ERK1/2 activation is required for resveratrol-induced apoptosis in MDA-MB-231 cells

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Abstract. Resveratrol (RSVL), a phytoalexin found in abundance in grapes and other grape-related products, has been shown to be antiproliferative and protective against various types of cancers, including breast cancer. However, the precise underlying mechanisms are not well understood. In this study, we show that treatment with RSVL induces growth inhibition and apoptosis in a highly invasive and metastatic breast cancer cell line MDA-MB-231. Cleavage of caspase-3 and PARP and fragmentation of DNA were observed following exposure to RSVL. Co-treatment with pan-caspase inhibitor completely prevents cell death induced by RSVL. We found that RSVL-induced apoptosis correlates with sustained activation of ERK1/2 and suppression of Bcl-2 expression. Inhibition of ERK1/2 activation by its specific inhibitor or small interfering RNA reverses the effect of RSVL on Bcl-2 suppression and inhibits apoptosis, while overexpression of MEK1, which is directly upstream of both ERK1 and ERK2, enhances apoptosis induced by RSVL. Moreover, ERK1/2 was found to act upstream of caspase-3 to induce apoptosis, while it was not directly involved in caspase-3 cleavage. The other closely related MAPK members, p38 and JNK are not involved in apoptosis induced by RSVL in MDA-MB-231 cells. These results suggest that activation of ERK1/2 is required for RSVL-induced apoptosis in MDA-MB-231 cells.

Introduction

Breast cancer is one of the most common malignancies and a frequent cause of death in women worldwide (1). Although early stage breast cancer is well managed by surgery and conventional treatments, there are few curative options beyond early detection. The metastasis rate found at presentation remains high and many of those without initial apparent clinical metastasis ultimately develop distant metastases (2). Metastasis has always been the most serious turning point of breast cancer since the majority of women with metastasis will die of their disease (3). Therefore, new treatments for breast cancer, especially invasive tumors, are needed.

Naturally occurring compounds have recently attracted attention since nutrition and diet have been found to influence the development and progress of breast cancer (4). A number of natural substances, particularly those existing highly in our daily diet, have been reported to prevent cancer development and inhibit cell growth (5,6). Resveratrol (RSVL) is one such dietary natural compound that has chemopreventive properties and inhibitory effects on cancer cells both in vitro and in vivo (7-9; reviewed in ref. 10). RSVL, found abundantly in grapes and grape-related products, was shown to inhibit cancer initiation, promotion and progression (7). As it is structurally similar to diethylstilbestrol, many studies have suggested that RSVL binds to and inhibits estrogen receptor (ER)α (11,12). However, RSVL has been shown to have growth inhibitory effects on ERα negative cell lines and blocking ERα does not prevent its anti-proliferative action, suggesting that antagonism of ERα is not a unique mechanism for its growth inhibitory effects (13). Although it has been shown RSVL exerts its inhibitory effects by inducing cell cycle arrest and apoptosis in a variety of cancer cell lines and more importantly in animal models of carcinogenesis (7,8,14-16), the mechanisms by which RSVL exerts its anti-tumor effects on highly metastatic and unresponsive breast cancer cells such as MDA-MB-231 (MDA231) are not fully understood.

Recent studies suggested that signal transduction pathways via ERK signaling play important roles in the formation, progression and metastasis of breast cancer (17,18). The Ras/MEK/ERK pathway regulates mammalian cell growth by conveying extracellular signals to the nuclear via a cascade of specific phosphorylation events which begin with Ras activation and center on ERK activation (18-20). Aberrant signaling through the ERK pathway could promote cell...
immortalization via telomerase induction, growth factor-independent proliferation by cell cycle activation, invasion and metastasis via stimulation of cellular motility and extracellular matrix remodeling (17,20). Highly metastatic breast cancer cell lines, such as MDA231 and MDA-MB-468, have constitutively higher ERK activity when compared to the non-invasive MCF-7 breast cancer cells and enforced induction of ERK activation enhances the mobility and invasion of these cells (18). Interestingly, recent studies implicate that activation of ERK may play a role in cancer treatment (reviewed in ref. 21). However, the available data are contradictory. While inhibition of ERK1/2 signaling leads to cell death, many recent studies suggest that ERK activation induced by anticancer drugs might be required for growth inhibition and apoptosis (22-24), prompting us to investigate the role of ERK activation in cell death induced by RSVL in the MDA231 metastatic breast cancer cells.

