

Resveratrol-induced apoptotic death in human U251 glioma cells

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Abstract

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a naturally occurring polyphenolic compound highly enriched in grapes, peanuts, red wine, and a variety of food sources. Resveratrol has antiinflammatory and antioxidant properties, and also has potent anticancer properties. Human glioma U251 cells were used to understand the molecular mechanisms by which resveratrol acts as an anticancer agent, since glioma is a particularly difficult cancer to treat and eradicate. Our data show that resveratrol induces dose- and time-dependent death of U251 cells, as measured by lactate dehydrogenase release and internucleosomal DNA fragmentation assays. Resveratrol induces activation of caspase-3 and increases the cleavage of the downstream caspase substrate, poly(ADP-ribose) polymerase. Resveratrol-induced DNA fragmentation can be completely blocked by either a general caspase inhibitor (Z-VAD-FMK) or a selective caspase-3 inhibitor (Z-DEVD-FMK), but not by a selective caspase-1 inhibitor. Resveratrol induces cytochrome *c* release from mitochondria to the cytoplasm and activation of caspase-9. Resveratrol also increases expression of proapoptotic Bax and its translocation to the mitochondria. Resveratrol inhibits U251 proliferation, as measured by MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt], and induces G₀/G₁ growth arrest, as determined by flow cytometry. The cyclin-dependent kinase inhibitor, olomoucine, prevents cell cycle progression and resveratrol-induced

apoptosis. These results suggest that multiple signaling pathways may underlie the apoptotic death of U251 glioma induced by resveratrol, which warrants further exploration as an anticancer agent in human glioma. [Mol Cancer Ther 2005;4(4):554–61]

Introduction

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a naturally occurring polyphenolic compound highly enriched in grapes, peanuts, red wine, and a wide variety of food sources (1). Resveratrol has been reported to elicit many cellular responses including cell cycle arrest, differentiation, and apoptosis (2), and it has antiinflammatory, antileukemic, neuroprotective, and antiviral properties (3–6). Resveratrol can also function as an antioxidant (7) and reduces the risk of developing coronary heart disease, likely through its modulation of lipid metabolism and prevention of the low-density lipoprotein oxidation (8), as well as inhibition of eicosanoid production and platelet aggregation (9). Most recently, it has been shown that resveratrol can act as a cancer-chemopreventive agent (10). Resveratrol has been shown to inhibit tumor initiation, promotion, and progression in a variety of cell culture systems and animal models (11). Resveratrol can inhibit several important enzymes involved in carcinogenesis, including ribonucleotide reductase (12), NADH:ubiquinone oxidoreductase (13), and human cytochrome P450 (14).

Resveratrol has been shown to induce apoptotic cell death in a number of cancer cell lines, including hormone-sensitive LNCaP prostate cells (15), hormone-insensitive DU 145 prostate cells (16), mouse myeloid leukemia cells (5), human B cell chronic leukemia cells (17), as well as several other human cancer cell lines such as MCF7, SW480, HCE7, Seg-1, and HL60 (2). Alteration of expression of Bcl-2 family of proteins, loss of mitochondrial function, release of cytochrome *c*, and activation of caspases may be involved in resveratrol-induced death, as shown in Bcl-2 overexpressing U937 cells (18), pancreatic carcinoma cells (19), mouse myeloid leukemia cells (5), normal and leukemic hematopoietic cells (17), and human Caco-2 colonic adenocarcinoma cells (20). Resveratrol can induce p53-independent apoptosis in human HCT116 colon carcinoma cells (21). However, p53 is required for resveratrol-induced apoptosis in mouse JB6 epidermal (22) or Hep G₂ cells (23). Therefore, multiple apoptotic signaling cascades may be activated by resveratrol, depending on the specific cell type and cellular environment.

Deregulation of the cell cycle has been closely associated with the development of cancer (24), and anticancer agents such as resveratrol may act by modulating cell cycle-associated proteins, such as cyclins, cyclin-dependent kinase (CDK), and CDK inhibitors (20). CDK inhibitors

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have been shown to be the downstream targets of caspase-3 activation and loss of these inhibitors can result in the aberrant up-regulation of CDKs that have been associated with apoptotic cell death (25). It has been shown that ectopic expression of a dominant-negative mutant of Cdk2 can partially suppress the apoptosis of human endothelial cells after growth factor deprivation (26). Olomoucine, a selective inhibitor of the cell cycle regulators, p34/cdc2 and CDK2, prevents camptothecin-induced neuronal apoptosis in PC12 cells, sympathetic and cortical neurons (27), or tetrahydrobiopterin-induced apoptosis in PC12 cells after trophic factor withdrawal (28).

Although resveratrol has been shown to inhibit different stages of tumor growth, the molecular mechanism of its anticancer activity is still not well defined, especially in brain tumors that have proven difficult to treat. We now report that resveratrol can induce apoptotic death in human glioma U251 cells, likely through deregulation of the cell cycle machinery and activation of mitochondria-mediated caspase-3-dependent apoptotic signaling cascades. These findings show that resveratrol is an attractive candidate for use in brain cancer prevention and treatment. Understanding the molecular signaling mechanisms of this naturally derived compound will facilitate the development of therapeutic interventions for the prevention and treatment of brain tumors.

