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# Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity

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**Resveratrol has been shown to induce anti-proliferation and apoptosis of human cancer cell lines. In the present study, we determined the effect of high intracellular levels of the anti-apoptosis protein Bcl-2 on caspase-3 activation, PLC-γ1 degradation and cytochrome *c* release during resveratrol-induced apoptosis. For this, we used U937/vector and U937/Bcl-2 cells, which were generated by transfection of the cDNA of the Bcl-2 gene. As compared with U937/vector, U937/Bcl-2 cells exhibited a 4-fold greater expression of Bcl-2. Treatment with 60 or 100 μM resveratrol for 24 h produced morphological features of apoptosis and DNA fragmentation in U937/vector cells, respectively. This was associated with caspase-3 activation and PLC-γ1 degradation. In contrast, resveratrol-induced caspase-3 activation and PLC-γ1 degradation and apoptosis were significantly inhibited in U937/Bcl-2 cells. Bcl-2 overexpressing cells exhibited less cytochrome *c* release and sustained expression levels of the IAP proteins during resveratrol-induced apoptosis. In addition, these findings indicate that Bcl-2 inhibits resveratrol-induced apoptosis by a mechanism that interferes with cytochrome *c* release and activity of caspase-3 that is involved in the execution of apoptosis.**

## Introduction

Apoptosis is essential for development, maintenance of tissue homeostasis and elimination of unwanted or damaged cells from multicellular organisms (1,2). The aberrant regulation of apoptosis has been observed in many forms of human disease such as neuronal disease, autoimmune disease, AIDS and cancer (2). Many therapeutic agents eliminate tumor cells by inducing apoptotic cell death (2). Therefore, understanding the mechanism of apoptosis has important implications in the prevention and treatment of many diseases.

Several genes have been identified as either inducers or repressors of apoptosis. Among these is Bcl-2, which was initially identified at the breakpoint of the t(14;18) chromosomal translocation that occurs in the majority of non-Hodgkin's B-cell lymphomas (3,4). Bcl-2 can protect against apoptosis induced by such diverse stimuli as viral infection, hypoxia, ionizing radiation or chemotherapeutic agents (5–9). The precise biochemical mechanisms by which Bcl-2 proteins influence cell survival and death, however, remain unclear. *In vivo* and *in vitro* studies have shown that Bcl-2 regulates intracellular $Ca^{2+}$ levels and prevents the loss of mitochondrial membrane potential induced by pro-apoptotic stimuli (10). Recent evidence suggests that Bcl-2 may act as an ion channel and regulate the release of cytochrome *c* from mitochondrial (11,12). The release of cytochrome *c* triggers the formation of a complex containing Apaf1 and procaspase-9 in the presence of dATP, resulting in caspase-9 activation (13,14). Once activated, initiator caspases in turn activate the effector caspases, caspases-3 and -7 (13,14). The active effector caspases promote apoptosis by cleaving cellular substrates leading to the morphological and biochemical features of apoptosis.

Resveratrol (3,5,4′-trihydroxystibene) is a phytoalexin present in grapes and a variety of medicinal plants (15–17). Recently, Jang *et al.* (18) demonstrated that resveratrol is a potent chemopreventive agent in assays representing the three major stages (e.g. tumor initiation, promotion and progression) of carcinogenesis. This effect has been attributed to the antioxidant activity, anti-inflammatory activity and inhibition of platelet aggregation by resveratrol (19,20). Resveratrol was also shown to inhibit the *in vitro* growth of a number of human cancer cell lines (21). However, conflicting results regarding its anti-cancer effect have been reported. Clement *et al*. (22) and Surh *et al.* (23) showed that resveratrol induced apoptosis in HL60 cells, whereas Ragione *et al*. (24) reported that resveratrol induced differentiation, but not apoptosis (22–24). The purpose of the present study was to further characterize the biologic consequences of resveratrol treatment in monocytic leukemia U937 cells and Bcl-2 overexpressing U937 cells.

In the present study, we focus on two major findings. One is the potential mechanism of resveratrol-induced apoptosis and the other is the mechanism of the inhibition effect of Bcl-2 on resveratrol-induced apoptosis. We have confirmed that caspase-3 activation, PLC-γ1 degradation and cytochrome *c* release are associated with resveratrol-mediated apoptosis. In particular, the relationship between cytochrome *c* release, caspase-3 activity and expression levels of XIAP family proteins was analyzed. Our results indicate that resveratrol triggers cytochrome *c* release as an early event preceding caspase activation. In addition, overexpression of Bcl-2 attenuates resveratrol-induced apoptosis, which prevent the accumulation of cytochrome *c* in the cytosol, thereby preserving caspase-3 in the inactive zymogen state and blocking the molecular cascade of apoptosis.

