Resveratrol induces pro-apoptotic endoplasmic reticulum stress in human colon cancer cells

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Abstract. Resveratrol (3,4',5 tri-hydroxystilbene), a naturally occurring polyphenolic compound highly enriched in grapes and red wine, has been shown to induce anti-proliferation and apoptosis of human cancer cell lines. Resveratrol-induced dose-dependent apoptotic cell death in colon carcinoma cells, was measured by FACS analysis. Treatment of HT29 human colon carcinoma cells with resveratrol was found to induce a number of signature ER stress markers; phosphorylation of eukaryotic initiation factor-2α (eIF-2α), ER stress-specific XBP1 splicing and CCAAT/enhancer-binding protein-homologous protein (CHOP). In addition, resveratrol induced up-regulation of glucose-regulated protein (GRP)-78, suggesting the induction of ER stress. Furthermore, the inhibition of caspase-4 activity by z-LEVD-fmk significantly reduced resveratrol-induced apoptosis. Taken together, the present study therefore provides strong evidence to support an important role of ER stress response in mediating the resveratrol-induced apoptosis.

Introduction

The endoplasmic reticulum (ER) is a principal site for protein synthesis and modification prior to directing protein delivery to other secretory organelles. These proteins are correctly folded in the ER under normal conditions, but protein folding in the ER is impaired under various physiological and pathological conditions. These abnormalities in the ER are collectively called ER stress (1,2). Cells display various adaptive responses to relieve ER stress. The unfolded protein response (UPR), one of these adaptive responses, induces the expression of ER-resident chaperones, such as glucose-regulated protein (GRP)-78 and (GRP)-94 (3). In addition, protein kinase-like ER-resident kinase (PERK), inositol requiring 1 (Ire1) and activating transcription factor-6 (ATF-6) serve as proximal sensors that regulate components which act to upregulate the capacity of the ER to fold newly synthesized proteins and degrade misfolded/unfolded proteins (4-7). Several mechanisms and ER stress-induced cell death modulators have been proposed (8,9). The ER stress-induced apoptosis modulators include caspase-12, C/EBP homologous protein/growth arrest and DNA damage-inducible gene 153 (CHOP/GADD153) and Bcl-2 family. CHOP is apparently a key proapoptotic transcription factor that is induced during ER stress and subsequently activated by p38 mitogen-associated protein kinase (9,10).

Apoptosis is one mechanism by which resveratrol expresses its anticancer effect (11,12). The goal of the present study is to test the hypothesis that resveratrol induction of apoptosis is associated with ER stress. Resveratrol (3,4',5 tri-hydroxystilbene) is a phytoalexin produced in grapes and a variety of medicinal plants (13,14). Resveratrol was shown to inhibit tumor initiation, promotion and progression in a variety of cell culture systems and animal models (15). Resveratrol is also able to activate apoptosis, to arrest the cell cycle or to inhibit kinase pathways (11,16,17). Resveratrol has also been demonstrated to induce apoptotic cell death and nonapoptotic cell death (autophagocytosis) (11,16,18). Down-regulation of anti-apoptotic proteins, loss of mitochondrial function and activation of caspases may be involved in resveratrol-induced apoptotic cell death in a number of cancer cell lines (11,19). However, the involvement of ER stress in resveratrol-induced apoptosis has not been studied in detail. The present study clearly demonstrats that resveratrol-induced apoptosis is associated, at least in part, with its ability to cause ER stress.