Materials and Methods

Reagents

Resveratrol was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in DMSO as a stock solution of 100 mmol/L. Resveratrol was further diluted in DMEM plus 10% fetal bovine serum to appropriate final concentrations. The general caspase inhibitor Z-VAD-FMK [Z-Val-Ala-Asp(OMe)-CH₂F] and selective inhibitors of caspase-1 [Ac-YVAD-CMK, Ac-Tyr-Val-Ala-Asp-CH₂Cl], caspase-3 [Z-DEVD-FMK, Z-Asp-(OH₃)-Glu(OH₃)-Val-Asp(OH₃)-CH₂F] and caspase-9 [Z-LEHD-FMK, Z-Leu-Glu(OMe)-His-Asp(OMe)-CH₂F] were obtained from Calbiochem (La Jolla, CA). Stock solutions of the caspase inhibitors (10 mmol/L each) were prepared in DMSO and diluted in DMEM plus 10% fetal bovine serum to a final concentration of 100 μmol/L. Monoclonal antibody against Bax (B-9) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the cleaved caspase-3 (Asp¹⁷⁵), caspase-7 (Asp¹⁹⁸), and caspase-9 (Asp³¹⁵) were obtained from Cell Signaling (San Diego, CA). Antibodies against poly(ADP-ribose) polymerase and actin were purchased from Oncogene Research Products (Boston, MA) and Santa Cruz Biotechnology, respectively. DMEM, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA) and fetal bovine serum was purchased from Hyclone (Logan, UT). The 2.5% trypsin/EDTA solution was purchased from Invitrogen and diluted to 0.5% for trypsinizing the attached cells.

Cell Culture

Human glioma U251 (kindly provided by Dr. S.A. Rempel at Henry Ford Health System, Detroit, MI) and

U87 cells (American Tissue Culture Collection, Rockville, MD) were cultured in DMEM, supplemented with 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified incubator containing 5% CO₂ and 95% air.

MTS Assay

MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] was done in 96-well plates using a CellTiter 96 nonradioactive cell proliferation colorimetric assay kit (Promega, Madison, WI). Cells (10,000 cells in 100 μL medium per well) were plated in 96-well plates the day before the experiment. At the end of various treatments, 20 μL of MTS solution was added to each well and further incubated for 1 hour and samples were read by a microplate reader at wavelength of 490 nm.

Lactate Dehydrogenase Release Assay

This assay was done using a CytoTox 96 nonradioactive cytotoxicity assay kit from Promega. Cells (2 × 10⁵ cells per well) were plated in 24-well plates the day before the experiments. After various treatments, medium from each well was collected to measure the amount of released lactate dehydrogenase (LDH), whereas separate wells exposed to lysis buffer (9% Triton X-100) and media collected to measure the total amount of cellular LDH. The amount of cell death was calculated as a percentage of released LDH over total intracellular LDH.

DNA Fragmentation

DNA fragmentation was done as previously reported (28). After various treatments, cells were collected and lysed with lysis buffer [0.5% Triton X-100, 5 mmol/L Tris Buffer (pH 7.4), 20 mmol/L EDTA], and RNA was removed by incubation with RNase A (0.8 mg/mL) at 37°C for 30 minutes. DNA was extracted with phenol/chloroform and precipitated with 1/10 volume of 3 mol/L sodium acetate (pH 5.2) and two volumes of 100% ethanol. DNA pellets were obtained by centrifugation at 10,000 × g for 20 minutes at 4°C, dried and resuspended in 25 μL of 1× TAE (40 mmol/L Tris-acetate and 1 mmol/L EDTA). The samples were separated on 2% agarose gels and the appearance of DNA laddering was detected by incubation of gels with ethidium bromide (1 μg/mL) for 20 minutes followed by destaining with distilled H₂O.

Cell Cycle Analysis

Cells (1 × 10⁶) were split into 6 cm dishes 1 day before the experiments. After resveratrol treatment for 24 or 48 hours, cells were collected and fixed overnight in 70% ethanol at 4°C. Samples were labeled with propidium iodide at 37°C for 30 minutes and analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ). The relative percentage of cells at G₀/G₁, G₂-M, and S phase, as well as the appearance of sub-G₁ phase, was determined with the software from BD Biosciences. All data acquisition and analyses were done with the Becton Dickinson instrument.

Western Blot Analysis

Western blot analysis was done as previously reported (29). After treatments, cells were collected by trypsinization

and centrifugation, washed once in $1 \times$ PBS, and lysed in lysis buffer [20 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 1% Nonidet P-40, 0.1% SDS, 1% deoxycholic acid, 10% glycerol, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaVO_3 , 50 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{mL}$ leupeptin, and 10 $\mu\text{g}/\text{mL}$ aprotinin] for 30 minutes on ice. Soluble protein was obtained by centrifugation at $10,000 \times g$ for 10 minutes at 4°C . The protein concentration of each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). For immunoblotting, equal amounts of cell lysate were subjected to electrophoresis on 10% or 18% SDS-polyacrylamide gels. Separated proteins were then electrotransferred to Immobilon membranes (Millipore, Bedford, MA). After exposure to specific antibodies, proteins were visualized by using an enhanced chemiluminescence protein detection kit from Pierce Chemical Co. (Rockford, IL).