## Materials and methods

### *Cells*

Human leukemia U937 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The culture medium used throughout

these experiments was Dulbecco's modified Eagle's medium, containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml gentamicin. Bcl-2-overexpressing U937 cells were generated using a pMAX vector containing the human *Bcl-2* gene (provided by Dr Rakesh Srivastava, NIH/NIA). U937 cells (400 μl) in RPMI 1640 ($20\times10^6$ cells/ml) were transfected by pre-incubating with 15 μg Bcl-2 plasmid for 10 min at room temperature and then electroporating at 500 V, 700 μF. The sample was immediately placed on ice for 10 min and then 10 ml complete medium was added and the cells incubated at 37°C for 24 h. The cells were selected in a medium containing 0.7 μg/ml geneticin (G418) for 4 weeks. Single cell clones were obtained by limiting dilution and subsequently analyzed for an increase in Bcl-2 protein expression relative to identically cloned empty vector control.

*Drugs and materials*

Resveratrol was directly added to cell cultures at the indicated concentrations. Anti-cdk2, anti-cdc2, anti-cdk4, anti-cyclin D3, anti-cyclin E, anti-cyclin A, anti-cyclin B1, anti-p21, anti-p27, anti-cIAP1, anti-cIAP2 and anti-HSP70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the following proteins were purchased from the indicated suppliers: PARP from Boehringer Mannheim (Indianapolis, IN), cytochrome *c* from PharMingen (San Diego, CA), actin from Sigma (St Louis, MO) and XIAP from R&D systems (Minneapolis, MN). Resveratrol was purchased from Biomol (Plymouth Meeting, PA).

*Western blotting*

Cellular lysates were prepared by suspending $1\times10^6$ cells in 100 ml lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). Cells were disrupted by sonication and extracted at 4°C for 30 min. Proteins were electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA). Detection of specific proteins was carried out with an ECL western blotting kit according to the manufacturer's instructions.

*Cell count and flow cytometry analysis*

Cell counts were performed using a hemocytometer. Approximately $1\times10^6$ U937 cells were suspended in 100 μl PBS and 200 μl 95% ethanol were added while vortexing. Cells were incubated at 4°C for 1 h, washed with PBS and resuspended in 250 μl 1.12% (w/v) sodium citrate buffer (pH 8.4) together with 12.5 μg RNase. Incubation was continued at 37°C for 30 min. Cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

*DNA fragmentation assay*

After treatment with resveratrol, U937 cells were lysed in a buffer containing 10 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10 000 *g* for 20 min. Fragmented DNA in the supernatant was extracted with a 25:24:1 (v/v/v) equal volume of neutral phenol:chloroform:isoamyl alcohol and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml ethidium bromide.

*Nuclear staining assay*

After treatment with resveratrol, U937 cells were washed once with ice-cold PBS and fixed with 3:1 (v/v) methanol:acetic acid solution for 30 min. Fixed cells were placed on slides and stained 0.1 μg/ml propidium iodide for 15 min. Nuclear morphology of cells was examined by fluorescence microscopy.

*Caspase 3 activity assay*

To evaluate caspase-3 activity, cell lysates were prepared after their respective treatment with resveratrol. Assays were performed in 96-well microtitre plates by incubating 20 μg cell lysates in 100 μl reaction buffer (1% NP-40, 20 mM Tris–HCl pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspase 3 substrate (DEVD-pNA) at 5 μM. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

*Analysis of cytochrome c release*

Cells ($2\times10^6$) were harvested, washed once with ice-cold phosphate buffered saline and gently lysed for 2 min in 80 μl ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 20 mM Tris–HCl pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin). Lysates were centrifuged at 12 000 *g* at 4°C for 10 min to obtain the supernatants (cytosolic extracts free of mitochondria) and the pellets (fraction that contains mitochondria). The resulting cytosolic fractions were used for western blot analysis with an anti-cytochrome *c* antibody.

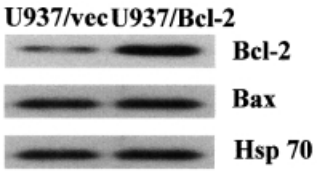


**Fig. 1.** Overexpression of Bcl-2 in U937 cells. Immunoblot analysis of cell lysates (40 μg) from control (U937/vector) or Bcl-2 transfected (U937/Bcl-2) cells. The bolt was probed with anti-Bcl-2 and anti-Bax. To confirm equal loading, the blot was stripped of the bound antibody and reprobed with anti-Hsp70 antibody.