Targeting apoptosis appears to be a promising choice in cancer treatment. Regulation of apoptosis is a complex process involving many cellular genes, including Bcl-2 and Bcl-2 related family members such as Bcl-XL, Bax and Bad. The balance between anti-apoptotic and pro-apoptotic proteins of the Bcl-2 family has been shown to play a critical role in maintaining cell survival. ERK activity has long known to be pro-survival because of its ability to inhibit pro-apoptotic proteins (25). However, other studies suggest ERK could also have possible pro-apoptotic properties upon activation following drug treatment by promoting the activities of pro-apoptotic proteins such as p53 and Bak (reviewed in ref. 21).

In this study, we show that RSVL inhibits cell growth and proliferation by mainly causing apoptosis in the ERα negative, highly metastatic breast cancer cell line MDA231. RSVL-induced apoptosis correlates with the activation of ERK1/2 and suppression of Bcl-2 expression. Inhibition of ERK activity by small molecule inhibitor or small interfering RNAs almost completely inhibits apoptosis and reverses Bcl-2 suppression, while enforced activation of ERK renders cells more sensitive to apoptosis induced by RSVL. Thus this is the first demonstration that ERK1/2 is required for apoptosis induced by RSVL in MDA231 cells.

Materials and methods

Reagents. Resveratrol (RSVL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4,6-diamidino-2-phenylindole (DAPI), bovine cytochrome c and dATP were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). MEK1/2 specific inhibitor U0126 were purchased from Cell Signaling Technology (Beverly, MA, USA). The pan-caspase inhibitor (Z-VAD-FMK) and p38 MAPK kinase inhibitor SB203580 were obtained from Calbiochem (La Jolla, CA, USA). c-Jun N-terminal kinase specific inhibitor SP600125 was kindly provided by Professor Natalie Ahn (University of Colorado, Boulder, CO, USA). HA-MEK1 (R4F) (17), was kindly provided by Professor Natalie Ahn (University of Colorado, Boulder, CO, USA). Pre-designed ON-TARGET plus SiRNA duplexes against ERK1 and ERK2, control (scrambled) SiRNA duplexes and Dharmacon transfection reagent were purchased from Dharmacon, Inc. (Lafayette, CO, USA). All ERK1, ERK2 and scrambled SiRNAs represented a mixture of four distinct RNA duplexes (smart pool). Hygromycin was purchased from Invitrogen (Carlsbad, CA, USA). RNeasy Protect Mini Kit, One-Step RT-PCR kit and SuperFect Transfection Reagent was obtained from Qiagen Inc. (Germany).

Cell culture and drug treatment. Human MDA-MB-231 (MDA231) cell line was obtained from ATCC and cultured in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 2 mM L-glutamine (growth medium) at 37°C in a 5% CO2 incubator. It has been documented that MDA231 cells are ERα negative, highly invasive and metastatic, express mutant-inactive p53, and have high activity of MAPK (16,18).

For SiRNA and stable transfection, 200,000 cells were seeded in 6-well plate 24 h, serum starved for 6 h before transfection. For treatment with RSVL and inhibitors, cells were seeded in growth medium at the same initial density, and were allowed to adhere and grow overnight (70-80% confluency). Cells were washed once with serum-free DMEM media (SFM) and serum starved for 6 hours (h) before treatment with indicated concentrations of RSVL in SFM. Control cells were treated with vehicle (0.1% DMSO). For combined treatment, inhibitors were added 1 h before RSVL.

Determination of cell growth rate and cell viability. The cell growth rate was determined by qualitative measurement of the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a water-insoluble formazan by the mitochondrial dehydrogenase enzyme in metabolically active cells. Cell growth rate was determined at 24 and 48 h after treatment using MTT assay as previously described (26). Cell viability was determined using trypan blue (0.4% in phosphate-buffer saline (PBS)) exclusion assay. Briefly, after 24 and 48 h of treatment, floating cells were collected by centrifuging the media. Adhered cells were trypsinized and pooled together with floating cells. An aliquot of suspension cells was mixed with an equal volume of trypan blue and counted by a light microscope. Dead cells were defined as those stained blue with the dye.
Analysis of nuclear staining with DAPI. Cells were treated with vehicle, 1, 10 and 50 μM of RSVL for 24 h. After treatment, cells were fixed with 3.7% formalin at room temperature for 1 h, washed with PBS and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml in methanol) for 5 min at 37°C. Cells were washed, examined and photographed using a Leica DC3000F fluorescence microscope equipped with a UV light filter. Apoptotic cells were defined on the basis of chromatin condensation and nuclear fragmentation (bead-like formation). Apoptotic percentage was obtained by counting the number of apoptotic cells among at least 500 cells/region. The data were expressed as the mean ± SD.

