

Caspase-2 Triggers Bax-Bak-dependent and -independent Cell Death in Colon Cancer Cells Treated with Resveratrol*

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Polyphenol phytoalexin (resveratrol), found in grapes and red wine is a strong chemopreventive agent with promising safety records with human consumption and unique forms of cell death induction in a variety of tumor cells. However, the mechanism of resveratrol-induced apoptosis upstream of mitochondria is still not defined. The results from this study suggest that caspase-2 activation occurs upstream of mitochondria in resveratrol-treated cells. The upstream activation of caspase-2 is not dependent on its anti-oxidant property or NF- κ B inhibition. The activated caspase-2 triggers mitochondrial apoptotic events by inducing conformational changes in Bax/Bak with subsequent release of cytochrome *c*, apoptosis-inducing factor, and endonuclease G. Caspase-8 activation seems to be independent of these events and does not appear to be mediated by classical death receptor processing or downstream caspases. Both caspase-2 and caspase-8 contribute toward the mitochondrial translocation of Bid, since neither caspase-8 inhibition nor caspase-2 inhibition could prevent translocation of Bid DsRed into mitochondria. Caspase-2 inhibitors or antisense silencing of caspase-2 prevented cell death induced by resveratrol and partially prevented processing of downstream caspases, including caspase-9, caspase-3, and caspase-8. Studies using mouse embryonic fibroblasts deficient for both Bax and Bak indicate the contribution of both Bax and Bak in mediating cell death induced by resveratrol and the existence of Bax/Bak-independent cell death possibly through caspase-8- or caspase-2-mediated mitochondria-independent downstream caspase processing.

Resveratrol (3,4,5-trihydroxy-*trans*-stilbene), a natural plant polyphenol widely present in foods such as grapes, wine, and peanuts is gaining acceptance as a potential antitumor agent because of its ability to inhibit various stages of carcinogenesis and eliminate preneoplastic cells *in vitro* and *in vivo* (1–4). The anticancer property of resveratrol is well documented using *in vivo* and *in vitro* models of skin, colon, and breast cancers (1–7). It is gaining much acceptance for its multifaceted mechanisms of action and safety records. The anticarcinogenic property of resveratrol is closely associated with its antioxidant activity and its ability to inhibit cyclooxygenase, hydroperoxidase, carcinogen conversion, protein kinase C, Bcl-2 phosphorylation, Akt, focal adhesion kinase, NF- κ B, and matrix metalloprotease-9 (4–9). Recent support to

develop this compound as a potential antitumor agent is based on its ability to induce programmed cell death in transformed cells with relative nontoxicity to normal cells as well as *in vivo* antitumor activity with clinically attainable concentration (10–12).

Most antitumor agents trigger apoptotic signaling via the involvement of mitochondria that mediate the essential caspase processing by the release of cytochrome *c* and other intermembrane proteins into the cytosol. The proapoptotic Bcl-2 family members, Bax and Bak, are essential for mediating cytochrome *c* release, and the loss of either one or both of these components often renders cancers resistant to drugs (13–15). Similarly, antiapoptotic Bcl-2 family members like Bcl-2 and Bcl-XL guard the mitochondria by preventing the release of cytochrome *c*, and a variety of cancers are resistant to drugs subsequent to overexpression of Bcl-2 or Bcl-XL (16–18). Various studies also suggest that resveratrol elicits apoptotic signaling primarily via mitochondria (2, 20–24). It is expected that modulators of apoptosis with different mechanisms of action independent of the classical apoptotic signaling cascade may have promising features of bypassing clinical drug resistance caused by impaired apoptotic signaling. Indeed, there are reports of cytochrome *c*-independent cell death induced by resveratrol that is not completely prevented by Bax deficiency, suggesting the complex nature of death signaling induced by resveratrol (25, 26). Processing of caspases independent of cytochrome *c* in certain cellular systems (25) and marked caspase-8 activation independent of classical death receptors (27, 28) add more credibility to the notion that resveratrol-mediated apoptosis involves more complex signaling events than the conventional pathways of apoptosis triggered by other known antitumor agents.

Recent findings from several laboratories are more in favor of caspase-2 than caspase-9 as being the apical caspase in stress-induced apoptosis (29, 30). Although the mechanisms of caspase-2 activation and its downstream events are not clearly defined, accumulating evidence indicates that caspase-2 primarily acts upstream of mitochondria (31). The present investigation was designed to address the precise mode of death signaling of resveratrol upstream of mitochondria with special emphasis on the specific role of caspase-2 and its downstream events. The results suggest that resveratrol induces cell death by both caspase-dependent and caspase-independent routes. The caspase-dependent cell death starts with the activation of caspase-2 that cooperates with Bax/Bak and subsequent mitochondrial amplification by the release of cytochrome *c* as well as caspase-independent effectors like apoptosis-inducing factor (AIF)⁴ and endonuclease G. The absence of

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⁴ The abbreviations used are: AIF, apoptosis-inducing factor; MEF, mouse embryonic fibroblasts; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; PMA, phorbol-12-myristate-13-acetate; NAC, *N*-acetyl-L-cysteine; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; RNAi, RNA interference; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; AS, antisense; AFC, 7-amino-4-trifluoromethylcoumarin.

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both Bax and Bak does not prevent cell death and caspase-3 activation completely in Bax- and Bak-deficient mouse embryonic fibroblasts (MEF). This suggests that resveratrol-induced apoptosis involves caspase-2/caspase-8-mediated processing of downstream caspases independent of cytochrome *c*.

EXPERIMENTAL PROCEDURES

Cell Lines and Expression Vectors—The human colon adenocarcinoma cell line HCT 116 with one intact Bax allele (Bax^{+/-}) and its Bax-deficient derivative (Bax^{-/-}) were kindly provided by Dr. Bert Vogelstein (32). The cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. SV40-immortalized MEF deficient for Bax (Bax knock-out), Bak (Bak knock-out), and both Bax and Bak (double knock-out) as well as wild type were generous gifts from Dr. Stanley Korsmeyer (13). These cells were routinely maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Bid DsRed vector was purchased from Clontech. Bax GFP expression vector and pC DNA₃-CrmA were obtained from Drs. Clark Distelhorst and V. M. Dixit, respectively. Antisense caspase-2 vector was kindly provided by Dr. Eric Solary (33). The p65 GFP, I κ B- α superrepressor, and NF- κ B-Luc constructs were obtained from Drs. Michael R. H. White, Dean Ballard, and Heiner Schaefer, respectively. Resveratrol was dissolved in Me₂SO and diluted with complete medium to get the final concentration. The cells were pretreated with the different caspase inhibitors for 1 h before the addition of the drug at a concentration of 100 μ M.

Chemicals and Reagents—The fluorescent labeled peptide substrates Ac-LDESD-7-amino-4-methylcoumarin for caspase-2, Z-DEVD-AFC for caspase-3, Z-IETD-AFC for caspase-8, and Ac-LEHD-AFC for caspase-9 and their respective caspase inhibitors Z-VDVAD-fmk, Z-DEVD-fmk, Z-IETD-fmk, and Z-LEHD-fmk, antibodies to endonuclease G (PC684) and conformation-specific Bak (AM03) and intracellular caspase-3 assay kit II, PhiPhiLux G2D2 (235432), were obtained from Calbiochem. Resveratrol, 4',6-diamidino-2-phenylindole (DAPI), [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromide] (MTT), and all secondary antibodies were procured from Sigma. Rabbit polyclonal antibodies to cytochrome *c* (sc-7159), PARP (sc-7150), actin (H-196), caspase-2 (sc-2121); mouse monoclonal antibodies to Hsc70 (sc-7298), Hsp90 (sc-13119), Hsp70 (sc-24), and HSF1 (sc-17757); and the FITC-annexin kit (sc-4252FITC) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal antibodies to caspase-9 (9502), Bid (2002), and mouse monoclonal antibody to caspase-8 (9746) were obtained from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies for cytochrome *c* (7H4.2C12; 556433) and conformationally active Bax (6A7; 56467) were from BD Pharmingen. Rabbit polyclonal antibodies for AIF (IMG-303) and monoclonal antibodies for caspase-3 (IMG-144) and caspase-8 and RNAi vector (IMG-805) were procured from Imgenex. The rat monoclonal antibody that recognizes mouse caspase-2 (10C6) was from Chemicon.

Cytotoxicity and Apoptosis Measurement—Cytotoxicity was assessed by a modified version of the MTT reduction assay (34). For analyzing chromatin condensation by DAPI staining, the cells were seeded on 12-mm coverslips and, after the indicated treatment, fixed in paraformaldehyde and permeabilized with Triton 0.2%. The monolayer was washed and stained with 0.5 μ g/ml DAPI in PBS for 5 min and viewed under a fluorescent microscope using the UV2A filter of an Epifluorescent microscope (Nikon TE 300). Apoptotic nuclei with intense condensed chromatin were scored in percentage from 200–300 cells/sample by at least two investigators.

Exposure of phosphatidylserine on the surface of plasma membrane, an important apoptotic marker, was analyzed using annexin V-FITC staining reagent as per standard protocol and viewed under a fluorescent microscope. Intracellular caspase-3 activity was monitored in MEF using cell-permeable fluorogenic peptide-based reagent PhiPhiLux as per instructions and viewed under a fluorescent microscope using a rhodamine filter or under a laser-scanning confocal microscope (TCS SP2; Leica). Fluorescing cells were counted from a total of 300–400 cells/sample to calculate the percentage of cells positive for active caspase-3.

Caspase Activity Analysis—The cells after the indicated treatment were lysed with a lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) for 30 min at 4 °C. 50 μ g of protein were incubated in assay buffer (100 mM HEPES, pH 7.0, 1 mM EDTA, 0.1% CHAPS, 10% glycerol, 20 mM dithiothreitol) in the presence of 50 μ M synthetic tetrapeptide fluorescent substrates for different caspases for 1 h at 37 °C. The fluorescence from the released free AFC (excitation wavelength of 405 nm and emission wavelength of 500 nm) and 7-amino-4-methylcoumarin (excitation wavelength of 360 nm and emission wavelength of 460 nm) was determined using spectrofluorimeter (LS-50B; PerkinElmer Life Science Products). The relative fluorescence intensity per mg of protein was compared with respective control to calculate -fold increase from the respective control value.