Cell Fractionation

Cells (5×10^6 cells per dish) were plated into 10 cm dishes the day before the experiments. After various treatments, the mitochondrial and cytosolic fractions were prepared using an Apo-Alert cell fractionation kit from BD Biosciences Clontech (Palo Alto, CA). Equal amounts of protein were used for Western blot analysis to detect the Bax and cytochrome *c* expression using the antibodies against Bax (Santa Cruz Biotechnology) and cytochrome *c* (BD Biosciences Clontech), respectively.

Caspase-3 Assay

This assay was done using an Apo-Alert colorimetric caspase assay kit from BD Biosciences Clontech. Cells (1×10^6 cells per well) were plated into 6 cm dishes and subjected to various treatments. Cells were then collected and lysed in lysis buffer provided in the kit. Caspase-3 activity was

measured at a wavelength of 405 nm using the detection of chromophore *p*-nitroaniline (pNA) after its cleavage by caspase-3 from the labeled caspase-3 specific substrate, DEVD-pNA.

Statistical Analysis

Data were presented as mean \pm SD. Student's *t* test was used for analyzing differences between groups, a *P* value < 0.05 was considered significant.

Results

Dose- and Time-Dependent Cell Death Induced by Resveratrol in Human Glioma U251 Cells

To examine whether resveratrol induces cell death in human glioma U251 cells, cells were treated with different doses of resveratrol (0, 10, 50, and 100 $\mu\text{mol}/\text{L}$). The extent of cell death was measured after 48 hours by LDH release assay (Fig. 1A), since the amount of LDH released from dying cells into the culture medium is proportional to the amount of cell death. LDH release was gradually increased to 149%, 261%, and 874% of control levels at concentrations of 10, 50, and 100 $\mu\text{mol}/\text{L}$ of resveratrol, respectively (Fig. 1A). Significant increases of resveratrol-induced LDH release were observed at the concentrations of 50 and 100 $\mu\text{mol}/\text{L}$. In addition, increased DNA fragmentation (laddering) was observed with increasing concentrations of resveratrol (Fig. 1B). To determine the time-dependent increase of cell death, cells were treated with 100 $\mu\text{mol}/\text{L}$ of resveratrol for 0, 6, 24, and 48 hours. LDH release was increased to 118%, 201%, and 324% of the control levels after 6, 24, and 48 hours of treatment, respectively. Significant increase of LDH release was observed at the 24- and 48-hour time points (Fig. 1C). These results suggest that resveratrol can induce dose- and time-dependent death in U251 cells.

