPKs) on resveratrol-induced cell death. Cells were exposed to 100 μM resveratrol for various times. (A) Representative expression of phospho-ERK1/2 (p-ERK), phosph-p38 (p-p38), and phospho-JNK (p-JNK) was evaluated using the specific antibodies. Total ERK (t-ERK) and GAPDH were employed as a loading control. (B) Quantitative data of densitometric analysis. Data are mean ± SEM of three independent experiments. (C) Effect of antioxidant on resveratrol-induced activation of MAPK subfamilies. Cells were treated with 100 μM resveratrol in the presence or absence of 5 mM N-acetylcysteine (NAC) for 24 hr. Activation of MAPK subfamilies were evaluated by Western blot analysis. (D) Effect of inhibitors of MAPKs on resveratrol-induced cell death. Cells were treated with 100 μM resveratrol in the presence or absence of each 10 μM of U0126 (U), SB203580 (SB), and SP6001252 (SP) for 48 hr. Cell viability was determined by MTT assay. Data are mean ± SEM of four independent experiments performed in duplicate. *p< 0.05 compared with respective control; $^{\#}$ p<0.05 compared with resveratrol alone.

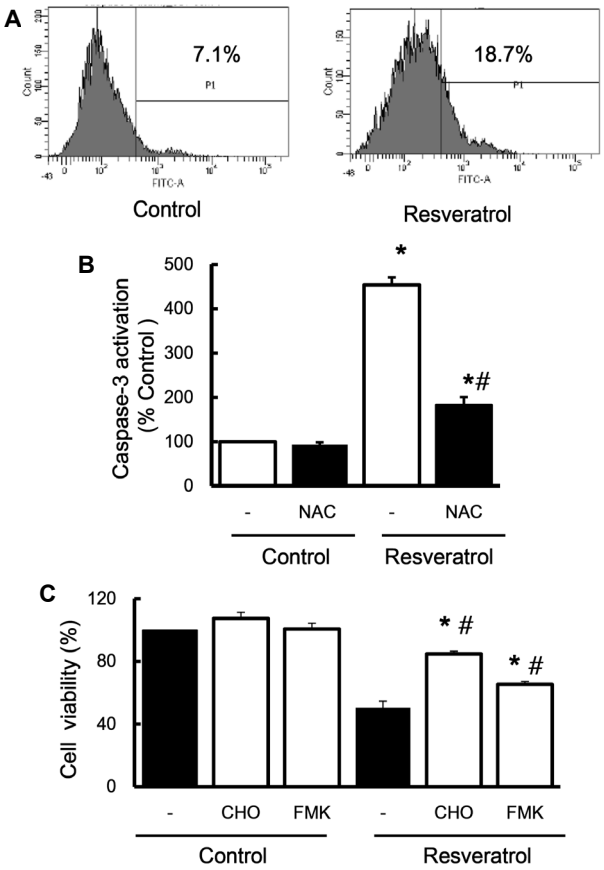

Fig. 5. Effect of resveratrol on caspase-3 activation. Cells were exposed to 100 μM resveratrol for 24 hr (A) and various times (B). Changes in caspase-3 activity were assayed using Flow Cytometer. (A) Representative results from five independent experiments are shown. (B) Effect of antioxidant on resveratrol-induced caspase-3 activation. Cells were treated with 100 μM for 24 hr in the presence or absence of 5 mM *N*-acetylcysteine (NAC). Changes in caspase-3 activity were assayed using Flow Cytometer. Data are mean ± SEM of three independent experiments performed in duplicate. *p<0.05 compared with respective control; $^{\#}p$<0.05 compared with resveratrol alone. (D) Effects of caspase inhibitors on resveratrol-induced cell death. Cells were exposed to 100 μM resveratrol for 48 hr in the presence or absence of each 10 μM of DEVD-CHO (CHO) and z-VAD-FMK (FMK). Cell viability was estimated by MTT assay. Data are mean ± SEM of five independent experiments performed in duplicate. * p<0.05 compared with respective control; $^{\#}$ p<0.05 compared with resveratrol alone.

MAPKs by reveratrol.

To evaluate whether activation of MAPKs is responsible for the resveratrol-induced cell death, the effect of inhibitors of these kinases on the cell death was evaluated. As shown in Fig. 4D, inhibitors of ERK (U0126), p38 (SB203580), and JNK (SP600125) prevented the reveratrol-induced cell death induced by resveratrol. These results suggest that activation of MAPKs is involved in the resveratrol-induced cell death.

Role of caspase activation in resveratrol-induced cell death

Caspases play a key role during the execution phase in various forms of apoptosis [5]. To explore whether the activation of caspase-3 was involved in the resveratrol-induced cell death, the effect of resveratrol on activation of caspase-3 was examined. Exposure of cells to resveratrol for 24 hr induced caspase-3 activation (Fig. 5A).

To demonstrate if caspase activation is associated with ROS generation, the effect of antioxidant NAC on activation of caspase-3 was examined. Resveratrol-induced caspase-3 activation was prevented by NAC (Fig. 5B), suggesting that resveratrol induces caspase-3 activation through ROS generation.