Western Blot—To prepare whole cell extracts for Western blot, cells were harvested, washed three times in PBS, and lysed in radioimmune precipitation lysis buffer (50 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 40 mM NaF, 10 mM NaCl, 10 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, and 1 μ g/ml each leupeptin and aprotinin). The cell lysates (50 μ g of protein) were resolved by SDS-PAGE, and the separated proteins were transferred to polyvinylidene difluoride membranes by the wet transfer method using a Bio-Rad electrotransfer apparatus. After blocking with 10% nonfat milk in Tris-buffered saline containing 0.2% Tween 20, the membrane was incubated with the primary antibody, followed by horseradish peroxidase-conjugated secondary antibody. Proteins were visualized by 3,3'-diaminobenzidine. For analyzing proteins released from mitochondria into cytosol, the cytosolic fraction was prepared from digitonin-permeabilized cells as per standard protocol. Briefly, the cells (untreated or after treatment) were harvested and washed two times with PBS, and the cell pellet was resuspended in digitonin lysis buffer (75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, 190 μ g/ml digitonin) containing protease inhibitors and incubated on ice for 5 min. The releasate was centrifuged at 15,000 rpm at 4 °C for 30 min and used for Western blotting as described above using antibodies to AIF or endonuclease G or cytochrome *c* and appropriate secondary antibodies.

Immunofluorescence and Confocal Imaging—Cells grown on 12-mm glass coverslips in 24-well plates and after drug exposure for different time periods were fixed with 4% paraformaldehyde in PBS. After permeabilization with 0.5% Triton X-100 in PBS for 10 min, the coverslips were incubated with primary antibody followed by fluorescence-labeled secondary antibody. For staining conformational specific Bax and Bak, the cells on coverslips after fixation were permeabilized with 0.0125% CHAPS in order to avoid artifactual epitope exposure (35). Subsequently, they were incubated with a conformational specific antibody and processed essentially as described above. All of the coverslips were mounted with glycerol and viewed either with a Nikon epifluorescent microscope (Nikon TE300) or imaged using a Leica laser-scanning confocal microscope (TCS SP2) attached to a Leica inverted fluorescent microscope DM IRE2. All images were obtained with 1.3 numerical

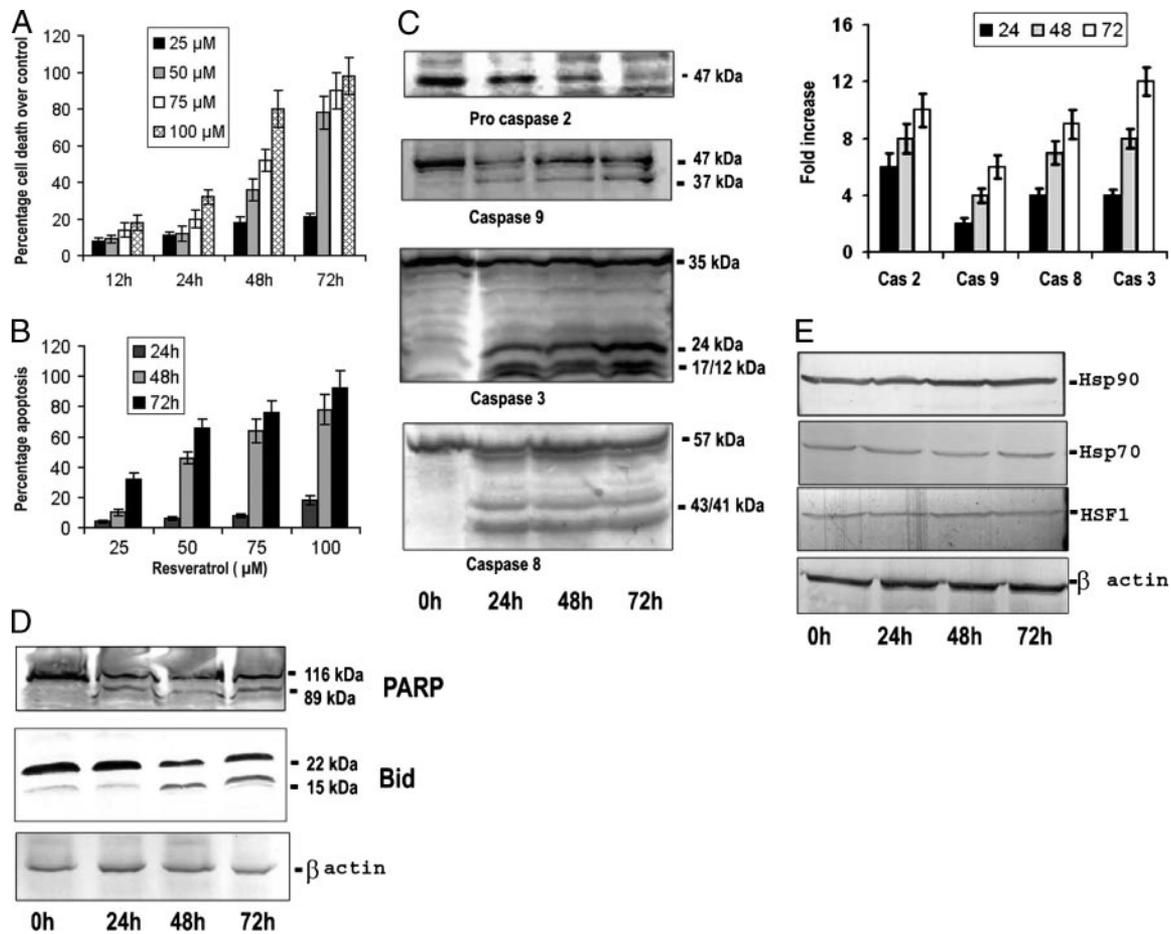


FIGURE 1. Resveratrol induces apoptosis in a concentration-dependent way involving processing of caspase-2, caspase-9, caspase-3, and caspase-8. A, HCT 116 cells were treated with 25–100 μ M resveratrol for 12, 24, 48, and 72 h. Cytotoxicity was analyzed by MTT assay. The percentage of cell death was calculated from the control. Mean \pm S.D. values are shown for four different experiments. B, HCT 116 cells were seeded onto 12-mm coverslips and treated with 25–100 μ M of resveratrol for 24, 48, and 72 h. The cells were fixed, permeabilized, and stained with DAPI to monitor the chromatin condensation under the UV2A filter of an epifluorescent microscope. The percentage positive from the total 300 cells counted for each sample was plotted as a graph. C, the whole cell extract prepared from untreated (Me_2SO) or resveratrol (50 μ M)-treated cells, was processed for Western blotting to detect the status of caspase processing using specific antibodies as described. The graph shows spectrofluorimetric determination of enzymatic activity of respective caspases analyzed using fluorescent labeled caspase substrates as described under "Experimental Procedures." The relative fluorescence intensity per mg of protein was determined, and the -fold increase was calculated from control. D, the cells treated as above were lysed, and the proteins were subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane and probed with antibody against PARP and Bid. The respective mother bands and cleaved fragments are shown. β -Actin served as loading control. E, the whole cell extract prepared as above was processed for Western blotting and probed with Hsp90, Hsp70, HSF1, or β -actin antibody.

aperture $\times 40$ Plan Neo Fluor objective. For DsRed, rhodamine, and PhiPhiLux, laser line 543 nm from the helium-neon laser was used, and for GFP/FITC, laser line 488 nm from the argon ion laser was used.

Transfection Experiments—HCT 116 cells were transiently transfected with the corresponding expression vectors using Lipofectamine 2000 (Invitrogen) to attain maximum transfection efficiency that ranged between 50 and 70%. The stable cells expressing Bax GFP and Bid DsRed were generated by growing transfected cells in 1 mg/ml of G418 (Invitrogen)-containing medium for 2 months. The resistant clones formed were picked up and analyzed for protein expression. The selected clones were maintained separately with 100 μ g/ml G418.

NF- κ B Reporter Gene Assay—The cells were transiently transfected with NF- κ B-Luc vector using Lipofectamine 2000 and after 24 h were exposed to 50 μ M hydrogen peroxide (H_2O_2), 10 nM phorbol-12-myristate-13-acetate (PMA) or 100 mM of *N*-acetyl-L-cysteine (NAC) alone or along with resveratrol. After 24 h, whole cell extract was prepared, and a luciferase assay was performed using the Dual-LuciferaseTM reporter assay system (Promega, Madison, WI) as per the instructions from the manufacturer. Luciferase values were normalized for differences in transfection efficiency on the basis of β -galactosidase activity.