Materials and methods

Cells and materials. HT29 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

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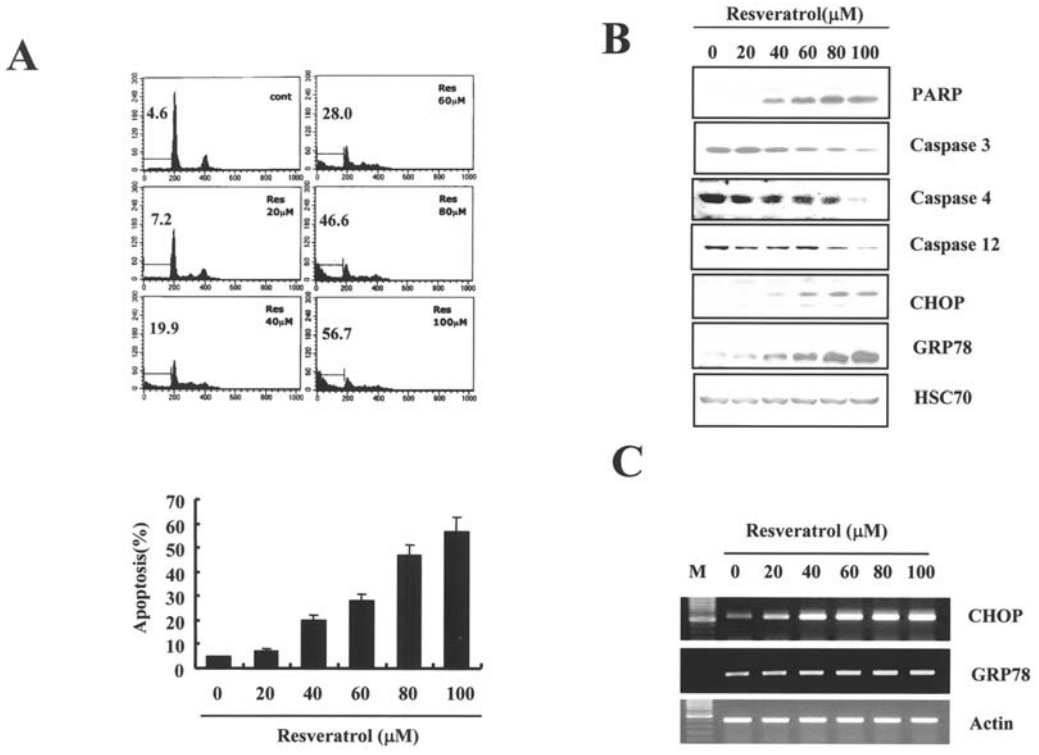

Figure 1. Resveratrol-induced apoptosis in HT29 cells. (A) Flow cytometric analysis of apoptotic cells. HT29 cells were treated with the indicated concentrations of resveratrol for 20 h. Apoptosis was analyzed as a sub-G1 fraction by FACS. (B) The expression levels of apoptosis and ER stress-related proteins in HT29 cells by treatment with resveratrol. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with specific antibodies, anti-PARP, anti-caspase-3, anti-caspase-4, anti-caspase-12, anti-CHOP, anti-GRP78 or with anti-HSC70 antibody to serve as a control for the loading of the protein level. A representative study is shown; two additional experiments yielded similar results. (C) Total RNA was isolated and RT-PCR analysis was performed using the CHOP- and GRP78-specific primers and also the internal control gene, ß-actin. A representative xperiment is shown; two additional experiments yielded similar results.