Western blot analysis. Treated cells were harvested at indicated times and lysed in a lysis buffer containing 75 mM NaCl, 10 mM Tris (HCl), 1 mM EDTA, 0.5% NP40, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na3VO4 and protease inhibitor cocktail (Pierce Biotechnology). Protein concentration was determined by the method of Bradford using protein assay kit (Bio-Rad). Proteins (30 μg) per sample were subjected to Western blot analysis. Blots were incubated with the antibodies specific for either cleaved caspase-3, cleaved PARP, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, phospho-Akt-1 (Thr308 and Ser473), Akt-1, phospho-p38 (Thr180/Tyr182), p38, phospho-JNK (Thr183/Tyr185), JNK, phospho-Bad (Ser112 and Ser136), Bad, Bax, phospho-Bcl2, Bcl-2, truncated Bid, Bim, Bcl-XL, cytochrome c, PI-3K and 1:7500 horseradish peroxidase-conjugated rabbit anti-mouse or mouse anti-rabbit secondary antibodies (Pierce Biotechnology). All the primary antibodies were used at the final concentration of 1 μg/ml. To detect phosphorylated proteins, the same blots were probed with phospho-specific antibodies, stripped and reprobed with antibodies against total proteins. All blots were incubated with mouse antibody against β-actin or α-tubulin to confirm equal protein loading. Bands were visualized with a Chemiluminescent Reagent Plus detection system as described by the manufacturer. For quantitation analysis of Western blotting, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated and normalized to the amount of β-actin using ImageJ software (National Institute of Health, USA). After normalisation to β-actin, the expression level of proteins under study between the samples was expressed in the fold changes.

SiRNA transfection. Pre-designed ON-TARGET plus SiRNA duplexes against ERK1/2 and control (scrambled) SiRNA duplexes were purchased from Dharmacon, Inc. (Lafayette). Both scrambled and ERK1/2 SiRNAs represented a mixture of four distinct RNA duplexes (Smart pool, Dharmacon). Cells were seeded in 6-well plates and transfected with 100 nM of SiRNAs using DharmaFECT reagent in antibiotic-free conditions according to the manufacturer’s instructions. At 6 h after transfection, cells were washed by DMEM media and supplemented with fresh DMEM containing 5% FCS. The cells were incubated 24 h prior to treatment. The transfected cells were then treated with either vehicle or indicated concentrations of RSVL for 24 h and harvested for Western blot analysis.
Statistical analysis. Each experiment was performed at least 3 times. Statistical significances between groups were determined by ANOVA test. A p<0.05 was considered significant.

Results

To investigate if RSVL had any growth inhibitory activity against highly metastatic MDA231 breast cancer cells, cells were treated with 0.1, 1, 10, 50 and 100 μM of RSVL for 24 and 48 h. Cell proliferation rate and cell viability were assessed by MTT and trypan blue exclusion assays. RSVL induced a concentration-dependent reduction in cell viability and proliferation, which was maximal at 10-100 μM (Fig. 1). At 10 μM, 20±3.4% and 40±4.1% reductions in cell proliferation were observed at 24 and 48 h, respectively, as assessed by MTT assay (Fig. 1A). Trypan blue exclusion assay, which gives a direct count of cells that exclude trypan blue dye (including apoptotic cells), shows a more significant and clearer effect on cell viability; 50±3.4% and 69±3.1% reductions in cell viability were observed at 24 and 48 h, respectively (Fig. 1B). A significant reduction in cell proliferation and viability was observed as early as 24 h after treatment (p<0.01). The maximal effects were seen at higher concentrations of RSVL, where almost all cells were dead after 48 h. At low concentrations from 0.1-1 μM, RSVL has no significant effect on cell viability and proliferation. Concentrations of 50 and 100 μM of RSVL showed an almost equal inhibitory effect on cell growth. 10 and 50 μM concentrations of RSVL were chosen for subsequent experiments.