## Results

*Expression levels of Bcl-2 overexpressed cells*

In order to evaluate the functional role played by Bcl-2 in preventing apoptosis using the chemopreventive agent resveratrol, we first established Bcl-2 overexpressing cells. U937 cells were transfected with an expression vector containing Bcl-2 cDNA. After 4 weeks, geneticin (G418) resistant cells were isolated and the relative expression level of Bcl-2 was determined by western blot. Western analysis revealed that U937/Bcl-2 cells exhibited a ~4-fold increase in Bcl-2 expression compared with cells containing empty-vector (Figure 1). In contrast, levels of Bax and Hsp70 were equivalent in U937/Bcl-2 and U937/vector cells.

*Bcl-2 significantly reduces, but does not prevent, apoptosis induced by resveratrol*

In order to assess the nature of apoptosis induced by resveratrol, cells treated for 20 h with 60 μM resveratrol were examined after propidium iodine staining. Nucleic acid staining with propidium iodine revealed typical apoptotic nuclei in resveratrol-treated cells, but control cells did not show any features of apoptosis (Figure 2A). In order to quantify the degree of apoptosis, we analyzed the amount of sub-$G_1$ DNA by flow cytometry of fixed nuclei. U937/Bcl-2 and U937/vector cells were exposed to various concentrations of resveratrol for 24 h. As shown in Figure 2B, resveratrol treatment in U937/vector cells resulted in a markedly increased accumulation of sub-$G_1$ phase, which occurred in a dose-dependent manner. In contrast, overexpression of Bcl-2 reduced resveratrol-induced accumulation of sub-$G_1$ phase (Figure 2C).

Another hallmark of apoptosis is the degradation of chromosomal DNA at internucleosomal linkages. We analyzed DNA fragmentation induced by resveratrol in U937/Bcl-2 and U937/vector cells. Following agarose gel electrophoresis of U937/vector cells treated with 100 μM resveratrol for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed. In contrast, DNA fragmentation in U937/Bcl-2 cells was significantly inhibited (Figure 2E).

Low dose resveratrol treatment induces S phase arrest in human cancer cell lines (Figure 2D) (24). To examine whether Bcl-2 interferes with cell cycle distribution, we compared cell cycle distribution and expression levels of the cell cycle regulatory proteins after exposing the U937/Bcl-2 and U937/vector cells to resveratrol. As expected, U937/Bcl-2 and U937/vector cells have very similar cell cycle distribution (Figure 2B). As shown in Figure 3, the expression levels of cell cycle regulatory proteins revealed no difference between U937/Bcl-2 cells and U937/vector cells after resveratrol treatment. However, cdc2, cyclin B1 and cyclin E slightly increased after resveratrol treatment in both U937/vector and U937/Bcl-2 cells compared with control cells.