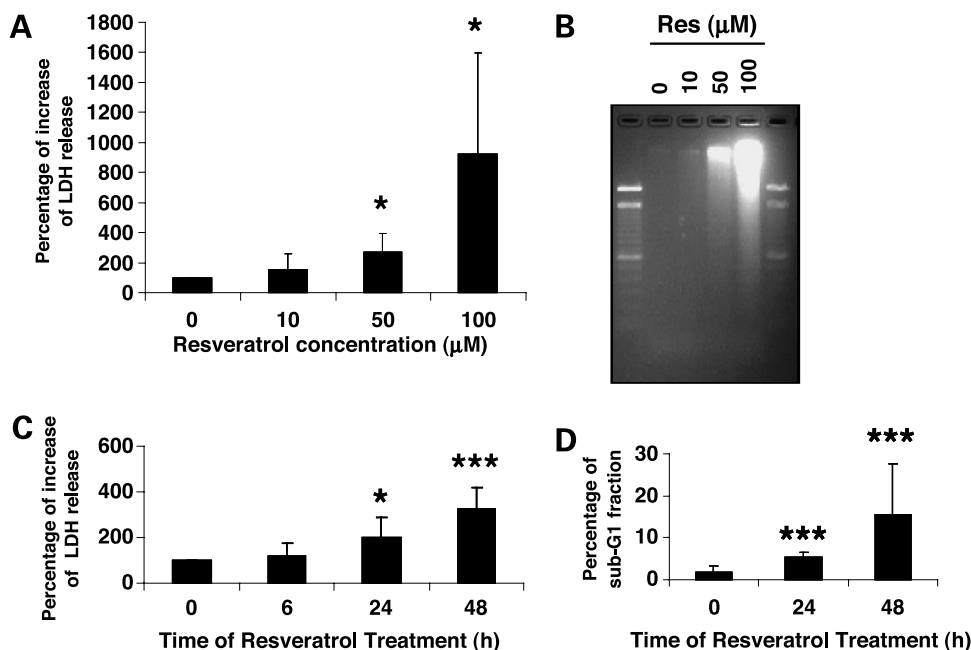


Figure 1. Dose- and time-dependent effects of resveratrol on U251 cell death. **A**, LDH release assay of media from U251 cells treated with resveratrol (0, 10, 50, and 100 $\mu\text{mol}/\text{L}$) for 48 h; columns, mean; bars, \pm SD; *, $P < 0.05$ compared with control ($n = 4$). **B**, DNA fragmentation analysis of U251 cells treated with resveratrol (0, 10, 50, and 100 $\mu\text{mol}/\text{L}$) for 48 h. **C**, LDH release assay of media from U251 cells treated with resveratrol (100 $\mu\text{mol}/\text{L}$) for 0, 6, 24, and 48 h; *, $P < 0.05$ compared with control ($n = 10$); ***, $P < 0.001$ compared with control ($n = 12$). **D**, the appearance of sub-G₁ fraction was determined by flow cytometry analysis of propidium iodide-stained DNA. Significant differences between control and 100 $\mu\text{mol}/\text{L}$ of resveratrol treatment for 24 or 48 h were observed; ***, $P < 0.001$ ($n = 12$); columns, mean; bars, \pm SD.

To examine the appearance of the sub-G₁ fraction, an indicator of apoptotic cell death, propidium iodide staining of DNA and flow cytometry were done in resveratrol-treated U251 cells. Resveratrol significantly increased the appearance of sub-G₁ fraction from 1.7% under control conditions to 5.4% and 15.6% after 24 and 48 hours of treatment, respectively, indicating an increase of resveratrol-induced apoptotic cell death (Fig. 1D).

Resveratrol-Induced Apoptotic Death

To further determine the apoptotic cell death induced by resveratrol, U251 cells were left untreated or treated with 100 $\mu\text{mol/L}$ of resveratrol for 48 hours, and caspase-3 activity assay was done. Resveratrol increased caspase-3 activity by approximately 3-fold (Fig. 2A). Furthermore, Western blot analysis showed the increase of cleaved caspase-3 and its substrate poly(ADP-ribose) polymerase after resveratrol treatment (Fig. 2B). To examine the activation of specific caspases by resveratrol, DNA fragmentation analysis was done in U251 cells treated with 100 $\mu\text{mol/L}$ of resveratrol for 48 hours in the absence or presence of either a general caspase inhibitor (Z-VAD-FMK, 100 $\mu\text{mol/L}$), or a selective caspase-3 inhibitor (Z-DEVD-FMK, 100 $\mu\text{mol/L}$). Both inhibitors completely blocked the resveratrol-

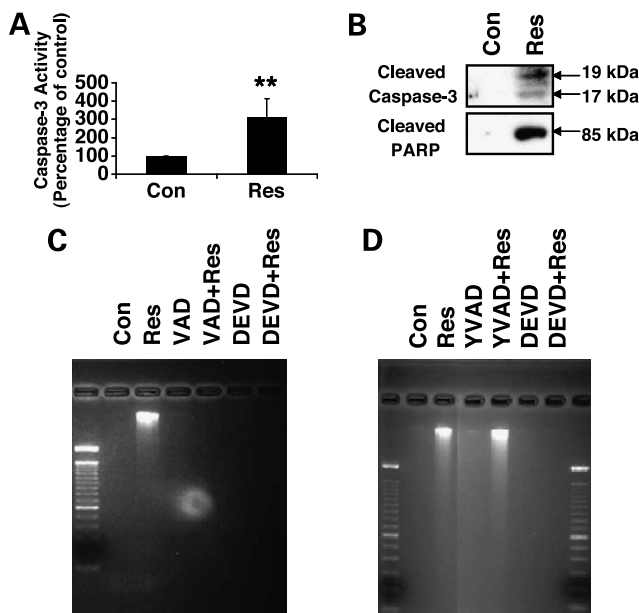


Figure 2. The effects of caspase inhibitors on resveratrol-induced DNA fragmentation. **A**, caspase-3 assay of U251 cells left untreated (*Con*) or treated with 100 $\mu\text{mol/L}$ of resveratrol for 48 h (*Res*); data are expressed as the percentage of caspase-3 activity under control conditions (57 pmol DEVD-pNA cleaved/ μg of protein/h); **, $P < 0.01$ compared with control ($n = 5$). **B**, Western blot analysis of cleavage of caspase-3 and poly(ADP-ribose) polymerase after 48 h of resveratrol treatment. **C**, U251 cells were left untreated or pretreated with the general caspase inhibitor (Z-VAD-FMK) or caspase-3 specific inhibitor (Z-DEVD-FMK) for 1 h and then treated with 100 $\mu\text{mol/L}$ of resveratrol for 48 h; DNA fragmentation was done. **D**, U251 cells were left untreated or pretreated with a selective caspase-1 inhibitor (Ac-YVAD-CMK) or caspase-3 inhibitor (Z-DEVD-FMK) for 1 h and then treated with 100 $\mu\text{mol/L}$ of resveratrol for 48 h. DNA fragmentation was done.

induced DNA fragmentation (Fig. 2C). However, the specific caspase-1 inhibitor, Ac-YVAD-CMK, had no effect on resveratrol-induced DNA fragmentation (Fig. 2D). These results suggest that resveratrol can induce caspase-3-dependent apoptotic death in U251 cells.