To determine if RSVL inhibits cell proliferation and viability by inducing apoptosis in MDA231 cells, DNA-specific fluorescent DAPI staining was performed. Morphological apoptotic features were characterized by condensed chromatin and fragmented nuclei. Apoptotic cells were indeed detected in RSVL-treated cells. Vehicle and 1 μM of RSVL treated cells exhibited round nuclei with well-distributed chromatin while apoptotic cells were observed with condensed chromatin and fragmented DNA after 24 h exposure to RSVL. RSVL significantly induced apoptosis at 10 μM (14.2±1.3%) and 50 μM (70.2±2.1%) (p<0.01) (Fig. 1C).

To examine if the apoptosis machinery is activated by RSVL treatment, we used specific antibodies against cleaved caspase-3 and cleaved PARP. RSVL induced the cleavage of caspase-3 and PARP in a dose-dependent manner, where the 17 and 20 kDa cleaved caspase-3 fragments and 89 kDa cleaved PARP product (Fig. 2A) were detected in samples treated with
10, 50 and 100 μM of RSVL. The data indicate that RSVL activates caspase-3 and further confirms the observation of DNA morphological changes after RSVL treatment.

As programmed cell death in mammalian cells has been shown to be regulated by the balance of proapoptotic proteins such as Bad, Bax and anti-apoptotic proteins like Bcl-2 and Bcl-XL (28), we determined if RSVL-induced apoptosis in MDA231 cells was also regulated by these proteins. As shown in Fig. 2A, no significant change in the expression of Bcl-XL, Bax and Bad was observed following RSVL exposure. We also observed that Bad was not phosphorylated at Ser112 and Ser136 in MDA231 cells using anti-phospho-specific antibodies (data not shown). However, treatment of RSVL significantly suppressed Bcl-2 expression in a dose-dependent manner, suggesting that the suppression of Bcl-2 might be responsible for cell death induced by RSVL.

The Raf/MEK/ERK signaling pathway has been shown to play important roles in cell invasion and metastasis (18,20), but activation of this pathway has also been implicated in the response of cancer cells to drug treatment (reviewed in ref. 21). RSVL has recently been shown to activate ERK signaling in breast cancer cells through a receptor within integrin αVβ3 (29). We wished to investigate if RSVL-induced apoptosis was also mediated by the Raf/MEK/ERK pathway in highly metastatic MDA231 cells. Treatment with RSVL up-regulated phosphorylated ERK1/2 (mainly ERK2) in a dose-dependent manner. Interestingly, the increase of phosphorylated ERK1/2 was observed concurrently with cleavage of caspase-3 and PARP (Fig. 2A). The other two main members of MAPK family, JNK and p38 MAPK were also investigated. As shown in Fig. 2B, there were no clear changes in the expression and activation of these kinases and phospho-p38 MAPK was not detectable under the experimental conditions (data not shown), suggesting that JNK and p38 MAPK may not be activated by RSVL in MDA231 cells.

Since RSVL has been reported to inhibit Akt signaling in prostate cancer cell line LnCap (30), we studied if RSVL has any effect on the PI-3K/Akt pathway in MDA231 cells. As
shown in Fig. 2B, RSVL did not produce a significant effect on the expression level of the p85 subunit of PI-3 kinase. The same blot was probed with antibodies against phosphorylated Akt-1 (Ser473 and Thr308) showing that these phosphorylation sites of Akt-1 are undetectable under the experiment conditions (data not shown) and no significant change in the expression of Akt-1 was observed. The data suggest that PI-3K/Akt pathway may not play a major role in apoptosis induced by RSVL in MDA231 cells.

To study the time course of ERK activation and apoptosis, MDA231 cells were treated with either vehicle or 50 μM of RSVL and were harvested at indicated time-points (1, 3, 6, 8, 10, 12, 16 and 24 h) for Western blot analysis. Significant activation of ERK was observed only after 10-h exposure to RSVL and remained highly activated through the course of experiment (24 h). Cleavage of PARP was detected after 16 h and detected at high levels after 24 h of treatment. Suppression of Bcl-2 occurred as early as 10 h after RSVL treatment (Fig. 3A), when apoptosis is not detected. This suggests that the sustained activation of ERK and suppression of Bcl-2 may trigger apoptosis induced by RSVL.