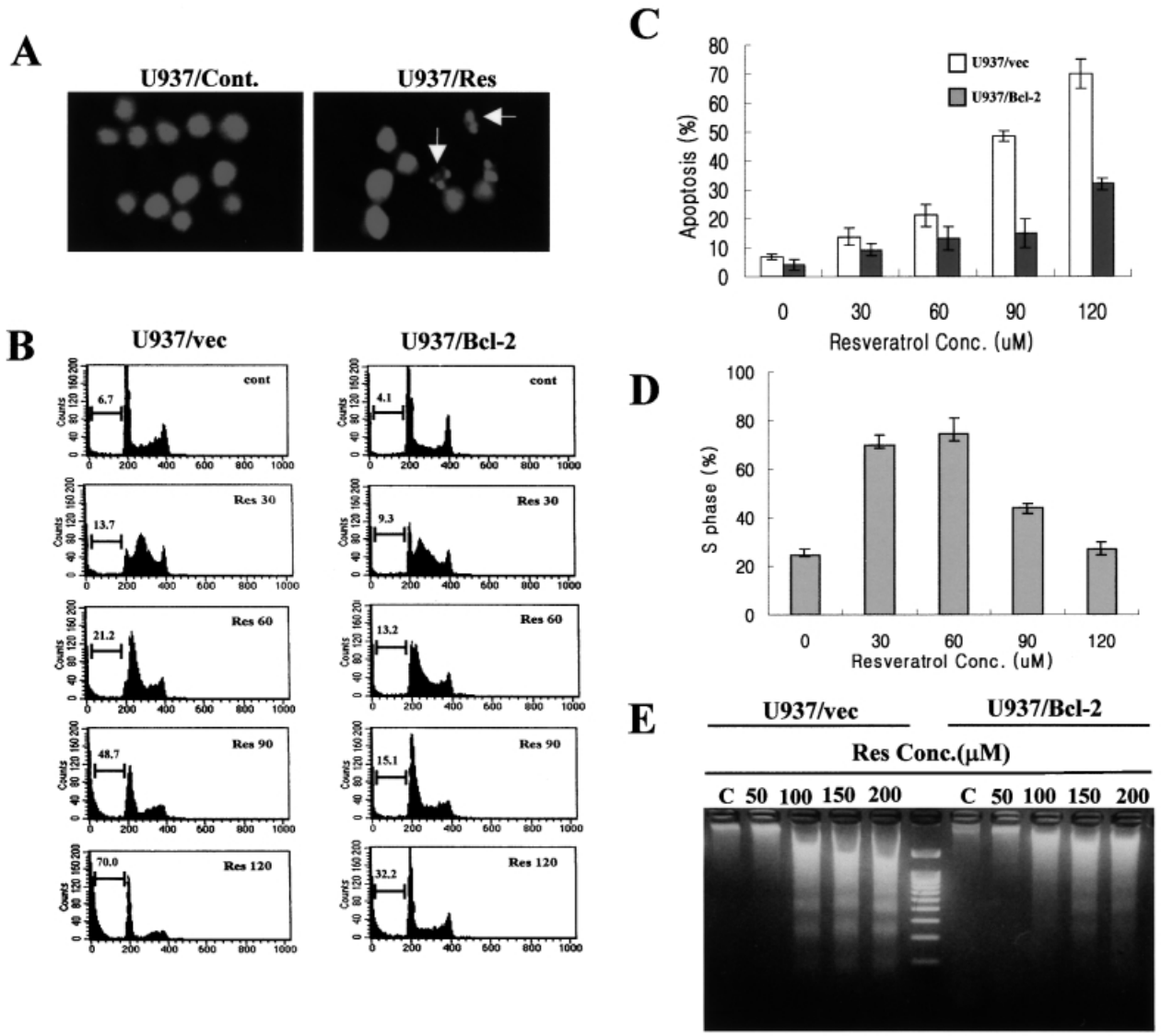


**Fig. 2.** Resveratrol-induced apoptosis in U937/vector and U937/Bcl-2 cells. (**A**) Morphological aspects of propidium iodide stained cells. U937 cells were treated with 100 μM resveratrol for 24 h and then stained with propidium iodide. Arrows indicate condensed or fragmented nuclei. (**B**) FACS analysis of apoptotic cells. Cells were treated for 24 h with the indicated concentrations of resveratrol and then evaluated for DNA content after propidium iodide staining. (**C**) The fraction of apoptoic cells as indicated. Data are mean values obtained from three independent experiments and bars represent standard deviations. (**D**) The fraction of cells in the S phase is indicated. Data are mean values obtained from three independent experiments and bars represent standard deviations. (**E**) Fragmentations of genomic DNA in cells after treatment for 24 h with indicated concentrations of resveratrol. Fragmented DNA was extracted and analyzed on 2% agarose gel.

Taken together, these results indicate that apoptosis induced by resveratrol in U937 cells is reduced by the overexpression of Bcl-2 protein. Cell cycle distribution of both cell lines might be independent of Bcl-2 expression levels.

*Resveratrol-induced caspase-3 activation and PLC-γ1 and PARP degradation*

Previous studies demonstrated that the exposure to high doses of resveratrol for 24 h induces apoptosis of U937 cells. To determine whether resveratrol-induced apoptosis was associated with the activation of caspase-3, we determined the level and activity of caspase-3 in U937/Bcl-2 and U937/vector cells that had been exposed to various concentrations of resveratrol. Caspase-3 is activated by proteolytic processing of the 32 kDa form into two smaller subunits. Activity of caspase-3 during resveratrol-induced apoptosis was measured by a decrease in pro-enzyme level using western blot analysis and a proteolytic activity with a chromogenic substrate. As shown in Figure 4A, treatment with resveratrol resulted in a decrease in the level of caspase-3 in U937/vector cells exposed to 100–200 μM resveratrol for 24 h. In contrast, the levels of caspase-3 were not significantly altered in resveratrol-treated U937/Bcl-2 cells.