Caspase-3 activation is regulated by its upstream caspase-9. To examine the effect of resveratrol on caspase-9 activation, Western blot analysis of cleaved caspase-9 was done. Resveratrol induced an increase in cleaved caspase-9 after 24 hours of resveratrol treatment (Fig. 3A). A selective inhibitor to caspase-9, Z-LEHD-FMK, blocked the resveratrol-induced increase of cleaved caspase-3 and caspase-7, two downstream caspases of caspase-9 (Fig. 3B). These results suggest that caspase-9 is acting upstream of caspase-3.

Resveratrol-Induced Up-Regulation of Bax and Release of Cytochrome *c*

Caspase activation is often associated with the alteration of expression of Bcl-2 family of proteins and required the release of cytochrome *c* from the mitochondria to cytoplasm. The extent of cytochrome *c* release was examined in the cytosolic fractions derived from cells treated with resveratrol for 0, 2, 4, 8, 24, and 48 hours. Figure 3C showed the Western blot analysis of cytosolic cytochrome *c* release at different time points. Increase of cytochrome *c* release was observed after 2 hours of resveratrol treatment and maintained similar levels after 24 hours of treatment. Figure 3D showed that significant increase of cytochrome *c* release was observed at 2, 4, 8, and 24 hours of resveratrol treatment. One of the proapoptotic members of the Bcl-2 family of proteins, Bax, can be translocated into mitochondria in response to various apoptotic stimuli and facilitate the release of cytochrome *c* and subsequent activation of caspases. Induction of Bax expression as well as its translocation from the cytosolic to the mitochondrial fraction following resveratrol (100 $\mu\text{mol/L}$) was detected as early as 4 hours and peaked between 4 and 8 hours (Fig. 3E). These results suggest that resveratrol-induced apoptotic death may be mediated by mitochondria-dependent signaling pathways.

Resveratrol-Induced Inhibition of Cell Proliferation and G₀/G₁ Growth Arrest

Cell cycle deregulation and apoptosis are closely related events, and disruption of cell cycle progression may ultimately lead to apoptotic death. The effects of resveratrol on U251 proliferation was determined using the MTS assay, which measures the appearance of a formazan product that is directly proportional to the number of living cells in the culture. Resveratrol (100 $\mu\text{mol/L}$) reduced the percentage of proliferating cells to 30% of the control levels after 48 hours (Fig. 4A). The expression of cyclin D1 was decreased with increasing time of resveratrol treatment (Fig. 4B), indicating that resveratrol may interfere with cell cycle progression. Since resveratrol inhibits U251 cell proliferation and increases the fraction of sub-G₁, we examined the effects of resveratrol on cell cycle progression using flow cytometry. Resveratrol increased the fraction of G₀/G₁ from 58% in control to ~70% at the 24- and 48-hour time points (Fig. 4C). The G₂-M and S phases were slightly

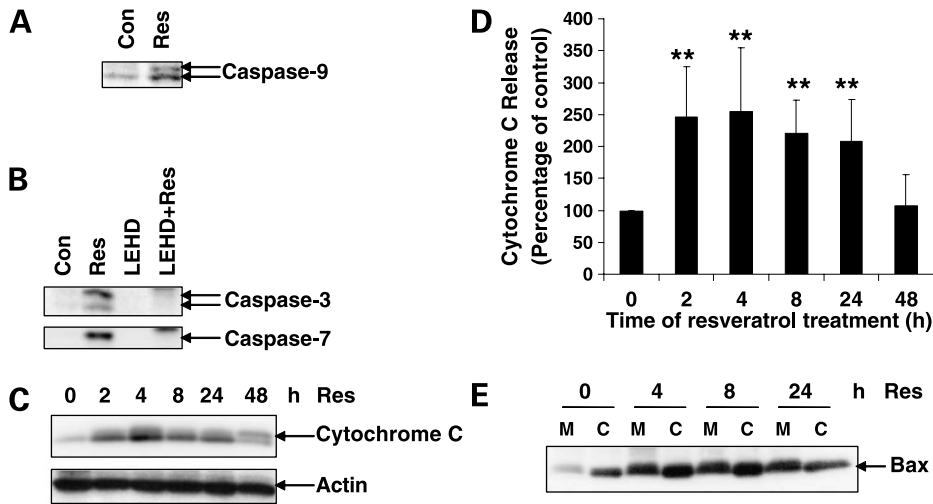


Figure 3. The involvement of Bax and cytochrome *c* release in resveratrol-induced apoptosis. **A**, Western blot analysis of cleaved caspase-9 after 24 h of 100 μmol/L resveratrol treatment. **B**, Western blot analysis of cleaved caspase-3 or caspase-7, in the absence or presence of 100 μmol/L of resveratrol or 100 μmol/L of caspase-9 inhibitor, Z-LEHD-FMK. **C**, cytosolic fractions were obtained from U251 cells treated with 100 μmol/L of resveratrol for 0, 2, 4, 8, 24, and 48 h using a kit from BD Biosciences Clontech. Western blot analysis was done using an antibody against cytochrome *c*. **D**, average cytochrome *c* release after 0, 2, 4, 8, 24, and 48 h of 100 μmol/L resveratrol treatment; **, $P < 0.01$ ($n = 4$). **E**, Western blot analysis of Bax expression using cytosolic and mitochondrial fractions and a monoclonal antibody against Bax.

decreased after resveratrol, indicating that resveratrol may induce the G_0/G_1 growth arrest in U251 cells. These results indicate that resveratrol-induced apoptosis is likely to involve the modulation of cell cycle progression.