To study the role of apoptotic cell death induced by RSVL, cells were pretreated with the pan-caspase inhibitor Z-VAD-FMK followed by RSVL treatment. Pretreatment with Z-VAD-FMK resulted in complete blockage of inhibitory effects of RSVL on cell growth (data not shown) and cleavage of caspase-3 and PARP (Fig. 3B), indicating that apoptosis is the main mechanism of cell death following RSVL treatment. Although co-treatment with caspase inhibitor inhibited apoptosis induced by RSVL, it did not affect phosphorylation of ERK1/2 (Fig. 3B). The data further suggest that ERK1/2 acts upstream of caspase-3 to exert its apoptotic influence on the RSVL-treated MDA231 cells.

To investigate if RSVL-induced apoptosis is mediated by ERK activation, MDA231 cells were treated with UO126, a well-known inhibitor of MEK1/2, a signaling molecule directly upstream of ERK1/2, to block ERK1/2 activation and its downstream substrates. Nuclear staining with DAPI showed that RSVL treatment induced DNA condensation and fragmentation by 69.4% while co-treatment of UO126 and RSVL significantly reduced the DNA fragmentation (to 11.3%, p<0.01) (Fig. 4A). The results were further strengthened by Western blot data, which show that RSVL at 10 and 50 μM alone increased ERK1/2 phosphorylation, caspase-3 and PARP cleavage, while co-treatment with UO126 almost completely blocked the activation of ERK1/2, and cleavage of caspase-3 and PARP induced by RSVL (Fig. 4B). To further investigate the possible mechanism of this prevention of apoptosis, the same blot was used to probe with Bcl-2, Bcl-XL, Bax, Bad and Bim antibodies. Interestingly, blocking MEK/ERK signaling does not only abrogate the actions of RSVL to suppress Bcl-2, but in fact increases the protein level of Bcl-2.

Figure 3. Time course and apoptosis induced by RSVL in MDA231 cells. A, Time-dependent phosphorylation of ERK1/2 and effects of RSVL on PARP cleavage and Bcl-2 expression. Cells were cultured and treated with either vehicle or 50 μM of RSVL for indicated times. Cells were harvested and lysed for Western blot analysis as described in Materials and methods. The blots were probed with specific antibodies against phospho-ERK1/2, ERK1/2, cleaved PARP and Bcl-2. The blots represented the time-points that significant change of phospho-ERK1/2 was observed. B, Effect of Z-VAD-FMK on RSVL-induced apoptosis. Cells were treated with either vehicle or 50 μM of RSVL or combination of 50 μM of Z-VAD-FMK and 50 μM of RSVL for 24 h. Cells were harvested and lysed for Western blot analysis. Blots were probed with antibodies against phospho-ERK1/2, cleaved caspase-3 and cleaved PARP. Representative blots of three experiments with similar results are shown.
Neither RSVL nor UO126 treatment had any effect on the levels of Bax and Bad expression. Bim was not detectable under experiment conditions (data not shown). The data indicate that activation of ERK1/2 is critical for apoptosis induced by RSVL and its possible role in suppressing Bcl-2 expression.

Since it has been reported that Bcl-2 is located on the outer membrane of mitochondria and plays an important role in the release of cytochrome c, which is necessary for the initiation of the apoptotic process (31), we hypothesized that RSVL may induce apoptosis by regulating Bcl-2 expression through ERK1/2 activation and thus the upregulation of Bcl-2 following incubation with UO126 may be involved in the release of cytochrome c. To address this hypothesis, cytosolic proteins (S-100) fractions extracted from cells treated with 50 μM of RSVL and 10 μM of UO126 both alone or in combination were subjected to Western blotting to detect cytochrome c and cleaved caspase-3. D. Induction of in vitro apoptosis using dATP and bovine cytochrome c. S-100 fractions (50 μg) from vehicle- or UO126-treated cells were incubated with dATP (1 mM) and/or bovine cytochrome c (0.3 μg) and subjected to Western blotting for detection of caspase-3 cleavage and phospho-ERK1/2. Experiments were repeated three times with similar results.
To address the question if ERK1/2 may directly affect caspase-3 cleavage as previously suggested (reviewed in ref. 21), S-100 fractions extracted from the vehicle or UO126-treated cells (for 3 h) were used to induce in vitro apoptosis using dATP and cytochrome c. As shown in Fig. 4D, phosphorylated ERK1/2 were undetectable after 3-h treatment with UO126 and in the presence of dATP and cytochrome c, cleavage of caspase-3 was observed virtually equally in both S-100s of vehicle and UO126 treated cells. Cleavage of caspase-3 was observed at a low level when only cytochrome c was added to the cytosol from UO126 treated cells. The results suggest that ERK1/2 may not directly affect caspase-3 cleavage during apoptosis.