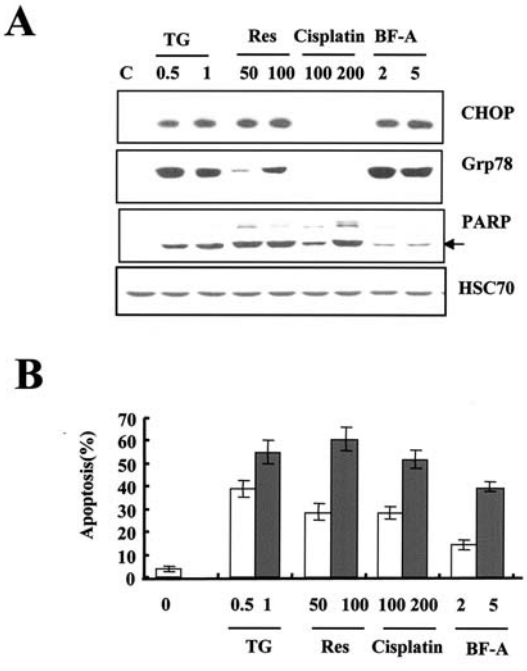

Figure 2. Up-regulation of the GRP78 and CHOP by ER stress and resveratrol treatment. (A) HT29 cells were treated with thapsigargin (TG; 0.5 and 1 μM), resveratrol (Res; 50 and 100 μM), cisplatin (100 and 200 μM) and brefeldin-A (BF-A; 2 and 5 μM) for 20 h. The whole cell lysates were analyzed by immunoblot analysis using specific CHOP, GRP78 and PARP antibodies. Anti-HSC70 antibodies serve as a control for the loading of the protein level. (B) HT29 cells were treated with indicated agents for 20 h. Apoptosis was analyzed as a sub-G1 fraction by FACS.

The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml gentamycin. Resveratrol was directly added to cell cultures at the indicated concentrations. Anti-CHOP, anti-PARP, anti-caspase-3, anti-caspase-4, anti-caspase-12, anti-GRP78, anti-phospho-eIF-2α and anti-HSC70 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Resveratrol was purchased from Biomol (Biomol Research Laboratories, Inc., PA, USA). Other chemicals were obtained from Sigma Chemical Co.

Western blotting. Cellular lysates were prepared by suspending 1x10^6 cells in 100 μl of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 100 μM phenylmethylsulfonyl fluoride and 20 μM leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4˚C for 30 min. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA, USA). The detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

Cell count and flow cytometry analysis. Cell counts were performed using a hemocytometer. HT29 cells (~1x10^6) were suspended in 100 μl of PBS and 200 μl of 95% ethanol were added while vortexing. The cells were incubated at 4˚C for 1 h,

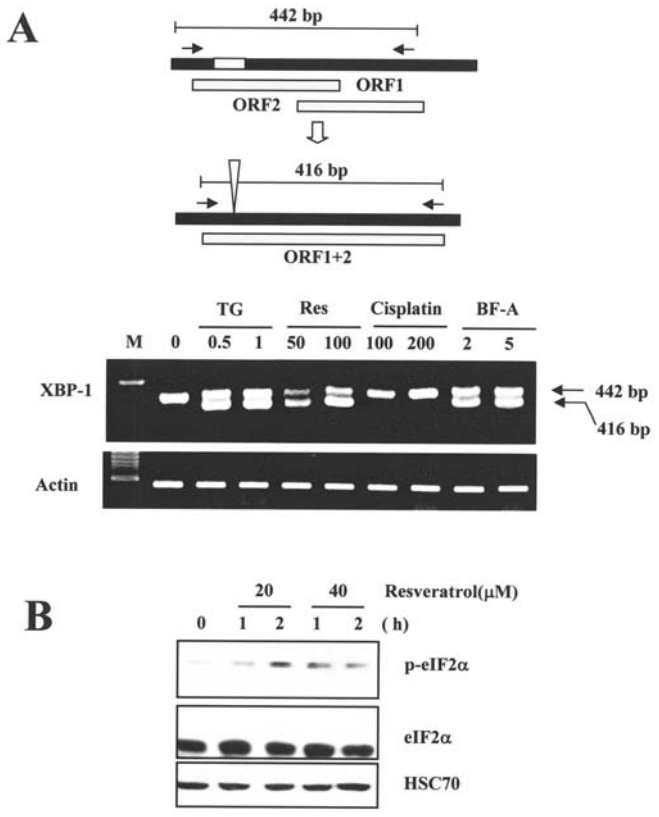

Figure 3. Resveratrol induces splicing of XBP1 mRNA and phosphorylation of eIF-2α. (A) HT29 cells were treated with thapsigargin (TG; 0.5 and 1 μM), resveratrol (Res; 50 and 100 μM), cisplatin (100 and 200 μM) and brefeldin-A (BF-A; 2 and 5 μM) for 20 h. Total RNA was isolated and RT-PCR analysis was performed as described in Materials and methods. The 416-bp form appearing in treated cells represents the spliced form of the mRNA. (B) HT29 cells were incubated for indicated times with indicated concentrations of resveratrol. The whole cell lysates were analyzed by immunoblot analysis using specific phospho-eIF-2α antibodies. Anti-HSC70 antibodies serve as a control for the loading of the protein level.