Effects of Cyclin-Dependent Kinase Inhibitor Olomoucine on Resveratrol-Induced Cell Death

Blocking cell cycle progression and rapidly up-regulating CDK activity may induce apoptotic cell death. To examine the effects of CDK inhibition on resveratrol-induced death, cells were treated with resveratrol (100 μmol/L) in the absence or presence of olomoucine (100 μmol/L), a p34/cdc2 and CDK2 selective inhibitor. LDH release assay showed that resveratrol increased LDH release to 336% of

control after 48 hours treatment (Fig. 5A). Olomoucine itself showed slight increase of LDH release, which was 148% of the control levels, but it significantly reduced resveratrol-induced LDH release from 336% back to ~136% of control (Fig. 5A). In addition, resveratrol-induced DNA fragmentation was greatly reduced by olomoucine after 48 hours treatment (Fig. 5B). Olomoucine also partially reduced the caspase-3 activity induced by resveratrol treatment (Fig. 5C). These results suggest that resveratrol induces apoptotic U251 death through deregulation of the cell cycle.

Effects of Resveratrol in Human Glioma U87 Cells

To examine if the effects of resveratrol we observed in U251 cells were cell-specific, we also examined another human

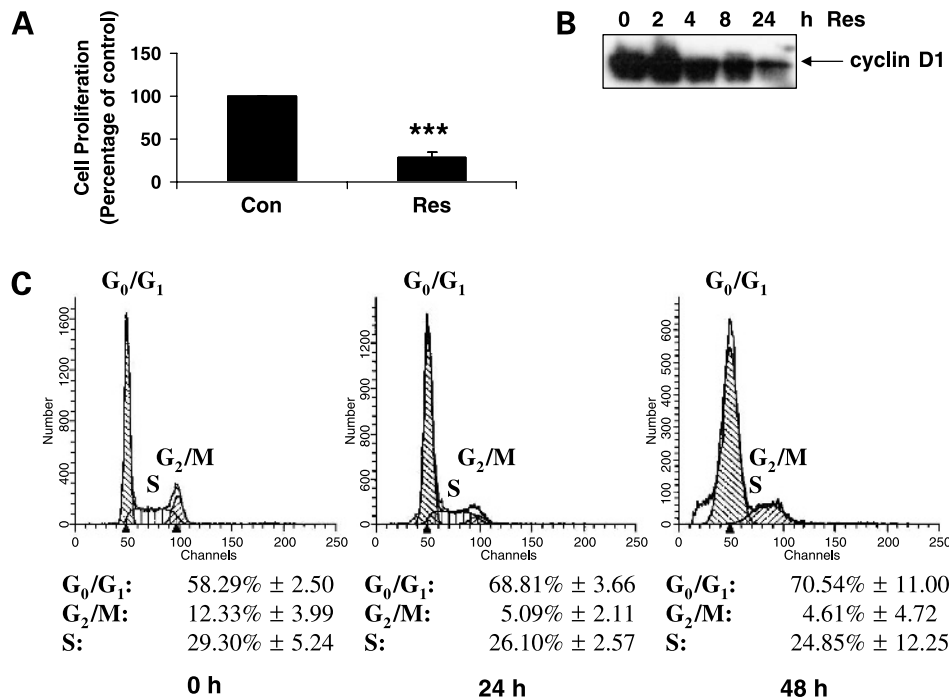


Figure 4. The effects of resveratrol on cell cycle progression in U251 cells. **A**, proliferation of U251 cells after 48-h treatment with 100 μmol/L resveratrol (*Res*) was measured by MTS assay using a nonradioactive cell proliferation assay kit from Promega; ***, $P < 0.001$ compared with control (*Con*); columns, mean; bars, ± SD ($n = 6$). **B**, Western blot analysis of cyclin D1 expression after 0, 2, 4, and 24 h of treatment with 100 μmol/L of resveratrol. **C**, cell cycle analysis of U251 cells treated with 100 μmol/L of resveratrol treatment for 0, 24, and 48 h. Points, mean; bars, ± SD ($n = 13$).