Recent studies suggested that biological actions previously thought to be responsible by ERK1/2 might, in fact, be mediated by MEK5/ERK5 signaling as UO126 and PD98059, another MEK1 inhibitor, inhibit ERK1/2 as well as ERK5 (32,33). To determine if ERK1/2 activation is specifically required for apoptosis induced by RSVL, specific knockdown of ERK1/2 by siRNA was employed for this study. As predicted, silencing ERK1/2 inhibits the cleavage of caspase-3 and PARP (Fig. 5A). Furthermore, upregulation of Bcl-2 protein was also observed when ERK1/2 was silenced. As phosphorylation of Bcl-2 at Ser70 has been shown to be critical for its anti-apoptotic functioning (34), we wished to study this specific phosphorylation site. The same set of samples was blotted with phospho-Bcl-2 (Ser70) antibody. The result showed that knockdown of ERK1/2 upregulated phospho-Bcl-2 (Ser70) (Fig. 5A). Interestingly, RT-PCR of Bcl-2 showed that inhibition of ERK1/2 activity by UO126 (data not shown) or siRNA slightly reduced the mRNA level of Bcl-2 (Fig. 5B), suggesting that abrogation of ERK1/2 activity might induce phosphorylation of Bcl-2 at Ser70 and this, in turn, stabilizes the Bcl-2 protein. Since previous studies including ours have suggested that duration and amplitude of ERK activation may be important in apoptosis induced by anticancer drugs (22,23,26), we determined if the intensity of this constitutively active signaling would affect cell sensitivity to RSVL. MDA231 cells were stably transfected with either empty vector pMCL (mock) or
pMCL-HA-MEK1 (R4F), which constitutively expresses MEK1 and activates both ERK1 and ERK2 (17). The mock transfected and two clones of constitutively activated MEK1-transfected cells (R4F12 and R4F18, which have the high expression level of MEK1 and phospho-ERK1/2) were treated with either vehicle, 10 or 50 μM of RSVL for 20 h and harvested for Western blot analysis. Fig. 6 shows that at 20-h incubation, a very low level of cleaved PARP was detected in RSVL-treated mock-transfected MDA231 cells by Western blotting, while much higher level of and cleaved PARP was detected in the two MEK1-transfected clones R4F12 (Fig. 6A) and R4F18 (Fig. 6B). Especially, at the low concentration of 10 μM of RSVL, significant amounts of cleaved caspase-3 and cleaved PARP were seen in the RSVL-treated MEK1-transfected cells by Western blotting, while much higher level of and cleaved PARP was detected in the two MEK1-transfected clones R4F12 (Fig. 6A) and R4F18 (Fig. 6B). Especially, at the low concentration of 10 μM of RSVL, significant amounts of cleaved caspase-3 and cleaved PARP were seen in the RSVL-treated MEK1-transfected cells, which were not clearly detected in the RSVL-treated mock-transfected cells. Combined treatment of UO126 and RSVL inhibited PARP cleavage and DNA fragmentation (data not shown). In addition, MEK1-transfected cells exhibited more DNA fragmentation than the mock-transfected cells when treated with RSVL (data not shown). These data further support the critical role of activation of MEK/ERK pathway in RSVL-induced apoptosis in MDA231 cells.

**Discussion**

In this study, we demonstrated that RSVL inhibits cell proliferation and induces apoptosis in highly invasive breast cancer MDA231 cells and this is critically regulated by the activation of ERK1/2. Our data suggest that in addition to promoting apoptosis, ERK1/2 activities could play an important role in regulating Bcl-2 expression through phosphorylation that tilts the balance of mitochondrial anti-apoptotic and pro-apoptotic proteins and eventually leads to cell death induced by RSVL in MDA231 cells.