To evaluate if caspase is involved in the resveratrol-induced cell death, the effect of caspase inhibitors on the cell viability was examined. Cells were exposed to resveratrol in the presence of the caspase-3 specific inhibitor DEVD-CHO and the general caspase inhibitor z-DEVD-FMK. The resveratrol-induced cell death was prevented by these inhibitors (Fig. 5C). These data indicate that resveratrol induces cell death through a caspase-dependent mechanism.

Discussion

Previous studies have shown both the chemopreventive and chemotherapeutic potential of resveratrol [1, 21]. It suppresses the proliferation of a wide variety of human tumor cells [1]. The antitumor activities of resveratrol are mediated through several cell signaling pathways including cell cycle arrest, suppression of tumor cell proliferation, induction of apoptosis, and differentiation, reduction of inflammation and angiogenesis, and inhibition of adhesion, invasion, and metastasis [1, 16, 21]. However, the underlying mechanisms by which reveratrol incues apoptosis in human glioma cells are not clearly defined.

The present study demonstrated that resveratrol-induced cell viability and suppression of cell proliferation were largely attributed to apoptotic cell death. These findings are consistent with seeral previous reports showing the cytotoxic effects of resveratrol in human glioma cells [3, 9].

Our previous studies have shown that flavonoids causes ROS generation associated with induction of apoptosis in human glioma cells [8, 13, 14]. The resveratrol-induced cell death also seems to be associated with ROS generation considering the findings in the present study showing that resveratrol increased ROS generation and the antioxidants NAC and catalase prevented the resveratrol-induced cell death (Fig. 3).

Members of the MAPK family constitute important mediators of signal transduction pathways that serve to coordinate the cellular response to a variety of extracellular stimuli. Based on structural differences, they have been classified into three major subfamilies: ERK, JNK, and p38 kinase. Modulation of these subfamilies has been demonstrated to be involved in apoptosis induced by flavonoids in human glioma cells [8, 13, 14]. Therefore, we examined if subfamilies of MAPKs are involved in the resveratrol-induced cell death. The results suggested that ROS-induced activation of MAPKs may be involved in the resveratrol-induced cell death. Prevention of the resveratrol-induced cell death by inhibitors of ERK, p38, and JNK further supported the role of MAPKs. From these data, we concluded that activation of MAPKs plays an important role in the reveratrol-induced cell death in human glioma cells.

Although growth inhibition and cell death induced by flavonoids are associated with caspase activation in human cancer cells [8, 14, 23] it is unclear whether resveratrol induces cell death through a caspase-dependent mechanism in human glioma cells. In the present study, resveratrol-induced activation of caspase-3 and its inhibition by the antioxidant NAC (Fig. 5A and B) suggested the involvement of ROS generation in the reveratrol-induced activation of caspase-3. Our data also show that the resveratrol induces apoptosis through a caspase-dependent mechanism, evidenced by prevention of the reveratrol-induced cell death by caspase inhibitors (Fig. 5C).

In conclusion, the present study demonstrated that resveratrol results in human glioma cell death through a caspase-dependent mechanism involving up-regulation of ERK, p38, and JNK. These events are initiated by ROS generation. Induction of cell death may be a promising therapeutic approach in cancer therapy. Our results suggest that resveratrol may be considered a potential candidate for induction of apoptosis in glioblastoma. Further investigation is needed to validate the contribution of resveratrol to tumor therapy *in vivo*.

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초록 : 사람의 신경교모세포종 기원 세포에서 레스베라트롤에 의한 활성산소종 생성 증가와 MAPK 활성화를 통한 세포 사멸 효과

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다형성 신경교모세포종은 사람의 원발성 뇌종양 중 가장 흔하면서 악성이 높은 종양의 하나로 수술과 방사선 치료, 화학치료 등 집중적 치료에도 불구하고 사망률이 높은 종양이다. 레스베라트롤은 다양한 자연산 물질에 포함되어 있는 폴리페놀의 일종으로 여러 종류의 암세포들에서 항암 작용이 있음이 보고되어 있으나 그 기전은 명확하게 밝혀져 있지 않다. 본 연구에서는 사람의 신경교모세포종 기원 세포인 A172 세포에서 레스베라트롤에 의한 세포 사멸 효과와 그 기전을 확인하고자 하였다. 레스베라트롤은 A172세포에서 활성산소종의 생성을 촉진하였으며 N-acetylcystein 혹은 catalase 등의 항산화제들을 전처치시 레스베라트롤의 세포 사멸 효과가 차단되었다. 레스베라트롤은 ERK와 p38 kinase, JNK 등의 인산화를 촉진하였으며 이들 인산화 효소의 억제제들을 전처치하면 레스베라트롤의 세포 사멸 효과가 차단되었다. Caspase 억제제를 전처치시 레스베라트롤에 의한 caspase-3의 활성화와 세포 사멸이 차단되었으며, N-acetylcystein을 전처치시 레스베라트롤에 의한 ERK의 활성화와 caspase-3의 활성화가 차단되었다. 이들 결과를 종합하면 레스베라트롤은 A172 세포에서 활성산소종의 생성을 촉진하며 이는 ERK와, p38 kinase, JNK 등의 활성화를 통해 caspase-의존성 기전으로 세포사멸을 유도하는 것으로 사료된다.