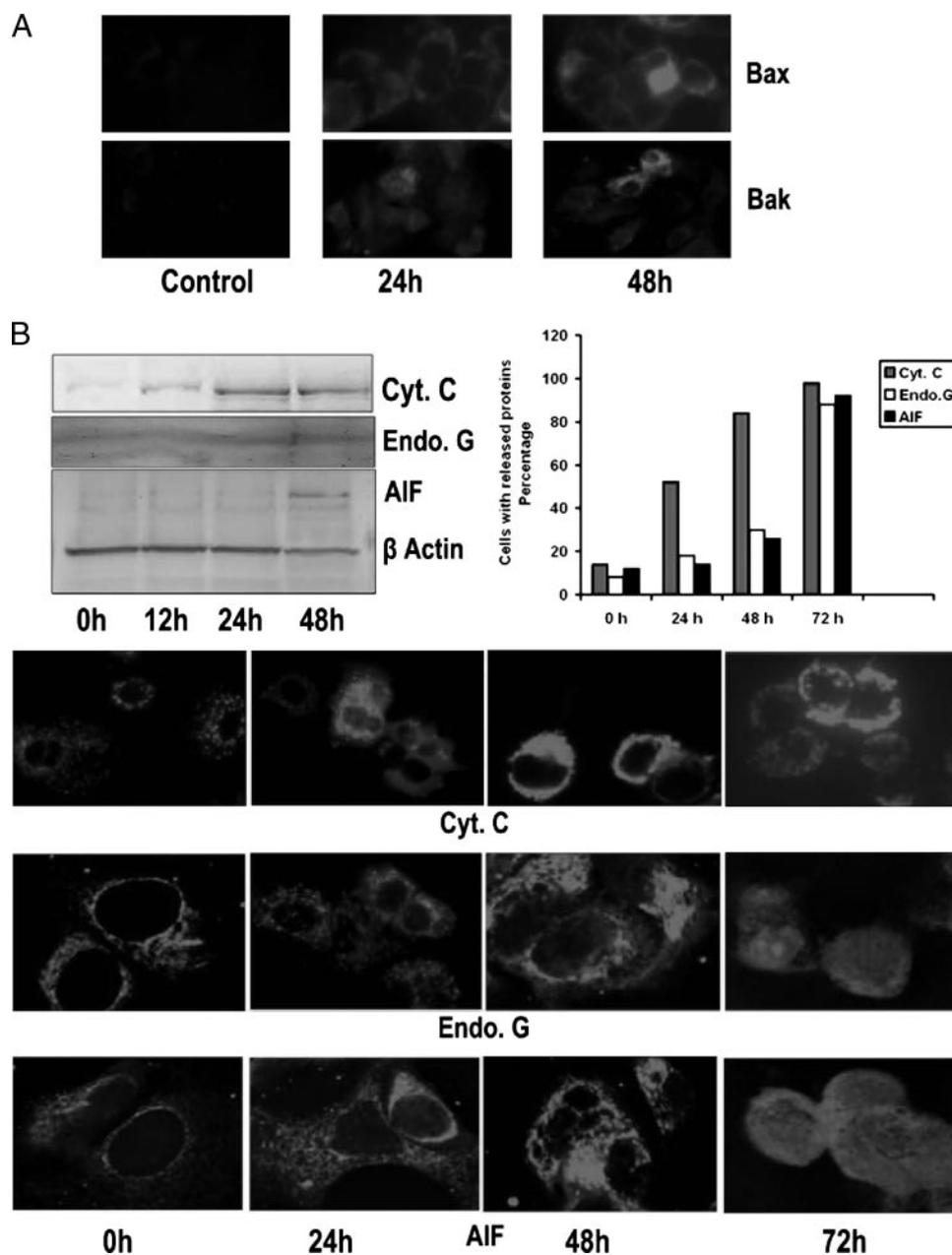
Translocation of Bid DsRed, Bax GFP, and p65 EGFP—The transfected cells were seeded onto 8-well-chambered coverglass and allowed to grow for 24 h. After a fixed time of incubation with resveratrol or the indicated treatment, the pattern of fluorescence was monitored under a fluorescent microscope using specific filters and photographed.

RESULTS

Resveratrol-induced Cell Death Involves Processing of Caspase-2, -9, -8, and -3 and Cleavage of PARP and Bid—Exposure of colon cancer cells (HCT 116) to 25, 50, 75, and 100 μ M resveratrol induced marked cytotoxicity, as evidenced from the MTT assay in a time- and concentration-dependent manner (Fig. 1A). Changes in chromatin condensation upon resveratrol treatment were then analyzed using DAPI to see whether the observed cytotoxicity is mediated through the induction of apoptosis. Resveratrol did not induce notable changes in nuclear morphology below 25 μ M concentration, but at 100 μ M for 72 h, it induced massive chromatin condensation in almost 92% of cells and at 48 h in 78% of cells (Fig. 1B). Since 50 μ M resveratrol was enough to induce nearly 50% cell death at 48 h, we used this concentration for subsequent experiments.

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FIGURE 2. Resveratrol induces the release of cytochrome *c*, AIF, and endonuclease G from mitochondria into cytosol with conformational change in both Bax and Bak and translocation of BAX GFP to mitochondria. *A*, the cells after treatment with resveratrol (50 μ M) for 24 and 48 h were fixed, permeabilized with 0.0125% CHAPS, and immunostained using antibody that recognized conformationally active Bax or Bak and rhodamine-conjugated secondary antibodies. The samples were viewed under a Nikon epifluorescent microscope. *B*, cells after treatment with resveratrol (50 μ M) for 0, 24, 48, and 72 h were washed with PBS, and cytosolic fraction free of mitochondria was prepared as described using the digitonin lysis method. 50 μ g of cytosolic fraction was separated on SDS-PAGE and transferred onto polyvinylidene difluoride and immunoblotted for cytochrome *c* (Cyt. *C*), AIF, or endonuclease G (*Endo G*). β -Actin served as loading control. Representative samples grown on glass coverslips after the indicated time of resveratrol treatment were processed for immunolocalization for cytochrome *c*, AIF, and endonuclease G. The samples were viewed under a Leica inverted fluorescent microscope equipped with a TCS SP2 scanning confocal unit. The cells were scanned using a 456-nm argon laser. The percentage of cells with released cytochrome *c*, AIF, and endonuclease G for different time intervals is shown in the graph. *C*, HCT 116 cells stably expressing Bax GFP were treated with resveratrol (50 μ M) for 12, 24, and 48 h, and the translocation of Bax to mitochondria was analyzed by viewing for perinuclear granular green fluorescence using an FITC filter under an epifluorescent microscope. The graph shows the percentage of cells with Bax GFP aggregates at mitochondria.



Next, the processing of caspase-2, -9, -3, and -8 was analyzed after incubation of the HCT 116 cells with or without 50 μ M resveratrol for 24, 48, and 72 h. All of the caspases analyzed were processed after 24 h of resveratrol treatment, as is evident from the appearance of cleaved fragments of caspase-3, -9, and -8 and loss of the mother band of caspase-2 with time in Western blot experiments (Fig. 1C). Spectrofluorimetric quantitative analysis of enzymatic activity of caspases also substantiates their processing upon resveratrol treatment (Fig. 1C). The nuclear apoptosis, as determined by DAPI staining shows that apoptotic nuclear morphology was noticeable from 24 h of resveratrol treatment. However, by 72 h, nearly 78% cells were apoptotic, consistent with increased activation of caspase-3 and pronounced cleavage of its substrate, PARP, with resveratrol treatment (Fig. 1D). We also analyzed the cleavage status of a proapoptotic Bcl-2 family protein, Bid, by Western blotting, and the 15-kDa cleavage fragment was generated by 48 h of resveratrol treatment (Fig. 1D).

Heat shock proteins are important regulators of apoptosis with a capacity to block caspase processing, and its down-regulation has been

reported with a variety of antitumor agents. Hence, we analyzed the expression levels of heat shock proteins 70 and 90 and the heat shock transcription factor, HSF1, with or without resveratrol treatment by Western blotting for the indicated time intervals. Expression levels of heat shock proteins or HSF1 were not altered by resveratrol up to 72 h (Fig. 1E). These results suggest that resveratrol is capable of inducing chromatin condensation and cleavage of PARP primarily through the processing of caspase-2, -9, -3 and -8 without significant changes in heat shock proteins.

*Resveratrol Induces Release of Cytochrome *c*, AIF, and Endonuclease G from Mitochondria to Cytosol and Translocation of Bax GFP to Mitochondria in a Time-dependent Manner*—The proapoptotic Bcl-2 family proteins, Bax and Bak, are activated by conformational changes that are considered essential for the release of cytochrome *c* and other apoptosis-enhancing proteins (13, 14). Immunofluorescent staining of cells treated with resveratrol for 24 h showed that 85 and 92% of them were stained positive for the active forms of Bax and Bak, respectively (Fig. 2A). The control cells stained negative for both active Bax and Bak (Fig.

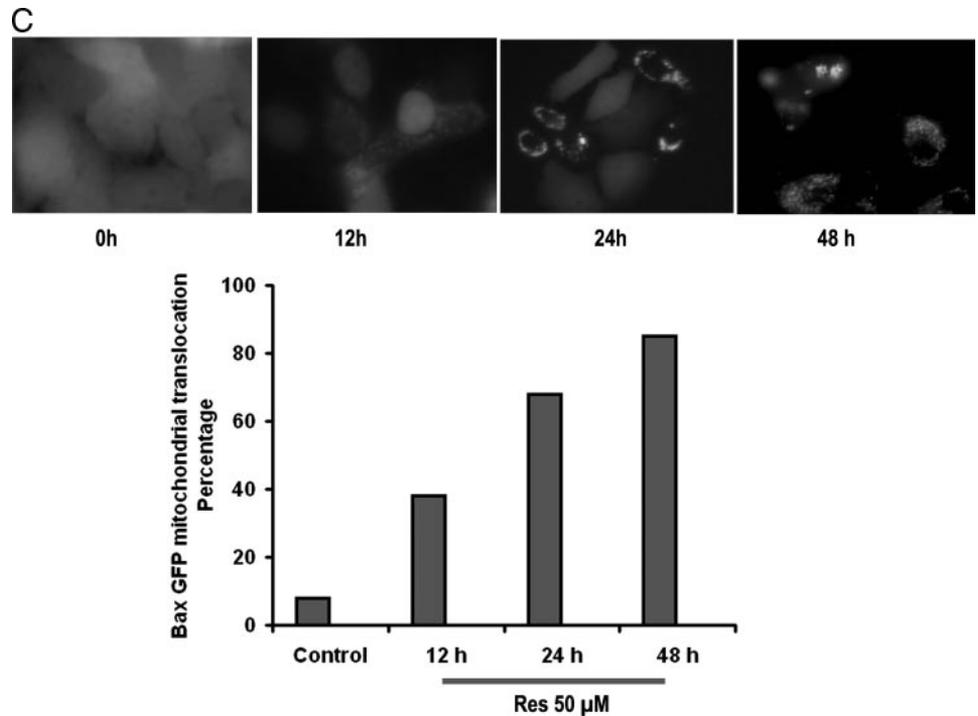


FIGURE 2—continued

2A). Bax and Bak can trigger mitochondrial apoptosis by the release of cytochrome *c* for subsequent caspase processing as well as certain caspase-independent death effectors like AIF and endonuclease G into cytosol (15). Hence, the release of cytochrome *c*, AIF, and endonuclease G was analyzed both by Western blotting and immunofluorescent staining in cells treated with or without resveratrol. In Western blot of digitonin-permeabilized cells, cytochrome *c* was released into cytosol after 24 h of resveratrol treatment, and it gradually increased up to 72 h, whereas the release of AIF and endonuclease G was a delayed event under the same conditions (Fig. 2B). The immunofluorescent staining of cells also shows that after 48 h of resveratrol treatment, almost all of the cells had a cytoplasmic diffuse pattern for cytochrome *c*, whereas only a few cells had nuclear or cytoplasmic positivity for AIF and endonuclease G (Fig. 2B). The reasons for the differences in the release kinetics of cytochrome *c*, AIF, and endonuclease G are not clear. The delayed release of AIF and endonuclease G upon resveratrol treatment supports the emerging notion that the release of mitochondrial membrane proteins is regulated differentially, and the release of AIF and endonuclease G requires caspase-processing downstream of cytochrome *c* release (36).