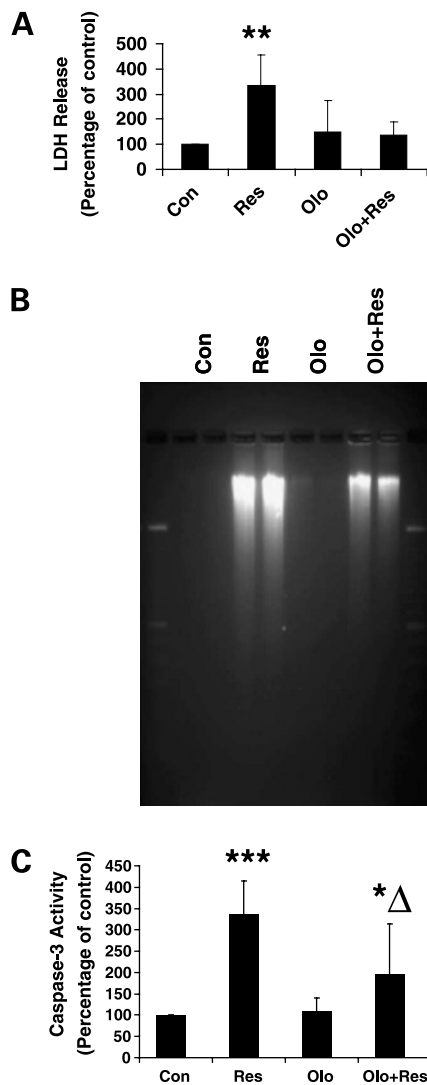


Figure 5. The effects of olomoucine on the resveratrol-induced cell death. Cells were left untreated or pretreated with 100 $\mu\text{mol/L}$ of olomoucine (*Olo*) for 1 h, followed by the treatment with 100 $\mu\text{mol/L}$ of resveratrol for 48 h. **A**, LDH release assay; **, significant difference between control (*Con*) and resveratrol treatment (*Res*), $P < 0.01$ ($n = 23$). **B**, DNA fragmentation. **C**, caspase-3 activity assay; data are expressed as the percentage of caspase-3 activity under control conditions (53 pmol DEVD-pNA cleaved/ μg of protein/h); ***, significant difference between control (*Con*) and resveratrol treatment (*Res*), $P < 0.001$ ($n = 6$); * Δ , significant difference between resveratrol treatment (*Res*) and olomoucine plus resveratrol (*Olo + Res*), $P < 0.05$ ($n = 6$).

glioma U87 cell line. Resveratrol induced similar increase of LDH release (Fig. 6A) as well as caspase-3 activation (Fig. 6B) after 48 hours of treatment, suggesting that resveratrol may induce the apoptosis in glioma cells in general.

Discussion

Resveratrol has been reported to induce the apoptotic cell death in numerous cancer cell lines. However, the effect of resveratrol in glioma cells is still not well understood. Our

study intends to examine the effect of resveratrol in a human glioma U251 cell line. Our results show that resveratrol can induce both dose- and time-dependent apoptosis in human glioma U251 cells. Resveratrol-induced apoptosis requires the activation of caspase-3 and involves the up-regulation of Bax expression, increased release of cytochrome *c* and activation of caspase-9. The temporal relationship between the induction of changes of apoptosis-related gene expression and the activation of caspases indicates that additional intracellular signaling pathways may be involved in resveratrol-induced apoptotic cell death process and it requires further investigation. Resveratrol has been shown to alter the expression of the Bcl-2 family of proteins, decrease mitochondrial membrane potential, increase cytochrome *c* release, and activate caspases in many tumor cell lines (19, 30). Our results are consistent with other published reports on the mechanisms of resveratrol-induced apoptotic cell death in other model systems, suggesting that resveratrol may employ common apoptotic signaling pathways in different cancer cell lines, including glioma cells.

Our results also show that resveratrol inhibits cell proliferation and induction of G_0/G_1 growth arrest through the suppression of cyclin D1 expression, which may contribute to the apoptotic cell death process. Cell cycle inhibitors can suppress the resveratrol-induced apoptosis, suggesting that disruption of regulation of cell cycle may be correlated with the resveratrol-induced apoptosis. Consistent with cell cycle involvement in resveratrol-induced apoptosis is a study showing that resveratrol induces apoptosis of human epidermoid carcinoma A431 cells through elevating the expression of the

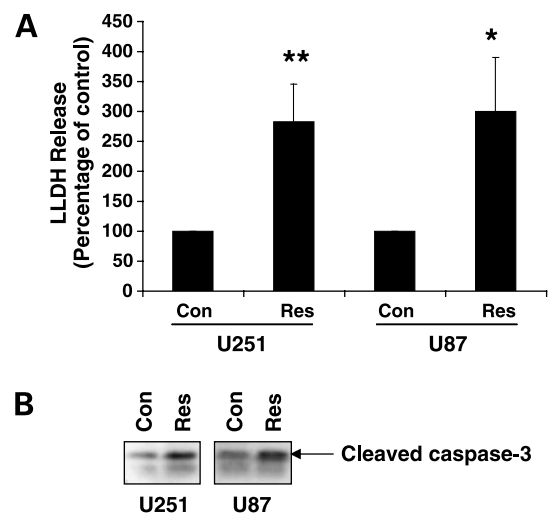


Figure 6. The effects of resveratrol on cell death in U251 and U87 glioma cells. **A**, LDH release assay in U251 and U87 cells after resveratrol treatment at 100 $\mu\text{mol/L}$ for 48 h; * and **, significant difference between control (*Con*) and resveratrol treatment (*Res*), $P < 0.05$ and 0.01, respectively ($n = 12$). **B**, Western blot of the active caspase-3 after resveratrol treatment using antibody against cleaved caspase-3 (Asp¹⁷⁵, Cell Signaling).