washed with PBS and re-suspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) together with 12.5 μg of RNase. Incubation was continued at 37˚C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from cells using the TRIzol reagent (Life Technologies). A cDNA was synthesized from 2 μg of total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The cDNA for CHOP, GRP78 and XBP1 were amplified by PCR with specific primers: CHOP (sense) 5'-CAACTGCAGAGAATT CAGCTGA-3' and (anti-sense) 5'-ACTGATGCTCTAGATT GTTCAT-3'; GRP78 (sense) 5'- GCTCGACTCGAATTCC AAAG-3' and (anti-sense) 5'-TTTGTCAGGGGTCTTTC ACC-3'; XBP1 (sense) 5'-CCTTGTAGTTGAGAACCAGG-3' and (anti-sense) 5'-GGGGCTTGGTATATATGTGG-3'. PCR amplification was carried out as follows: 1 x (94˚C, 3 min); 30 x (94˚C, 45 sec; 57˚C, 45 sec; and 72˚C, 1 min); and 1 x (72˚C, 10 min). PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

Results

Cellular features characteristic of apoptosis in HT29 cells exposed to resveratrol. To investigate the effect of resveratrol-induced apoptosis, human colon carcinoma HT29 cells were treated with various concentrations of resveratrol. Two established criteria were subsequently used to assess apoptosis in our system. Apoptosis was determined in HT29 cells using flow cytometry analysis demonstrating hypodiploid DNA. Fig. 1A shows treatment with resveratrol in HT29 cells resulted in a markedly increased accumulation of sub-G1 phase in a dose-dependent manner of resveratrol. Because cells undergoing apoptosis execute the death program by activating caspases and cleavage of PARP (20), we analyzed expression levels of pro-caspase-3, cleavage of PARP and other apoptosis related proteins. As Fig. 1B demonstrates, exposure to resveratrol led to a reduction of the 32-kDa precursor, accompanied by a concomitant revealed cleavage of PARP. In addition, one of the hallmarks of the ER stress responses involved in the activation of ATF6 and the subsequent induction of GRP78 and CHOP (2,9). We examined the effect of resveratrol on the expression of GRP78 and CHOP by Western blotting. As shown in Fig. 1B, treatment with resveratrol induced the expression of GRP78 and CHOP protein in a dose-dependent manner. It was suggested that the ER-specific apoptosis pathway was mediated by caspase-4 and -12 (21,22). As Fig. 1B shows, exposure to resveratrol led to a reduction of the caspase-4 and -12 precursors. We then investigated whether resveratrol-induced CHOP and GRP78 induction is controlled at the transcriptional level. RT-PCR analysis demonstrates that resveratrol induces GRP78 and CHOP mRNA levels in a dose-dependent manner (Fig. 1C).

Effects of resveratrol on ER stress. To examine whether the induction of CHOP and GRP78 was caused by ER stress, we analyzed the induction of CHOP and GRP78 in response to several apoptotic stimuli (Fig. 2A). We found that the induction of CHOP and GRP78 was induced in HT29 cells by treatment with thapsigargin (TG; inhibitor of the ER Ca^{+2}-ATPase) and brefeldin-A (BF-A; blocker of protein transport from ER to Golgi), both of which caused ER stress. As shown in Fig. 2A, resveratrol also induced the expression of CHOP and GRP78 protein. In contrast, when cells were exposed to a non-ER stress inducer such as cisplatin at a dose providing a similar extent of cell death to that by thapsigargin and BF-A, induction of CHOP and GRP78 was not observed (Fig. 2A and 2B). These results suggest that the induction of CHOP and GRP78 by apoptotic stimuli induces ER stress, but not by other stimuli that do not cause ER stress.