To visualize the movement of cytosolic Bax into mitochondria in live cells, we expressed the Bax protein fused with GFP in Bax knock-out HCT 116 cells. Treatment of cells that express Bax-GFP fusion protein with resveratrol resulted in Bax migration into mitochondria within 12 h, and after 48 h, most of the cells (85%) showed perinuclear granular fluorescence, indicating massive translocation of Bax (Fig. 2C). Taken together, the results suggest that resveratrol induces conformational change in Bax and Bak, triggers mitochondrial apoptosis by the immediate release of cytochrome *c*, and subsequently induces the release of AIF and endonuclease G in a delayed manner.

Essential Role of Caspase-2 in Downstream Processing of Caspases—The above observations suggest that Bax/Bak mediated mitochondrial amplification as the primary event in resveratrol-induced cell death. However, the upstream mitochondrial events in resveratrol-induced cell death are far from clear. Controversy exists as to how Bax mediates mitochondrial cytochrome *c* release into cytosol. Based on recent

reports that suggest that caspase-2 acts upstream of mitochondria in stress-induced apoptosis (29, 30), we decided to analyze whether caspase-2 is activated upstream of mitochondria. First, we preincubated cells with cell-permeable inhibitors for caspase-2, -3, -9, or -8 followed by resveratrol treatment and analyzed for nuclear apoptosis by DAPI staining and for externalization of phosphatidylserine by FITC-annexin V staining. Upon resveratrol treatment, 51 of 100 cells had condensed nuclei, and 72% cells showed positivity for annexin V after 48 h; however, caspase-3 and caspase-2 inhibitors marginally inhibited cell death up to 33 and 38%, respectively. Inhibition of caspase-8 also reduced cell death to 60% (Fig. 3A). Further, to determine the initiator caspase in resveratrol-induced cell death, we analyzed the activated caspases using fluorescent substrates 48 h after resveratrol treatment with or without caspase-2, -3, -9, and -8 inhibitors. Caspase-3, -9, or -8 inhibitors failed to reduce the processing of caspase-2 significantly; however, pretreatment with caspase-2 inhibitor reduced the processing of caspase-9, caspase-3, and caspase-8. Caspase-9 inhibitor reduced the processing of caspase-3 (Fig. 3B). These results substantiate that resveratrol-induced processing of caspase-9 and -3 is downstream of caspase-2 processing and a possible role of caspase-2 to process caspase-8.

To further validate the initiator caspases in resveratrol-mediated apoptosis, we silenced caspase-2 and caspase-8 by transient transfections with vectors for antisense caspase-2 and RNAi, respectively. The transient transfection of caspase-2 AS or RNAi caspase-8 reduced the expression of caspase-2 and caspase-8, respectively, in HCT 116 cells by nearly 50–60% from their corresponding vector control cells, consistent with their transfection efficiencies (Fig. 3C). Antisense down-regulation of caspase-2 matched with a reduction of apoptosis in HCT 116 cells when assessed by both DAPI (33%) and annexin V staining (45%) compared with the corresponding vector-transfected control cells (Fig. 3C). Antisense silencing of caspase-2 also markedly reduced the activities of caspase-2, -9, and -3, but caspase-8 processing was only slightly reduced (Fig. 3D). Silencing of caspase-8 also reduced the processing of caspase-9 and -3 but not that of caspase-2. These observations suggest that processing of procaspase-2 is the initiating event in resveratrol-

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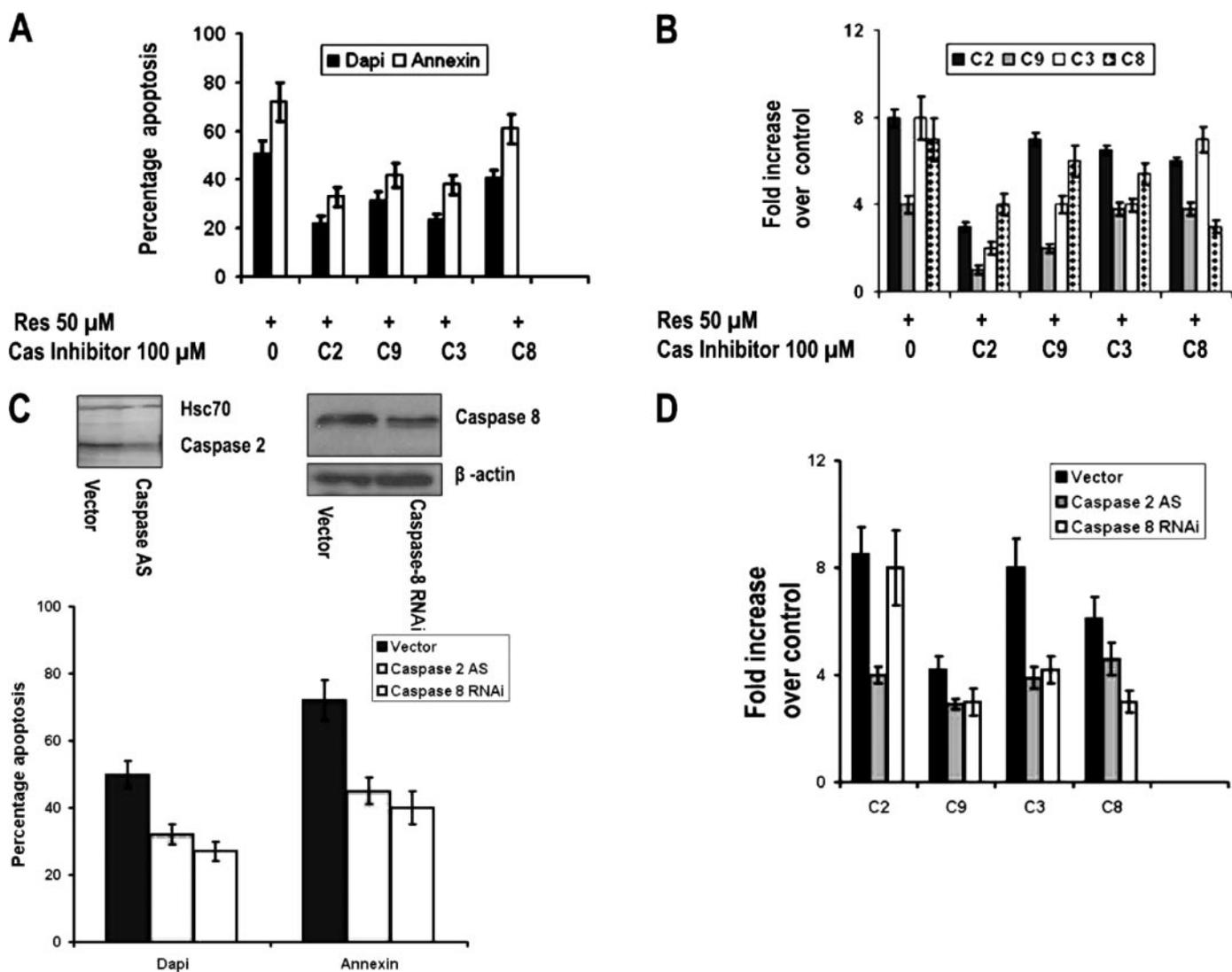


FIGURE 3. Essential role of caspase-2 in resveratrol-induced apoptosis. *A*, HCT 116 cells grown on glass coverslips were pretreated with respective caspase inhibitors (100 μ M) for 1 h followed by resveratrol (50 μ M) for 48 h. The cells were fixed with paraformaldehyde for 10 min, permeabilized, and stained with DAPI. The percentage of cells with intense condensed chromatin was calculated from the total number of cells counted in a well. Live cells were washed and stained with annexin V-FITC as per instructions, and positive cells were counted as above using an inverted fluorescence microscope. *B*, the cells prepared as earlier with different caspase-inhibitors and resveratrol were lysed in caspase-lysis buffer. 50 μ g of protein was used for determining enzymatic activity of different caspases with respective peptide fluorescent substrates as described. From the relative fluorescent intensity determined using spectrofluorimeter, the -fold increase from control was calculated. Values are mean \pm S.D. from three experiments. *C*, HCT 116 cells were transfected with pcDNA3 (Vector) or an expression vector for caspase-2 in antisense orientation (Caspase-2 AS) or RNAi vector for caspase-8 (Caspase-8 RNAi). 24 h after transfection, whole cell extract was prepared and Western blotted for caspase-2 or caspase-8. Hsc70 or β actin served as loading control (inset). The cells were treated with resveratrol (50 μ M) for 48 h, and cell death was analyzed by DAPI and annexin V staining as described. *D*, the vector control, caspase-2 AS, and caspase-8 RNAi cells were exposed to resveratrol (50 μ M) for 48 h, and the cells were lysed for the caspase activity assay as described. The -fold increase enzymatic activity for caspase-2 (C2), caspase-9 (C9), caspase-3 (C3), and caspase-8 (C8) from control are shown. Values are mean \pm S.D. from three experiments.

induced cell death, and a possible role exists for caspase-2 in activating caspase-8.