Caspase-3 represents one of the key proteases known to be responsible for cleavage of poly (ADP-ribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase C δ (PKC-δ) and other substrates (25–27). Among the downstream targets of activated caspase 3 *in vivo*, PLC-γ1 has recently been shown to be cleaved into a 60 kDa fragment (28). Subsequent western blotting demonstrated proteolytic cleavage of PLC-γ1 in U937/vector cells after 24 h of 50 μM resveratrol treatment. In contrast, in U937/Bcl-2 cells there was little detectable 60 kDa cleavage product following treatment with

50 μM resveratrol. The cleavage of PLC-γ1 was dose-dependent in both cells lines. In addition, treatment with 120 μM resveratrol for 24 h resulted in the cleavage of PARP with the appearance of the expected 85 kDa apoptotic fragment in U937/vector cells. In contrast, the overexpression of Bcl-2 significantly inhibited resveratrol-induced activation of caspase-3 and cleavage of PARP (Figure 4A).

To further investigate and quantitate the proteolytic activity of caspase-3, we performed an *in vitro* assay based on the proteolytic cleavage of DEVD-pN by caspase-3 into the chromophore *p*-nitroanilide (pNA). U937/vector cells showed a 6-fold increase in DEVD-pNA cleavage after 24 h exposure to 100 μM resveratrol, but U937/Bcl-2 cells showed a 2-fold increase.

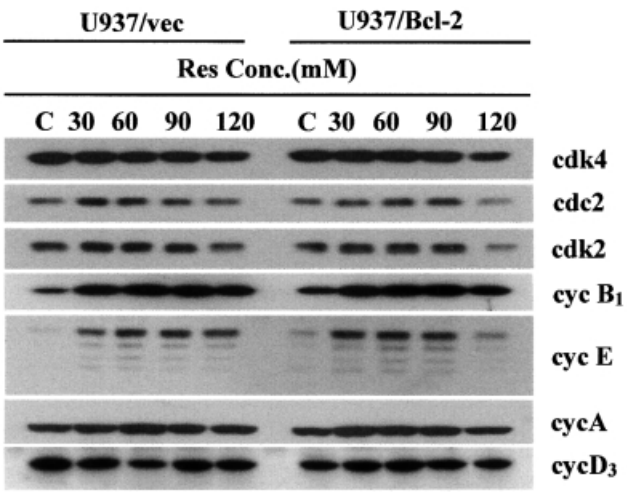


**Fig. 3.** The expression levels of cell cycle regulatory proteins by resveratrol. U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of resveratrol. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with specific antibodies. Proteins were detected by ECL. A representative study is shown; two additional experiments yielded similar results.

*Caspases mediate resveratrol-induced apoptosis*

To address the significance of caspase activation in resveratrol-induced apoptosis, we used a general and potent inhibitor of caspases, z-VAD-fmk (benzyloxycarbony-Val-Ala-Asp-fluoromethyl ketone). Blockade of caspase activities by pre-treatment of U937/vector cells with 50 μM z-VAD-fmk prevented resveratrol (100 μM)-induced genomic DNA digestion (Figure 5A). Resveratrol strongly stimulated caspase-3 protease activities, but z-VAD-fmk pre-treated cells abolished resveratrol-induced caspase-3 activities (Figure 5B). Furthermore, resveratrol treatment of U937/vector cells generated a

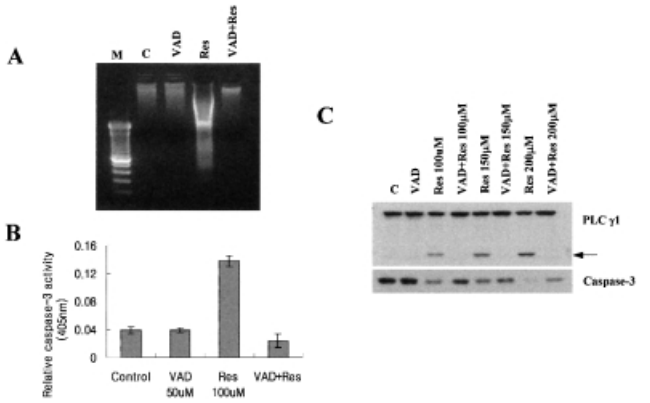


**Fig. 5.** Caspase-mediated apoptosis induced by resveratrol. (**A**) Inhibition of resveratrol-induced genomic DNA fragmentation by z-VAD-fmk. U937/vector cells were treated with z-VAD-fmk (50 μM) for 1 h before challenge with 100 μM resveratrol for 20 h. Cellular DNA was extracted and analyzed by 2% agarose gel electrophoresis. (**B**) Effects of z-VAD-fmk on resveratrol-induced caspase 3 activation. U937/vector cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with resveratrol. Caspase activity was determined as described in Figure 4. Data are mean values from three independent experiments and bars represent standard deviations. (**C**) Effects of z-VAD-fmk on cleavage of PLC-γ1. U937/vector cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with resveratrol. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot for caspase 3 and PLC-γ1. The proteolytic cleavage of PLC-γ1 is indicated by an arrow.