Resveratrol induces splicing of XBP1 mRNA and phosphorylation of eIF-2α. During ER stress, the mRNA encoding the transcription factor XBP1 is spliced by the endoribonuclease IRE1 that results in the removal of 26-nucleotide intron and a translational frame shift (7,23). The spliced XBP1 mRNA is translated into a functional transcriptional activator (7,23).

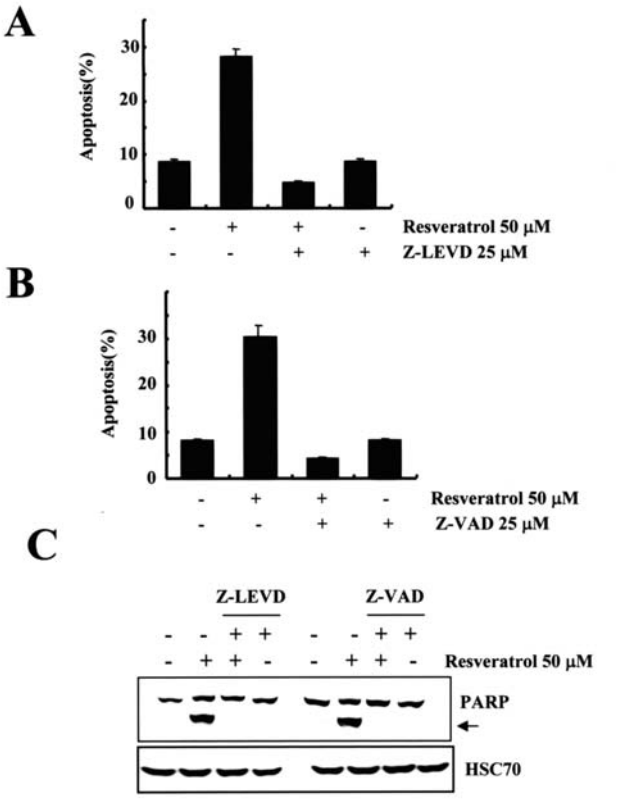

Figure 4. Effect of caspase-4 inhibitor on resveratrol-induced apoptosis. (A) HT29 cells were incubated with caspase-4 inhibitor z-LEVD-fmk (50 μM) or solvent for 1 h before challenge with resveratrol (50 μM) for 20 h. Apoptosis was analyzed as a sub-G1 fraction by FACS. (B) HT29 cells were incubated with pancaspase inhibitor z-VAD-fmk (50 μM) or solvent for 1 h before challenge with resveratrol (50 μM) for 20 h. Apoptosis was analyzed as a sub-G1 fraction by FACS. (C) Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti-PARP or with anti-HSC70 antibody to serve as a control for the loading of the protein level. The proteolytic cleavage of PARP is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results.

RT-PCR analysis demonstrated that resveratrol and other ER stress-inducing agents (TG and BF-A) induced splicing of XBP1 mRNA (Fig. 3A). However, non-ER stress inducer, cisplatin, did not induce splicing of XBP1 mRNA.

The eukaryotic initiation factor-2α (eIF-2α) is phosphorylated by the PKR-like ER-localized eIF-2α kinase (PERK) in response to ER stress leading to an attenuation of translational initiation and protein synthesis (4,5). To determine whether resveratrol-induced ER stress might be associated with PERK/eIF-2α kinase signaling pathway, we examined the phosphorylation of eIF-2α (the target of PERK). Fig. 3B shows resveratrol induced a significant increase in phosphorylated eIF-2α levels at 2 h when the cells were incubated with resveratrol.