Most biological properties of resveratrol are related to its strong antioxidant property and inhibition of the transcription factor NF- κ B (8, 9). NF- κ B is sequestered in the cytoplasm through interaction with an inhibitory protein I κ B that masks its nuclear localization signal. Upon activation, I κ B is released through the activation of an I κ B kinase (IKK) complex that induces I κ B phosphorylation and subsequent degradation. This allows the free NF- κ B to translocate to the nucleus for the transcription of target genes (9). We used cells expressing p65-EGFP fusion protein to study the translocation of p65 to the nucleus. Both H₂O₂ and PMA induced massive nuclear translocation of p65 that was remarkably reduced upon co-treatment of cells with resveratrol for 24 h (Fig. 4A). The antioxidant NAC also prevented the nuclear translocation of p65 induced by both H₂O₂ and PMA. Further, we analyzed the

p65 reporter gene assay to substantiate the findings. Transactivation of NF- κ B induced by H₂O₂ or PMA was reduced by resveratrol (Fig. 4B). However, H₂O₂ or PMA fails to activate caspase-2 at the concentration that induced p65 nuclear translocation (Fig. 4C).

To confirm whether caspase-2 activation in resveratrol-treated cells is subsequent to NF- κ B inhibition, we used HCT 116 cells overexpressing p65EGFP and cells transfected with superrepressor I κ B that blocks NF- κ B activation. Surprisingly, there was no marked difference in caspase-2 activation between vector-transfected and I κ B superrepressor-transfected cell populations (Fig. 4D). The findings suggest that caspase-2 activation observed in resveratrol-treated cells is independent of its antioxidant activity or NF- κ B inhibition.

Resveratrol-induced Conformational Changes in Bax and Bak Require Caspase-2—The initial trigger for inducing conformational change of Bax/Bak is controversial. Recently, Solary and co-workers (28)

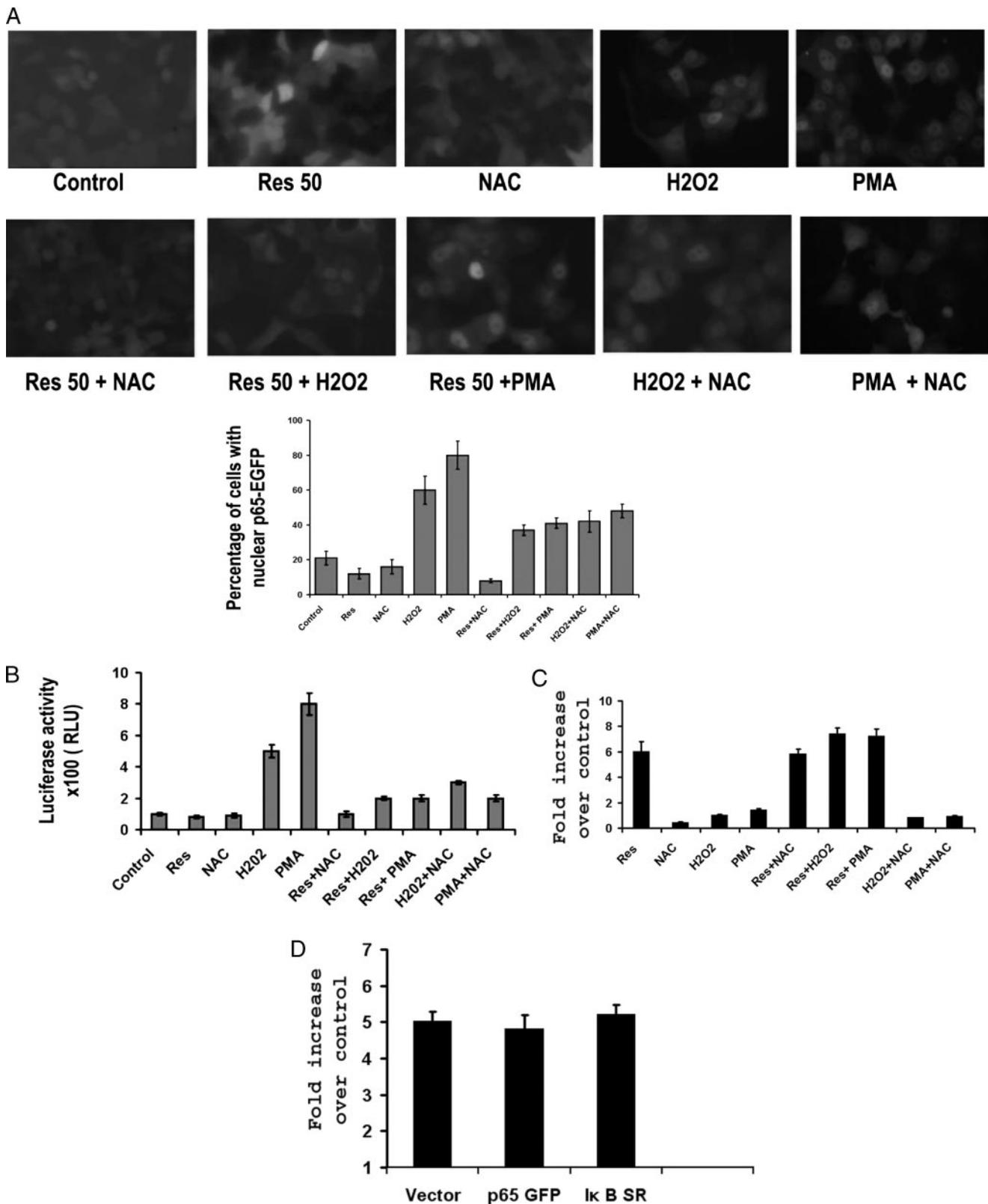


FIGURE 4. H_2O_2 - and PMA-induced NF- κ B activation is inhibited by resveratrol; however, caspase-2 activation is not dependent on NF- κ B inhibition. *A*, the HCT 116 cells were transfected with p65 EGFP vector. The transfected cells were exposed to H_2O_2 ($50 \mu M$), PMA ($10 nM$), and NAC ($100 nM$) alone or with resveratrol ($50 \mu M$). The representative fluorescent images are shown. The cells showing GFP nuclear translocation were counted from the total number of GFP cells in a total of 200 cells. The percentage of cells with nuclear GFP positivity is shown in the graph. *B*, HCT 116 cells were transiently transfected with NF- κ B-dependent luciferase reporter plasmids. After 24 h, the cells were either left untreated or treated with the indicated drugs. The whole cell extract was prepared after 24 h to measure luciferase activity. The relative luciferase activity (RLU) is shown. *C*, the cells were exposed to H_2O_2 , PMA, and NAC alone or in combination with resveratrol for 24 h, and the whole cell extract was prepared. Caspase-2 activity was measured in the samples using the spectrofluorimetric method as described earlier. -Fold increase of caspase-2 activity from control is shown. *D*, HCT 116 cells were transiently transfected with pcDNA3 (Vector) or an expression vector for p65 GFP or I κ B SR vector. 24 h after transfection, the cells were treated with resveratrol ($50 \mu M$) for 48 h. Whole cell extract was prepared for caspase-2 analyses as described earlier.

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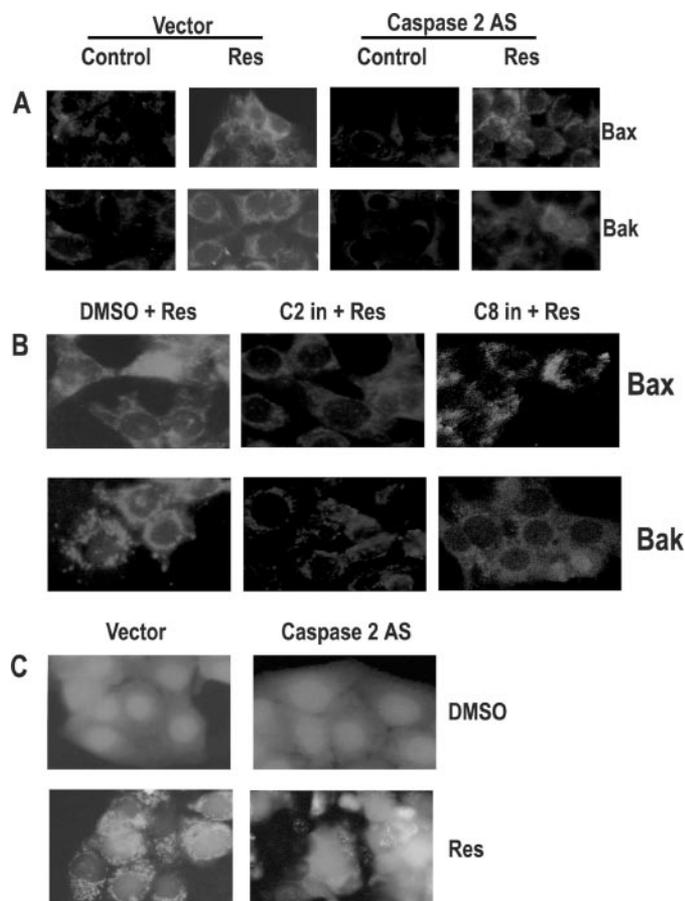


FIGURE 5. Caspase-2 contributes to the conformational change in Bax/Bak and translocation of Bax GFP to mitochondria. *A*, vector control and caspase-2 AS cells were treated with resveratrol (50 μM) for 48 h and immunostained for conformational active Bax or Bak as described. *B*, HCT 116 cells were either treated with Me_2SO (DMSO) alone or a 100 μM concentration of either caspase-2 inhibitor or caspase-8 inhibitor for 1 h followed by resveratrol (50 μM) for 48 h. The cells were then immunostained for conformational active Bax or Bak as above. *C*, HCT 116 cells stably expressing Bax GFP were further transfected with empty vector or caspase-2 AS vector and treated with resveratrol for 48 h, and translocation of Bax was analyzed under a fluorescent microscope.