RSVL has been shown to inhibit cell growth and proliferation in a variety of cancer cell lines by causing cell cycle arrest and apoptosis (13,15,16,29). Our study aimed at elucidating the possible mechanisms of apoptosis induced by RSVL in MDA231 highly metastatic breast cancer cells. Two major distinct apoptotic pathways for mammalian cells have been described (35). A previous study suggested that RSVL could trigger death receptors by clustering of Fas and its redistribution and result in activation of the initiator caspase-8, which can propagate the apoptosis signal by direct cleavage of downstream effector caspses such as caspase-3 in other cells (36). However, we did not observe any significant change in caspase-8 and Bid expression. Cleaved caspase-8
and truncated Bid were also not detected (data not shown). Therefore, it is unlikely that apoptosis in MDA231 cells was initiated by triggering death receptors. We did not observe a significant difference in Bad expression and its phosphorylated forms at Ser112 and Ser136, suggesting that Bad may not be important for RSVL-induced apoptosis in MDA231 cells, neither was any difference in Bax expression detected. Although the precise role of Bax was not examined in this study, Bax redistribution from its predominantly cytosol location to mitochondrial membrane, but not its expression has been shown to play a role in inducing the release of cytochrome c (37). Moreover, administration of RSVL significantly suppresses the anti-apoptotic protein Bcl-2.

Overall, there is a shift in the ratio of pro-apoptotic and anti-apoptotic proteins following RSVL treatment. It is possible that inhibition of Bcl-2 by RSVL allows more Bax to translocate to the mitochondrial membrane and induces the membrane permeability transition, a critical event for apoptosis (reviewed in ref. 38). The suppression of Bcl-2 also allows more free apoptosis-activating factor (Apa1-1) to trigger the release of cytochrome c, which is required to activate the two death proteases, caspases-9 and -3 (39). Two closely related proteins of Bcl-2 were observed unchanged (Bcl-XL) or undetectable (Bim). Although contribution of other anti- and pro-apoptotic proteins was not examined in this study, the evidence of apoptosis and suppression of Bcl-2 could suggest that there may be a shift that tilts the balance between the anti-apoptotic and pro-apoptotic proteins favoring apoptosis following RSVL treatment. Although the relationship between Bcl-2 and ERK activation has been implicated in many studies and growth factor-regulated pro-survival ERK1/2 has been shown to upregulate Bcl-2 expression, it is not known if proapoptotic activity of ERK1/2 regulates Bcl-2 functions. In this study, suppression of Bcl-2 by RSVL is reversed by inhibition of ERK1/2 by both U0126 and SiRNA. Moreover, inhibition or knockdown of ERK1/2 slightly decreases the transcript level of Bcl-2 but increases the Bcl-2 protein and its phosphorylated form at Ser70, suggesting that the posttranslational modification at Ser70 may render Bcl-2 more stable and thus enhance its anti-apoptotic function. The data are supported by previous study showing that phosphorylation of Bcl-2 at Ser70 is required for anti-apoptotic functioning of Bcl-2 (34). The data also suggest ERK1/2 activation by RSVL contributes to the suppression of Bcl-2 function. This result is similar to that observed by Xu et al that transfection of dominant negative ERK2 reversed the Bcl-2 suppression elicited by many highly effective anti-tumor iso(thio)cyanates in prostate cancer cells (41). It remains to be determined how ERK inhibition upregulates posttranslational modification of Bcl-2 at Ser70 and if ERK1/2 activation during RSVL treatment affects the degradation of Bcl-2.

Previous studies have demonstrated that the other two closely related members of ERK1/2, p38 MAPK and SAPK/JNK might be associated with cell proliferation or apoptosis depending on the cell types and stimuli (41-44). In this study, we did not observe any significant change of p38 MAPK and JNK activities after RSVL treatment in MDA231 cells. Moreover, our unpublished data showed that pretreatment with specific p38 MAPK inhibitor or JNK inhibitor neither prevented nor enhanced significantly apoptosis induced by RSVL. Therefore, it is unlikely that p38 MAPK or JNK signaling participates in RSVL-induced cell death of MDA231 cells.