The role of ER stress in resveratrol-induced apoptosis of HT 29 cells. Recently, our group reported that resveratrol-induced apoptosis was attenuated in cells transfected with CHOP siRNA (24). These data suggest that resveratrol-induced CHOP up-regulation may be involved, at least in part, in resveratrol-induced apoptosis. To further confirm, we blocked resveratrol-induced activation of ER stress-mediated caspase-4. Induction of apoptosis by resveratrol was significantly reduced not only by z-LEVD-fmk (a specific caspase-4 inhibitor), but also by z-VAD-fmk (pan-caspase inhibitor) (Fig. 4A and 4B). In addition, treatment of HT29 cells with z-LEVD-fmk and z-VAD-fmk prevented cleavage of PARP (Fig. 4C). Taken together, our results suggest that resveratrol-induced apoptosis is associated with its ability to cause ER stress.

Discussion

Although the cancer chemopreventive agent, resveratrol, was shown to induce apoptosis in many human cancer cell lines (11,12,19), the mechanisms associated with resveratrol-induced apoptosis are not well established. The central novel finding in this study, provides important evidence to support the involvement of ER stress in the induction of apoptosis by resveratrol in HT29 colon carcinoma cells. Resveratrol induced GRP78 expression and CHOP expression and XBP1 splicing and phosphorylation of eIF-2α, all of which are indicative of ER stress.

Known ER stresses include calcium store depletion, inhibition of glycosylation, reduction of disulfide bonds and overexpression of mutant proteins (3,25,26). We characterized the effects of resveratrol on markers of ER stress, a previously undescribed activity of this compound. Our results which demonstrate that resveratrol treatment induces apoptosis are in agreement with previous studies. The following experimental evidence in the present study demonstrates that the induction of ER stress-related proteins may be involved in resveratrol-induced apoptosis. i) Resveratrol induces GRP78 and CHOP expression. CHOP, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), is one of the highest inducible genes during ER stress; ii) Resveratrol induces phosphorylation of eIF-2α; iii) Resveratrol induces splicing of XBP1 mRNA. Under ER stress conditions, XBP1 mRNA is processed by unconventional splicing and translated into a functional transcription factor (7,23). iv) The inhibition of caspase-4 activity by z-LEVD-fmk significantly reduced resveratrol-induced apoptosis (4). Taken together, these observations suggest that resveratrol induces ER-mediated apoptosis.

CHOP protein was first identified to be a member of the CCAAT/enhancer binding proteins (C/EBPs) that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription (2). Expression of CHOP is mainly regulated at the transcriptional level through the PERK/eIF-2α/ATF6 pathway (2). CHOP$^{-/-}$ mice exhibit reduced apoptosis in response to ER stress (27,28). Therefore, CHOP is one of the components of the ER stress-mediated apoptosis pathway. Among the ER-associated apoptotic molecules, CHOP and caspase-4 are apparently major pro-apoptotic factors that are closely associated with ER stress (2,21). ER-resident caspase-12 was reported to play an important role in the rodent model (22). However, the human caspase-12 contains several mutations, which renders it non-functional (29). It was reported that human caspase-4, which is also a resident of the ER, is the counterpart of murine caspase-12 and is activated by ER stress (21). We recently observed that the suppression of

CHOP by CHOP siRNA attenuated resveratrol-induced apoptosis (24). In the present study, we provide evidence that the inhibition of caspase-4 activity by z-LEVD-fmk significantly reduced resveratrol-induced apoptosis. This is the first report to demonstrate the ability of resveratrol to induce ER stress and apoptosis. These data suggest that ER stress may be of importance for the cytotoxic activity of resveratrol. However, the manner in which resveratrol causes ER stress in HT29 cells is not clear. Therefore, further study on resveratrol is required to delineate exactly its reactions in cells and to explain its biological effects.

Acknowledgements

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