showed that caspase-8 could induce a conformational change in Bax/Bak that subsequently targets Bax/Bak to mitochondria to release cytochrome *c* in response to resveratrol. Since we observed caspase-2 as the apical caspase processed during resveratrol-induced apoptosis, we sought to analyze the precise role of caspase-8 or caspase-2 in inducing conformational changes in Bax/Bak. Exposure of caspase-2-silenced cells to resveratrol showed only 32 and 24% positivity for conformationally active Bax and Bak, respectively, whereas vector-transfected cells showed 94% for Bax and 83% for Bak. Almost all control cells showed negative staining for conformationally active Bax or Bak (Fig. 5*A*). Next, the cells were processed for conformationally active Bax/Bak after treatment with resveratrol for 24 h with or without an inhibitor of caspase-2 or -8. With resveratrol treatment, most of the cells were positive for Bak (80–90%) and Bax (70–80%), with intense red fluorescence indicating mitochondrial pattern (perinuclear staining). Pretreatment with caspase-8 inhibitor failed to reduce the percentage of cells positive for either Bax or Bak significantly. However, caspase-2 inhibitor prevented the conformational change in Bax from 80 to 54% and that of Bak from 92 to 78% (Fig. 5*B*). The translocation of Bax GFP to mitochondria in response to resveratrol was also reduced in caspase-2 AS cells compared with vector control cells (Fig. 5*C*). After 48 h of resveratrol treatment, almost 88% of cells showed mitochondrial granular appearance for GFP in vector-transfected cells; however, only 59% of cells were positive in

caspase-2 AS cells. These results suggest that caspase-2 targets mitochondria by inducing conformational changes in both Bax and Bak in the presence of resveratrol.

Role of Bid in Mitochondrial Amplification, Primarily through Caspase-2 and Caspase-8—The involvement of death receptor signaling in resveratrol-induced apoptosis is controversial, with reports of classical death-inducing signaling complex processing in certain cells (37), Fas-independent death signaling in others (27), and caspase-8 activation involving lipid rafts and FAS in colon cancer cells (28). Pronounced caspase-8 activation was noticed both spectrofluorimetrically and by Western blots (with cleavage of Bid) after resveratrol treatment in HCT 116 cells. In order to further understand the specific role of caspase-2 and -8 in mediating mitochondrial apoptosis through Bid, we stably transfected HCT 116 cells with Bid DsRed vector. They were treated with resveratrol for 48 h and analyzed for the translocation of Bid DsRed from cytosol to mitochondria as the index of its cleavage during apoptosis. Bid DsRed stable cells were further transiently transfected with caspase-2 AS and vector coding CrmA (which can prevent processing of caspase-8) and exposed to resveratrol for 48 h. Compared with vector-transfected cells, silencing of caspase-2 or CrmA transfection effectively reduced the translocation of Bid DsRed from 70% to 42 and 46%, respectively (Fig. 6*A*). Moreover, pretreatment of Bid DsRed cells with an inhibitor of either caspase-8 or caspase-2 alone failed to block the translocation of Bid DsRed to mitochondria markedly. However, pretreatment of cells with caspase-2 and caspase-8 inhibitors together reduced the translocation from 70 to 28% (Fig. 6*B*). The finding substantiates that both active caspase-2 and active caspase-8 can process Bid, and both of the events contribute to the mitochondrial amplification of cell death.

The Essential Role of Bax and Bak in Resveratrol-induced Cell Death: Possible Involvement of Bax/Bak-independent Cell Death via Caspase-2/Caspase-8—Bax knock-out HCT 116 cells failed to completely prevent cell death induced by resveratrol, suggesting Bax-independent cell death presumably operating through redundant Bak, consistent with an earlier report (26). However, silencing of Bak by RNAi in Bax-deficient cells also failed to prevent cell death completely in Bax-deficient cells (data not shown). For detailed analysis of the role of Bax and Bak in resveratrol-induced cell death, we used MEF deficient for either Bax or Bak or both and compared the cell death by analyzing chromatin condensation after resveratrol treatment. Absence of either Bax or Bak significantly reduced chromatin condensation to 39 and 30% compared with 78% observed with wild type cells at 24 h. Quite surprisingly, we failed to observe a complete prevention of cell death even in cells deficient for both Bax and Bak, indicating the existence of a Bax/Bak-independent cell death program in the presence of resveratrol (Fig. 7*A*). In order to understand whether caspases are contributing for the cell death in the absence of Bax or Bak or both, we stained the cells to locate intracellular active caspase-3 using PhiPhiLux reagent and viewed them under confocal microscopy. Fig. 7*B* suggests the presence of active caspase-3 in cells deficient in Bax or Bak or DKO. Caspase-2 inhibitor reduced the intracellular caspase-3 activities in all cells but still failed to block completely in DKO cells (Fig. 7, *B* and *C*). These findings suggest that even in the absence of Bax and Bak, resveratrol can trigger apoptosis through caspase-3 activation possibly through mitochondria-independent caspase-2- or caspase-8-mediated downstream caspase processing or by some uncharacterized route. For confirming caspase-2 activation by resveratrol in the absence of Bax/Bak, we measured it both by spectrofluorimetric and Western blot methods. In all of the cells, caspase-2 was processed after 24 h of resveratrol treatment (Fig. 7*D*). Surprisingly, the difference in the extent of caspase-2 activation was minimum between MEF WT and DKO cells, suggesting that caspase-2 activation

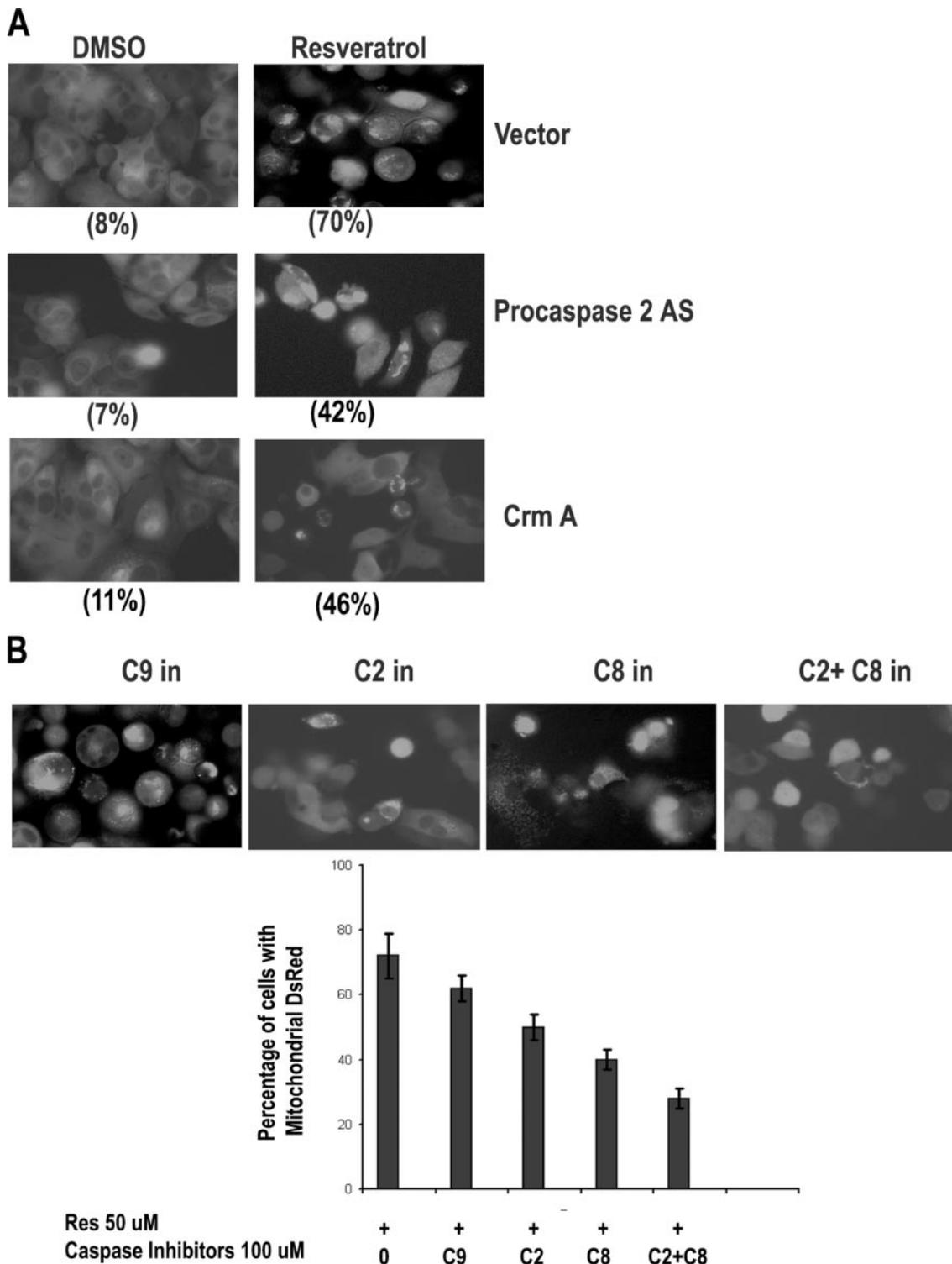


FIGURE 6. Both caspase-2 and caspase-8 contribute to the translocation of Bid DsRed in resveratrol-treated cells. *A*, HCT 116 cells were transfected with Bid DsRed, and stable cells were generated. The stable cells were further transiently transfected with empty vector or caspase-2 AS or CrmA. 24 h after transfection, cells were exposed to resveratrol (50 μ M) for 48 h. The translocation of Bid DsRed to mitochondria was visualized as granular perinuclear staining using an epifluorescent microscope and percentage positivity in a total of 300 cells is shown in parentheses. *DMSO*, dimethyl sulfoxide. *B*, Bid DsRed stable cells were pretreated with different caspase inhibitors (100 μ M) for 1 h followed by resveratrol (50 μ M) for 48 h. The translocation of DsRed to mitochondria was analyzed as earlier, and the percentage of cells with granular appearance was calculated from the total number of 300 cells counted per sample. Values are the mean \pm S.D. of three different experiments.

observed in resveratrol-treated cells is upstream of mitochondrial Bax and Bak.