CDK inhibitor, WAF1/p21, and suppressing expression of cyclin D1, D2, and E, or CDK2, 4, and 6 (31). Resveratrol has also been shown to inhibit cell proliferation and induce apoptosis in human breast carcinoma MCF-7 cells through the inhibition of cyclin D1 and CDK4 (30). Resveratrol has been reported to induce the cell cycle arrest at the S phase in human promyelocytic leukemia HL-60 cells (32), T cell-derived lymphocytic leukemia cell line CEM-C7H2 (33) and prostate LNCaP cells (34). Resveratrol can partially disrupt G₁-S transition in androgen-nonresponsive DU 145, PC-3, and JCA-1 human prostate cancer cells, but has no effects in androgen-responsive LNCaP cells (35). Resveratrol-induced arrest of the cell cycle at the G₁-phase followed by apoptosis has been reported in human epidermoid carcinoma A431 cells (31). In addition, it has been shown that the CDK-inhibitors, flavopiridol and olomoucine, can suppress camptothecin-induced neuronal apoptosis (27). Therefore, our observations are consistent with the observation that the deregulation of expression and/or activities of different isoforms of cyclins, CDKs, and CDK inhibitors may play an important role in resveratrol-induced cell death. However, the role of cell cycle regulation on the extent of resveratrol-induced apoptosis requires further demonstration of the correlation of cell cycle regulation and apoptosis.

Resveratrol and curcumin, a yellow pigment of turmeric, can regulate inflammatory and immune responses through the suppression of NF- κ B activation (6, 36) and enhances tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in LNCaP prostate cancer cells (6). The constitutive activation of NF- κ B has been observed in malignant glioblastoma and has been shown to be closely associated with the resistance to tumor necrosis factor- α immunotherapy (37). Resveratrol can induce apoptosis through *c-Jun*-NH₂-kinase and p53-dependent signaling pathways, in mouse JB6 epidermal cells (38), LNCaP (39), Hep G₂ (23), or a mutant p53 prostate cancer cell line, DU 145 (16), suggesting that resveratrol may induce apoptosis in both wild-type and mutant p53-containing cell lines, and it is also consistent with our observation that resveratrol induces apoptosis in both human glioma U251 and U87 cells. In addition, extracellular signal-regulated kinase and p38 mitogen-activated protein kinase have been suggested to mediate the resveratrol-induced activation of p53 and apoptosis through the phosphorylation of p53 at serine 15 (40). PI3-K/Akt signaling cascade has been shown to be involved in cell proliferation, survival, angiogenesis, migration, and invasion (41), as shown by several downstream intracellular targets which it regulates, such as Bad, caspase-9, and NF- κ B (42). In addition, PI3-K/Akt signaling through the mammalian target of rapamycin and the eukaryotic initiation factor-4E has been reported to be an important mechanism of oncogenesis and drug resistance (43), which may also represent a potential intracellular signaling target modulated by resveratrol. Therefore, the effect of resveratrol on the modulation of these intracellular signaling proteins in human gliomas needs to be further defined. The effect of

resveratrol in glioma cell lines was described only recently by a report showing that resveratrol could suppress angiogenesis and tumor growth in rat RT-2 glioma cells implanted s.c. or intracerebrally (44). It is true that our study shows that the mechanisms in which resveratrol mediates the apoptotic cell death in U251 glioma cells seem to be similar with the reported studies in other model systems. Nevertheless, this is one of the first studies to describe the signaling mechanisms of resveratrol-induced apoptosis in glioma cells. One of the concerns of using resveratrol is the potential toxicity at high dosages. In the study using rat RT-2 glioma cells, it was shown that 40 and 100 mg/kg/day (in 0.5 mL) are required to show significant antitumor effects and prolong the survival in s.c. and intracerebral glioma models, respectively (44). These dosages are compatible to the *in vitro* dosages used, taking into the consideration of the low bioavailability of resveratrol *in vivo*. In another reported study, resveratrol was given p.o. to rats for 28 days at 20 mg/kg/day, which is 1,000 times the amount consumed by a 70 kg person taking 1.4 g of *trans*-resveratrol per day (45). No differences in the body weight, water consumption, hematologic, or biochemical measurements were found between resveratrol-treated and control groups, suggesting that high dosages of resveratrol were not toxic *in vivo*.

Resveratrol, a compound found in many natural foods, is a promising, novel therapeutic agent for cancer prevention that warrants further investigation. Our results will likely aid in the development of therapeutic strategies for the prevention and treatment of brain tumors and indicate that resveratrol may be a useful adjunct to conventional anticancer therapies. Further studies are required to delineate the precise signaling pathways involved in resveratrol-induced apoptotic death of U251 glioma cells and the extent to which this compound is effective in suppressing tumor growth *in vivo*.

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