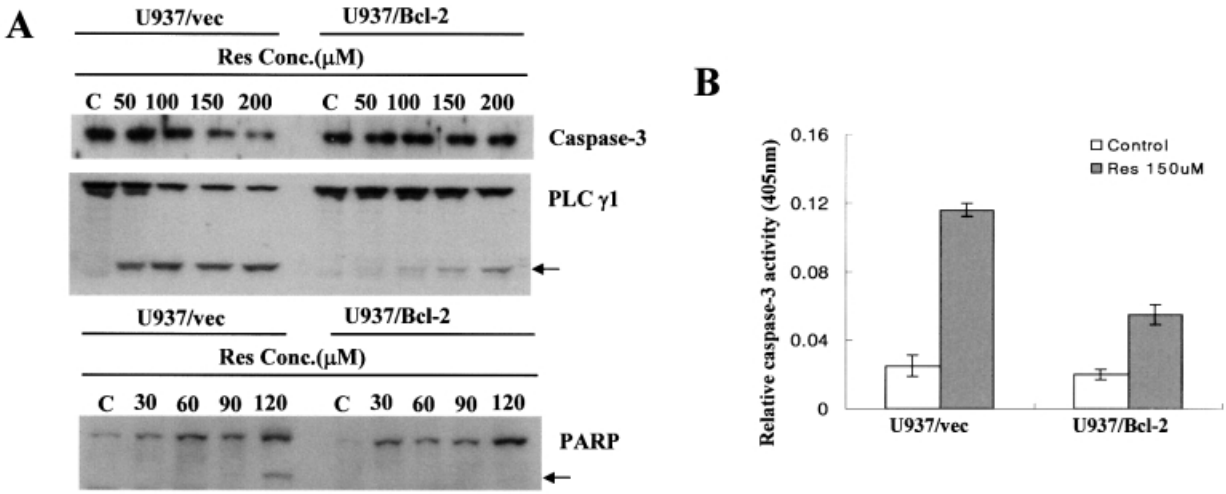


**Fig. 4.** Effect of resveratrol on caspase-3 activity and caspase-specific cleavage of PLC-γ1 and PARP. (**A**) Cells were treated with the indicated concentrations of resveratrol. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by western blot for caspase-3, PLC-γ1 and PARP. The proteolytic cleavage of PLC-γ1 and PARP is indicated by an arrow. (**B**) U937/vector and U937/Bcl-2 cells were treated with resveratrol (150 μM) for 24 h and harvested in lysis buffer. Enzymatic activities of caspase-3 were determined by incubation of 20 μg total protein with 200 μM chromogenic substrate (DEVD-pN) in a 100 μl assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (pNA) was monitored spectrophoto metrically (405 nm). Data are mean values from three independent experiments and bars represent standard deviations.

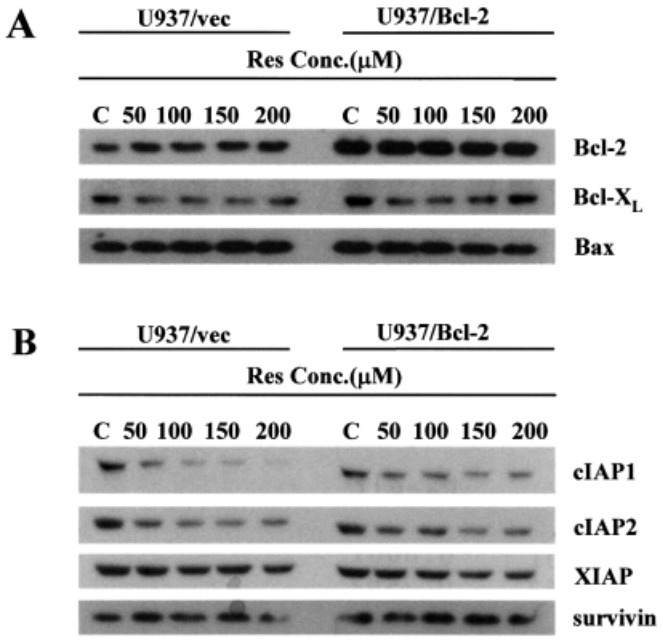


**Fig. 6.** The expression levels of apoptosis related proteins in U937/vector and U937/Bcl-2 cells after treatment with resveratrol. (**A**) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of resveratrol. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with specific antibodies (anti-Bcl-2, anti-Bcl-$X_L$ and anti-Bax). A representative study is shown; two additional experiments yielded similar results. (**B**) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of resveratrol. Equal amounts of cell lysates (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose and probed with specific antibodies (anti-cIAP1, anti-cIAP2, anti-XIAP and anti-survivin). A representative study is shown; two additional experiments yielded similar results.