Further, in order to delineate the specific roles of caspase-2 or caspase-8 on Bax/Bak-independent cell death, the cells were pretreated with a caspase-2, caspase-8, or caspase-9 inhibitor, followed

by annexin V staining. Pretreatment with a caspase-2 inhibitor reduced cell death to 44, 32, 30, and 22% from 82, 45, 42, and 34%, respectively, for wild type, Bax KO, Bak KO, and DKO cells. Caspase-8 inhibitor was also equally effective in DKO cells but failed to reduce cell death in others possibly because in the presence of

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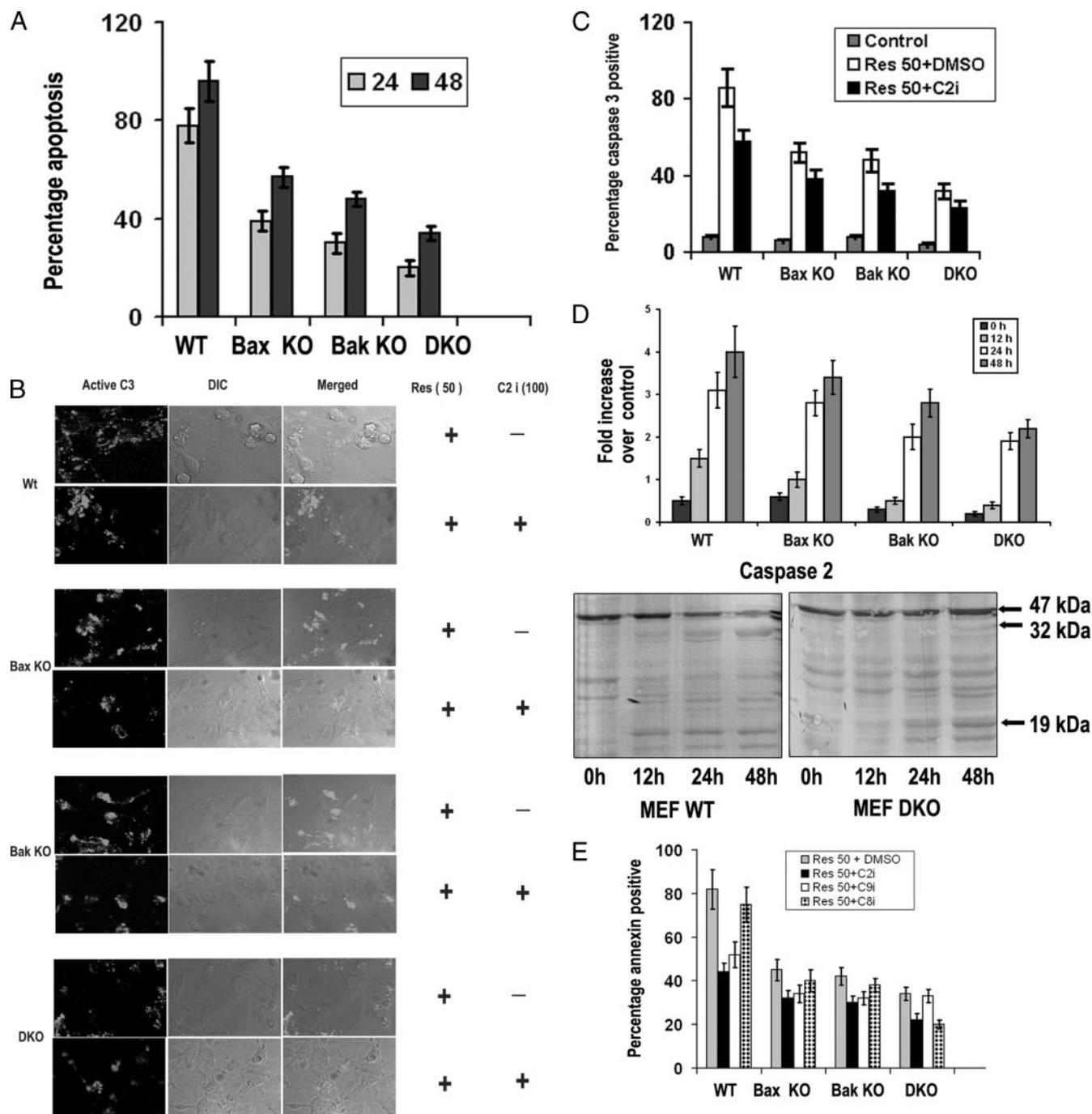


FIGURE 7. Resveratrol-induced cell death is reduced in MEF knock-out of either Bax or Bak or Bax plus Bak but not completely prevented. *A*, MEF deficient for Bax, Bak, or both and wild type were seeded onto glass coverslips and exposed to resveratrol (50 μ M) for 24 and 48 h. The cells were processed for DAPI to determine chromatin condensation as described. Values are the percentage of cells with condensed nuclei from three different experiments. *B*, the MEF cells were pretreated with caspase-2 inhibitor or Me2SO (DMSO) alone for 1 h followed by resveratrol (50 μ M) for 48 h. The cells were washed and incubated with caspase-3 intracellular assay reagent for 20 min. Then the cells were washed and viewed under a confocal scanning microscope as described. The differential interference contrast image was collected to see the total number of cells in a field. *C*, from the above experiments, the number of caspase-3-positive cells from a total of 300–400 cells/sample was counted to get the percentage of caspase-3-positive cells. Means of three different experiments were plotted as a graph. *D*, MEF WT, Bax KO, Bak KO, and DKO cells were exposed to resveratrol (50 μ M) for the indicated time periods, the whole cell extract was prepared, and caspase-2 activity was analyzed by spectrofluorimetry as described. The -fold increase from control is shown. The whole cell extract was prepared for Western blot to detect caspase-2 as described. *E*, the above cell lines were pretreated with different caspase inhibitors for 1 h followed by resveratrol treatment (50 μ M) for 48 h and processed for annexin V staining as described. The results from three different experiments were plotted as a graph.

Bax/Bak, residual caspase activity may be enough to amplify mitochondrial apoptosis. Pretreatment with any of the caspase inhibitors fails to completely prevent cell death even in DKO cells (Fig. 7E). These observations suggest that both Bax and Bak are required for the cell death induced by resveratrol. However, the evidence suggests the existence of Bax/Bak-independent cell death also possibly through an uncharacterized death signaling

or via caspase-2- or caspase-8-dependent signaling independent of mitochondria (Fig. 8).

DISCUSSION

The experiments presented in this work provide evidence for the first time that the activation of caspase-2 is the major determinant for the

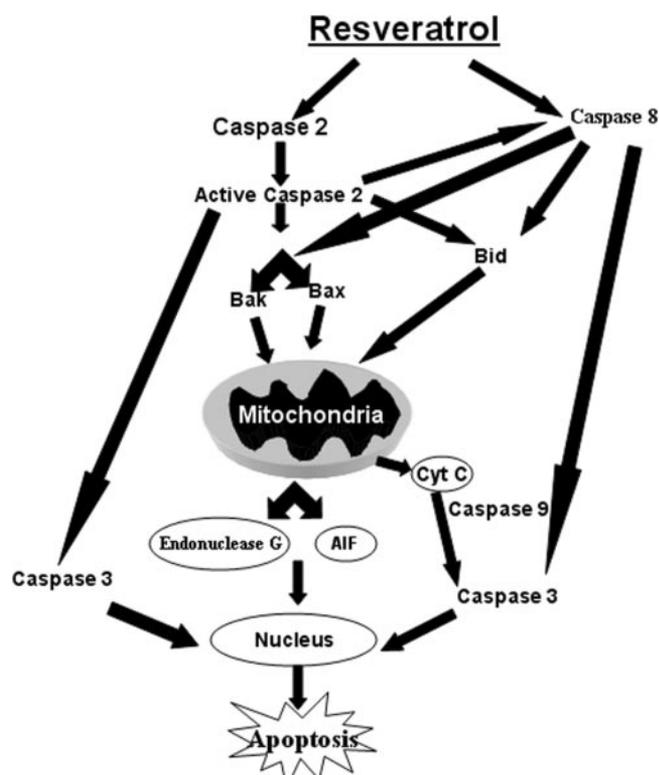


FIGURE 8. Schematic diagram showing apoptotic signaling induced by resveratrol. Resveratrol induces apoptosis by inducing a conformational change in Bax/Bak through activated caspase-2. Both caspase-2 and caspase-8 may proteolytically process Bid to amplify mitochondrial apoptosis. Caspase-2 or caspase-8 may also process downstream caspase-3 that may escape the mitochondrial apoptosis controlled at the level of Bax/Bak. A possible role for caspase-2 to process caspase-8 is also suggested for Bax/Bak-independent cell death. The caspase-independent death effectors AIF and endonuclease G released from mitochondria also mediate nuclear apoptosis.

cell death induction by resveratrol upstream of mitochondria and unravel the existence of Bax- and Bak-dependent as well as -independent death signaling. Release of cytochrome *c* from mitochondria into cytosol is recognized as the primary event of cell death signaling induced by genotoxic and other forms of cytotoxic insults. This event is mainly controlled at the level of Bcl-2 family proteins with an essential requirement of either Bax or Bak or both. The notion that most antitumor agents induce apoptosis by classical apoptotic signaling either through mitochondria or the death receptor pathway of apoptosis is challenged by several recent observations: flavopyridol induces p53 and caspase-independent apoptosis (38); resveratrol stimulates death receptor-independent caspase-8 processing (28, 39); cephalostatin 1-treated cells show a Smac (second mitochondria-derived activator of caspases)-mediated cell death independent of death receptor and cytochrome *c* or AIF release (40); and chlorophyllin induces AIF-mediated cell death independent of caspase-3 (41). These observations suggest that the very basic events of apoptosis differ from cell to cell and from one drug to another.