Our study demonstrates that ERK1/2 activation plays a critical role in the cleavage of executive caspase-3 and PARP, which is central in the process of cell death induced by RSVL in MDA231 cells. It is important to note that there are different mechanisms of ERK activation. While ERK activation induced by growth factors could be rapid (occurring within minutes) and transient, it can also be sustained depending on the stimuli. With RSVL, no significant activation occurs until 10 h, and the activity remains highly elevated through the remaining time period examined (24 h). It is worthy to note that cells were treated in SFM conditions in which no interfering factors, which are usually found in the serum, would affect the ERK pathway. It has been reported that RSVL binds to cell surface receptor integrin αvβ3 and activates ERK signaling through this receptor in breast cancer cells (29). Activation of ERK1/2 occurred before apoptosis could be detected and remains highly activated while cells were undergoing the intensified stage of apoptosis. This could suggest that the activation of ERK either signals or is required for the cleavage of executive caspases. Utilizing the specific inhibitor U0126 and SiRNAs against ERK1/2 to modulate the ERK activity, we found that the inhibition of ERK1/2 activation significantly reduced RSVL-induced cytochrome c release and cleavage of caspase-3 and PARP. Especially, inhibition of ERK reverts the RSVL-induced suppression of Bcl-2, which has been shown to be able to prevent apoptotic cell death (45) and increases the phosphorylation of Bcl-2 at Ser70, which was shown to be critical for its anti-apoptotic action (34). Our data also showed that cell free extracts from UO126 treated cells exhibited an equal cleavage of caspase-3 compared with vehicle-treated cells when inducing in vitro apoptosis using dATP and cytochrome c as previously described (27). Similar data were observed when using other techniques to remove ERK activity in the cytosol (S-100) of MDA231 cells (immuno-depleted, SiRNA transfection) before inducing in vitro apoptosis (data not shown). Thus, the data suggest that ERK activity may be required for suppressing anti-apoptotic functioning of Bcl-2 protein through its phosphorylation at Ser70, which leads to the release of cytochrome c and caspase-3 activation, rather than directly affecting caspase-3 cleavage as previously suggested (reviewed in ref. 21). This finding further emphasized the importance of ERK1/2 activation and its activity in RSVL-induced apoptosis of MDA231 cells by suppressing Bcl-2 and caspase-3 activation. Our results support the proapoptotic role of ERK1/2 during drug treatment and are in agreement with previous studies, which demonstrated that ERK activation is required for cisplatin-induced apoptosis in HeLa and A549 cells (46) and abrogation of ERK pathway prevents RSVL-induced apoptosis in human osteosarcoma S1SA1 cells (47). We also showed that transfection of the constitutively expressed MEK1, which activates both ERK1 and ERK2, renders the cells more invasive and able to survive longer in serum-free media (>48 h) than the mock-transfected cells (data not shown). This result is supported by the previous report that MEK1 transfected cells become more invasive...
and metastasized and highly metastatic breast cancer cells have higher constitutive activity of ERK compared to less invasive cancer cells (18). Moreover, constitutively active ERK has been shown to contribute to cell growth, proliferation, migration and transformation (17,18). Surprisingly, MEK1 transfected cells become more sensitive to RSVL-induced apoptosis. The mechanisms by which these highly invasive cells become more responsive to RSVL are unclear. However, it suggests that ERK activity could play a central role in apoptosis induced by RSVL in these cells. Similarly, RSVL could act more effectively on the highly proliferative cells. This highly proliferative cell-targeting property of RSVL is further supported by previous studies showing that RSVL does not induce apoptosis in normal cells treated at very high concentrations (48) but extremely effective against the highly invasive breast cancer cell line MDA-MB-435 (15). The ability of ERK to regulate cell death and proliferation appears to depend on cell types, timing of activation, duration of its activation, amplitude of its activity and the agents, which signal the activation. In this study, highly active status of ERK1/2 renders cells more vulnerable to RSVL exposure. The possible stabilization of Bcl-2 through its phosphorylation at Ser70 when ERK1/2 is inhibited suggests that the highly active status of ERK1/2 could render cells more sensitive to ERK-activating agents. The questions remaining are how posttranslational modification of bcl-2 at Ser70 is regulated by ERK1/2, whether ERK1/2 activity and its activation during RSVL treatment involved in Bcl-2 degradation, and what specifies cell death rather than survival when ERK1/2 activated.

In conclusion, this study contributes to clarification of the pro-apoptotic role of ERK1/2 in cancer treatment. Since many powerful anticancer drugs also activate ERK signaling and exert their functions through ERK activation (26,46,49) and a combination may need to be considered for treatment of cancer, especially of metastatic tumors, it raises the possibility that RSVL could act in synergy with these compounds in treatment of highly invasive, metastatic and unresponsive breast cancer.

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