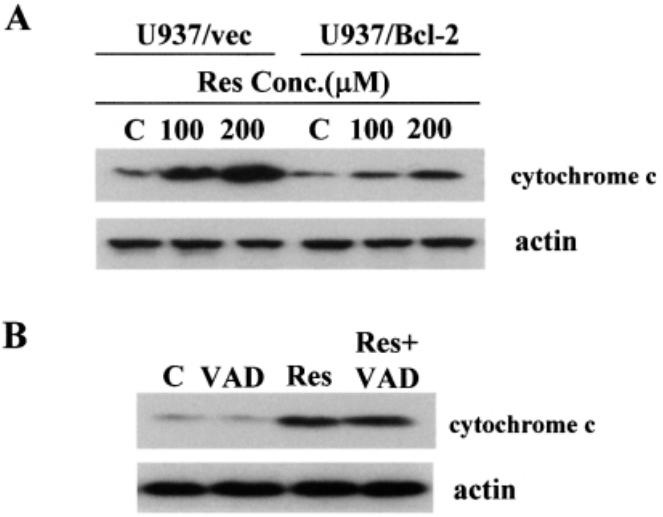


**Fig. 7.** Release of cytochrome *c* in resveratrol-treated U937 cells. (**A**) U937/vector and U937/Bcl-2 cells were treated with indicated concentrations of resveratrol. Cytosolic extracts were prepared as described under Material and methods. Cytosolic protein (30 μg) was resolved on 12% SDS–PAGE and then transferred to nitrocellulose, and probed with specific anti-cytochrome *c* antibody, or with anti-actin antibody to serve as control for the level of protein loaded. (**B**) Effects of z-VAD-fmk on resveratrol-induced cytochrome *c* release. U937 cells were incubated with z-VAD-fmk or solvent for 1 h before treatment with resveratrol (100 μM) for 20 h. Cytosolic extracts were prepared and analyzed as described. Experiments were repeated three times.

60 kDa cleavage product of PLC-γ1. However, z-VAD-fmk pre-treated cells significantly reduced 60 kDa cleavage product of PLC-γ1 (Figure 5C). These data clearly indicate that resveratrol-induced apoptosis is associated with caspase activation.

*Modulation of Bcl-2 and IAP protein families in resveratrol-induced apoptosis in U937/Bcl-2 cells and U937/vector cells*

We also examined whether resveratrol induces cell death by modulating the expression of Bcl-2 family members, which ultimately determine the cell's response to apoptotic stimuli. Treatment of U937/Bcl-2 cells and U937/vector cells with concentrations of resveratrol that are sufficient to induce apoptosis did not significantly alter the expression of the Bcl-2, Bcl-$X_L$ or Bax proteins after 24 h (Figure 6A). Bcl-$X_L$ levels showed an unexplained decrease after 50–150 μM resveratrol treatment in both U937/vector and U937/Bcl-2 cells, followed by a recovery to levels of the untreated cells after 200 μM resveratrol treatment. These results indicate that the expression levels of Bcl-2 family proteins had no effect on apoptosis induced by resveratrol in U937 cells.

To determine whether activity of caspase-3 was associated with the levels of caspase inhibitors in resveratrol-induced apoptosis, we determined the expression levels of IAP family proteins in U937/Bcl-2 cells and U937/vector cells that had been exposed to various concentrations of resveratrol. As shown in Figure 6B, treatment with resveratrol resulted in a decrease in levels of cIAP1 and cIAP2, but not XIAP and survivin in U937/vector cells exposed to 50–200 μM resveratrol for 24 h. In contrast, the decrease of cIAP1 and cIAP2 in U937/Bcl-2 cells was significantly inhibited (Figure 6B). Expression levels of XIAP and survivin revealed no difference between U937/Bcl-2 cells and U937/vector cells after resveratrol treatment. These results indicated that the elevated caspase-3 activity in resveratrol-treated U937 cells is correlated with down-regulation of cIAP1 and cIAP2, but not XIAP and survivin.