Resveratrol has been reported to induce caspase activation subsequent to mitochondrial release of cytochrome *c* (2, 4, 21), caspase activation independent of cytochrome *c* release (25), caspase-8-mediated Bax/Bak-dependent cell death (28), p53- or Bax-dependent and -independent cell death (26), ceramide-mediated cell cycle arrest and apoptosis (42), and death receptor (CD95)-dependent death signaling (38). Multiple and diverging death signaling events, all converging to apoptosis in different cells, suggest that resveratrol-induced cell death varies from cell to cell, indicating a possible role for regulatory molecules of apoptosis that govern the apoptotic threshold of a cell. In a variety of colon cancer cells, Bax/Bak-mediated cytochrome *c*-dependent caspase

activation has been reported as a contributing factor in resveratrol-induced cell death, involving FAS redistribution and processing of caspase-8 at the death-inducing signaling complex (28). However, the upstream events of mitochondrial apoptosis and whether Bax/Bak is absolutely necessary for death induction by resveratrol are not clear.

Our results in colon cancer cells (HCT 116) show the involvement of conformational changes in both Bax and Bak, followed by the release of cytochrome *c*, AIF, and endonuclease G together with the activation of caspase-2, -9, -3, and -8 in resveratrol-induced apoptosis. Except for the processing of procaspase-8, all other changes indicate a prominent role for mitochondria in resveratrol-induced cell death. Compared with cytochrome *c* release, the release of caspase-independent AIF and endonuclease G is slow and gradual with resveratrol treatment, suggesting that the kinetics of release of the latter molecules may need prior caspase processing or may be regulated independently. There is experimental evidence to support that caspase processing is required for the release of AIF and endonuclease G (36). The release of caspase-independent endonuclease G and AIF, in the later stages of cell death, indicates the potential use of this compound in bypassing drug resistance, where impaired caspase processing is common as is reported for tumors overexpressing inhibitors of apoptosis proteins or heat shock proteins (43–45). Our studies suggest that the heat shock transcription factor HSF1 and the heat shock proteins 70 and 90 are not modulated by resveratrol.

Studies by the group led by Aggarwal suggest that NF- κ B (involved in cell proliferation and cell survival) is also an important target of resveratrol both *in vitro* and *in vivo* (8, 10). Their *in vitro* experiments suggest that anticarcinogenic, anti-inflammatory, and growth-modulatory effects of resveratrol are associated with its ability to inhibit activation of NF- κ B, AP1, and associated kinases (8). Later, they elaborated the potential of resveratrol to suppress 7,12-dimethylbenz(*a*)anthracene-induced mammary carcinogenesis involving down-regulation of NF- κ B cyclooxygenase-2 and matrix metalloproteinase-9 expression (10). These studies substantiated *in vivo* preclinical efficacy of resveratrol and suggest that resveratrol can be considered as a candidate drug for cancer therapy. However, one recent study suggests that resveratrol can prevent hydrogen peroxide-induced apoptosis in rat pheochromocytoma cells (46). A lower concentration of resveratrol is also reported to inhibit caspase activation and release of cytochrome *c* induced by H₂O₂ (47). The strong antioxidant activity of the compound is attributed for its NF- κ B inhibition. Our studies involving p65 GFP-transfected cells and NF- κ B reporter gene assay suggest that resveratrol is a strong inhibitor of NF- κ B activation induced by both H₂O₂ and PMA, consistent with earlier reports. The antioxidant, NAC, is also equally effective in preventing NF- κ B activation. However, caspase-2 activation is not altered either by NAC or cotreatment with PMA or H₂O₂, suggesting that caspase-2 activation observed with resveratrol is independent of its NF- κ B inhibition. Moreover, overexpression of p65 or transfection with a superrepressor form of I κ B that normally blocks NF- κ B activation also fails to alter the caspase-2 processing. Further studies are needed to clarify whether downstream events of apoptosis are influenced by the inhibition of NF- κ B in resveratrol-treated cells.

Pretreatment of caspase-8 inhibitor or CrmA transfection or RNAi silencing of caspase-8 slightly reduced cell death and Bid DsRed translocation to mitochondria induced by resveratrol. It appears that both caspase-2 and caspase-8 contribute for the translocation of Bid to mitochondria in resveratrol-treated cells, since either caspase-2 or caspase-8 inhibitor alone was not enough to prevent the translocation of Bid to mitochondria. This is in agreement with the recent observation that caspase-2 can process Bid in a cell-free system (48). However, one

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recent study suggests that proteolytic activation of Bid and subsequent induction of mitochondrial apoptotic pathway through Bax/Bak is essential for apoptosis triggered by caspase-2 (49). Although several groups reported the activation of caspase-8 with resveratrol, the exact mechanism behind the processing is controversial. Classical death receptor processing involving up-regulation of FasL mRNA and FasL was reported in HL60 and T47D cells (37). However, subsequent experiments failed to substantiate classical death receptor processing in other cells (27). Caspase-8 can also be activated downstream of executioner caspases like caspase-3 (50). Caspase-2 inhibitor capable of reducing caspase-3 processing with resveratrol fails to prevent caspase-8 processing, indicating that caspase-3 or downstream caspases are not involved in caspase-8 activation. The clustering of FAS and its redistribution in cholesterol and sphingolipid-rich fraction together with FADD and procaspase-8 seems to be the trigger for caspase-8 processing by resveratrol (28).

The well established notion that caspase-9 is the initiator caspase was recently challenged by several lines of evidence that suggest caspase-2 as the prominent initiator caspase upstream of mitochondria in a p53-dependent way (29–31). A cytochrome *c*-independent processing of caspase-9, -2, -3, and -6 were reported in T-acute lymphoblastic leukemia cells treated with resveratrol (25). However, the mechanism of caspase-2 activation upstream of mitochondria is not well defined. In a recent work, activation of caspase-2 within a complex involving PIDD (p53-induced death domain-containing protein) and the adaptor protein RAIDD has been noted (51). However, it is not very clear how resveratrol induces caspase-2, and further studies are needed to know whether resveratrol modulates the expression level of PIDD or RAIDD in this cell line. Ceramide, etoposide, and other genotoxic agents are known to be the inducers of caspase-2 (52). Ceramide generation with resveratrol reported in MADA-MB-231 breast cancer cells may also be a possible trigger for caspase-2 activation (42).

Our experiments suggest that apart from caspase-8, caspase-2 also contributes to the conformational changes in Bax/Bak. Consistent with earlier reports, the absence of Bax fails to block apoptosis significantly in HCT 116 cells, deficient only for Bax, possibly indicating a role for Bak in substituting Bax function. However, we failed to see any marked difference in cell death even in Bak-silenced Bax knock-out cells, suggesting the involvement of Bax/Bak-independent cell death (data not shown). The results from MEF knock-out cells for both Bax and Bak substantiated the involvement of Bax/Bak-independent cell death and processing of caspase-2. The cell death was reduced in Bax, Bak, and double knock-out MEF cells but not completely prevented even in cells where both Bax and Bak are knocked out. It is reasonable to believe that caspase-2-mediated downstream caspase processing also contributes to cell death independent of mitochondria. A possible role for activated caspase-2 to target caspase-8 cannot be ruled out, since caspase-2 inhibitor also reduced processing of caspase-8 in these cells. One recent study suggests that activated caspase-2 can process procaspase-8 monomers between the large and small subunits, thereby priming cancer cells for TRAIL-mediated apoptosis (53). The possible role of lysosomes or cathepsins in caspase-2-mediated death processing independent of mitochondria needs to be clarified. Further studies are needed to unravel the role of cathepsins in resveratrol-induced cell death through the possible route of caspase-2 or caspase-8. The presence of detectable intracellular caspase activation in double knock-out Bax/Bak cells suggests a possible processing of executioner caspases by either caspase-2 or by caspase-8 without engaging the apoptosome or mitochondria. Further studies are needed to substantiate whether the activation of caspase-2 observed in the absence of Bax/Bak with resveratrol is a gen-

eral feature or unique to resveratrol, in light of the recent observation that initiator caspases are not activated in the absence of both Bax/Bak with DNA damage signals (54).

Evasion of apoptotic signaling subsequent to loss or deregulation of components of apoptotic machinery is the primary reason for clinical drug failure. Classical apoptosis inducers often fail in the treatment of such tumors. Newer generation plant-derived active compounds with a capacity to induce unique forms of cell death differing from the classical apoptosis signaling may act as alternatives to circumvent such drug resistance. The processing of caspase-8 independent of death receptor; the release of caspase-independent death effectors like AIF, endonuclease G from mitochondria, Bax, and Bak-independent death signaling, etc. indicate that resveratrol can be considered as an ideal candidate agent to circumvent inherent drug resistance. It is increasingly felt that such compounds are not potent as standard antitumor agents and require much higher concentration to eliminate tumors both *in vitro* and *in vivo*. However, they can be used as potent agents to bypass drug resistance along with other antitumor agents that primarily act through classical apoptosis. A study in this line suggests that resveratrol is an ideal candidate agent to enhance the apoptotic potential of antitumor agents like TRAIL and paclitaxel (55, 56). More preclinical studies are needed to clarify whether 50 μM concentration required for the desired biological effect can be achieved *in vivo*. Studies in animal models suggest that resveratrol is well absorbed and glucuronidated and sulfated both in the liver and intestinal epithelial cells. An oral dose of 50 mg/kg body weight of resveratrol in rat yielded peak plasma levels of unmetabolized resveratrol (6.6 μM) and its conjugate resveratrol glucuronide of 105 μM (57). The remarkable *in vivo* potency of the drug was demonstrated in a rat carcinogenesis model with a very low dosage starting from 200 $\mu\text{g}/\text{kg}/\text{day}$ to 2 mg/kg (10, 19). Further studies are ongoing in our laboratory to understand whether resveratrol can be used to bypass acquired drug resistance due to altered Bcl-2 family proteins or inhibitors of apoptosis protein with interventional approaches targeting other apoptotic regulatory molecules like heat shock proteins.

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