*Resveratrol induces cytochrome c release in U937 cells*

There is accumulating evidence that mitochondria play an essential role in many forms of apoptosis by releasing apoptogenic factors, such as cytochrome *c* and apoptosis-inducing factor (AIF). Cytochrome *c* directly activates caspases by binding to Apaf-1 in the presence of ATP (14). To examine the release of cytochrome *c* in resveratrol-treated U937/vector and U937/Bcl-2 cells, we conducted western blotting analysis with cytosolic fractions. Importantly, the overexpression of anti-apoptotic Bcl-2 in U937 cells significantly blocked resveratrol-induced release of cytochrome *c* from the mitochondria into the cytoplasm (Figure 7). To determine whether the observed cytochrome *c* release is indeed the trigger of cell death or a consequence of caspase activation, we used the irreversible broad-range caspase inhibitor z-VAD-fmk. Pre-incubation of U937 cells with z-VAD-fmk was sufficient to prevent DNA fragmentation. The release of cytochrome *c* from mitochondria, however, was not inhibited by z-VAD-fmk. These results are consistent with previous results showing that translocation of cytochrome *c* from mitochondria is independent of caspase activation (29).

## Discussion

Resveratrol has been reported to exert a variety of biological effects including antioxidant, anti-proliferative and cancer chemopreventive activity (18–20). Although resveratrol seems

to have a wide range of potential targets, the underlying mechanisms of apoptosis induction are not well understood. Recently, contradictory results were reported on the induction of apoptosis by resveratrol (22–24). Anti-apoptotic effects of resveratrol include inhibition of arachidonate-metabolizing enzymes in human erythrolukemia K562 cells by hydrogen peroxide (30). However, Clement *et al*. (22) reported that resveratrol treatment enhances CD95L expression on HL60 and T47D breast cancer cells, but not normal human peripheral blood lymphocytes. In this study, we evaluated the possibility that resveratrol induces apoptotic cell death in U937 cells. Resveratrol-induced apoptosis is mediated by caspase-3 activation and down-regulation of IAP family proteins, but not Bcl-2 family proteins. Furthermore, Bcl-2 overexpression attenuates resveratrol-induced apoptosis in U937 cells by inhibition of caspase-3 activity and sustained expression of the IAP caspase inhibitors.

Since the discovery of Bcl-2 as an anti-apoptotic protein, several theories concerning the Bcl-2 anti-apoptotic mechanism have been proposed (10–12). The Bcl-2 anti-apoptotic function may be explained by its ability to control several key steps of apoptosis signaling. Bcl-2 can form ion channels in biological membranes (31,32). This ion channel activity of Bcl-2 may control apoptosis by influencing the permeability of intracellular membranes and cytochrome *c* release from mitochondria (31,32). The release of cytochrome *c* induces the activation of caspase-3 and fragmentation of DNA. Caspase-3 activation and DNA fragmentation, but not cytochrome *c* release, were blocked by caspase inhibitor. Our data showed that the release of cytochrome *c* from mitochondria in U937 cells preceded caspase-3 activation by resveratrol. However, overexpression of Bcl-2 protein may rescue cells from apoptosis by regulating the permeability of the cellular membranes, blocking the release cytochrome c from mitochondria and maintaining membrane integrity, or by a yet unrecognized function of the Bcl-2 protein (11,12,33). The other reason for caspase-3 inactivation in resveratrol-treated Bcl-2 overexpression cells may be increased IAP expression. Human IAP proteins, including XIAP, c-IAP1, c-IAP2, NAIP and survivin, are characterized by the presence of one to three copies of a 70 amino acid motif, the BIR domain, which bears homology to sequences found in the baculovirus IAP proteins (34). IAPs have been reported to inhibit apoptosis due to their function as direct inhibitors of activated effector caspases, caspase-3 and caspase-7. Furthermore, cIAP1 and cIAP2 are also able to inhibit cytochrome *c*-induced activation of caspase-9 (34–36).

In summary, our studies demonstrate that resveratrol treatment of U937 cells induces cytochrome *c* release which activates pro-caspase-3 and DNA fragmentation. Moreover, overexpression of Bcl-2 attenuates resveratrol-induced apoptosis and prevents the release of cytochrome *c* from the mitochondria. Sustained expression levels of the IAP proteins, therefore, maintain caspase-3 in the inactive zymogen state and attenuate the molecular cascade of apoptosis. In view of accumulating evidence that resveratrol and Bcl-2 may be an important determinant of clinical response in leukemia, further efforts to explore this therapeutic strategy appear warranted.

## Acknowledgements

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