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Elevated *gadd153/chop* expression during resveratrol-induced apoptosis in human colon cancer cells

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ABSTRACT

Resveratrol (3,4′,5-tri-hydroxystilbene), a natural phytoalexin found at high levels 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, as measured by FACS analysis and internucleosomal DNA fragmentation assays. We demonstrate for the first time that resveratrol induce CCAAT/enhancer-binding protein-homologous protein (CHOP). Resveratrol-induced CHOP mRNA (and also protein) expression was inhibited by JNK specific inhibitor, but not ERK, p38 MAPK, PI3K and NF-κB inhibitors. Resveratrol-induced expression of CHOP involves the putative Sp1 site within the CHOP promoter region. Using a combination of the Sp1 cDNA transfection, the luciferase reporter assay and Sp1 inhibitor assay, we found that Sp1 site is required for resveratrol-mediated activation of the CHOP promoter. Suppression of CHOP expression by CHOP siRNA and treatment with mithramycin A attenuated resveratrol-induced apoptosis. Taken together, the present studies suggest that induction of CHOP protein may be involved, at least in part, in resveratrol-induced apoptosis.

1. Introduction

Resveratrol (3,4′,5-tri-hydroxystilbene) is a phytoalexin produced in grapes and a variety of medicinal plants [1,2]. Numerous studies have reported interesting properties of *trans*-resveratrol as a preventive agent against important pathologies, i.e. vascular diseases, cancers, viral infection or neurodegenerative processes [3–6]. Recently, Jang et al. [7] demonstrated that resveratrol acts on the process of carcinogenesis by affecting the three phases (tumor initiation, promotion and progression). Resveratrol is also able to activate apoptosis, to arrest the cell cycle or to inhibit kinase pathways [8–10].

Resveratrol has been shown to induce apoptotic cell death and nonapoptotic cell death (autophagocytosis) [9–11]. 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 [9,12]. Resveratrol can induce nonapoptotic cell death (autophagocytosis) in ovarian cancer cells [11]. However, the mechanisms associated with the anti-proliferative and chemopreventive effects of resveratrol are not well established.

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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 [13,14]. CHOP is also known as growth arrest- and DNA damage-inducible gene 153 (GADD153) [13,14]. Although low in normal proliferating cells, the expression of CHOP is induced by variety of stress stimuli, including genotoxic agent, nutrient depletion and endoplasmic reticulum (ER) stress [13–17]. Overexpression of CHOP has been reported to lead to cell cycle arrest and/or apoptosis [18,19]. Studies have also suggested that CHOP triggers the critical early events leading to the initiation of apoptosis, which are believed to be important targets for cancer drug development [14].

The reports of complex biological activities by resveratrol raise the question of whether this compound induces multifaceted biological responses. CHOP is one of the components of the ER stress-mediated apoptosis pathway. In addition, $CHOP^{-/-}$ mice exhibit reduced apoptosis in response to ER stress. To gain a better understanding of the molecular effects resveratrol on colon carcinoma cells, the aim of the present study was to determine whether resveratrol induces CHOP gene expression. We focus on two major findings. One is the potential mechanism of CHOP-mediated apoptosis in resveratrol treated cells and the other is the mechanism of the resveratrol-induced CHOP expression.

2. Materials and methods

2.1. Cells and materials

HT29 cells were obtained from the American Type Culture Collection (ATCC: Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100 μg/ml gentamicin. Resveratrol was directly added to cell cultures at the indicated concentrations. CHOP siRNA, anti-CHOP, anti-PARP, anti-caspase 3, anti-XIAP, anti-cIAP1, anti-cIAP2 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.

2.2. Western blotting

Cellular lysates were prepared by suspending 1×10^6 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). Detection of specific proteins was carried out with an ECL Western blotting kit according to the manufacturer's instructions.

2.3. Cell count and flow cytometry analysis

Cell counts were performed using a hemocytometer. Approximately 1×10^6 HT29 cells 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, washed with PBS, and resuspended 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.

2.4. DEVDase activity assay

To evaluate DEVDase activity, cell lysates were prepared after their respective treatment with/without resveratrol. Assays were performed in 96-well microtiter plates by incubating 20 μg of cell lysates in 100 μl of reaction buffer (1% NP-40, 20 mM Tris–HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspases substrate [Asp-Glu-Val-Asp-chromophore-*p*-nitroanilide (DVAD-*p*NA)] at 5 μM. Lysates were incubated at 37 °C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

2.5. DNA fragmentation assay

After treatment with resveratrol for 24 h, HT29 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at $10{,}000 \times g$ for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 μg/ml of ethidium bromide.

2.6. Plasmids, transfections, and luciferase gene assays

CHOP promoter constructs were generously provided by Dr. P. Fafournoux (U.R. 238 de Nutrition Cellulaire et Moléculaire, France). The human CHOP promoter-containing plasmids have been described previously [16]. For transfection, in brief, cells were plated onto 6-well plates at a density of 5×10^5 cells/well and grown overnight. Cells were co-transfected with 2 μg of various plasmid constructs and 1 μg of the pCMV-β-galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and β-galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for β-galactosidase activity in cell lysate and expressed as an average of three independent experiments.

2.7. 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 and actin were amplified by PCR with specific primers. The sequences of the sense and anti-sense primer for CHOP were 5′-CAACTGCAGAGAATTCAGCTGA-3′ and 5′-ACTGATGCTCTAGATTGTTCAT-3′, respectively. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

2.8. *Small interfering RNA (siRNA)*

The CHOP siRNA duplexes used in this study were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cells were transfected with siRNA oligonucleotides using LipifectAMINE 2000 (Invitrogen, Calsbad, CA) according to the manufacturer's recommendations.

3. Results

3.1. *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. Three established criteria were subsequently used to assess apoptosis in our system. First, apoptosis was determined in HT29 cells using flow cytometry analysis demonstrating hypodiploid DNA. As shown in Fig. 1A, treatment with resveratrol in HT29 cells resulted in a markedly increased accumulation of sub-G1 phase in a dose-dependent manner of resveratrol. Second, we analyzed DNA fragmentation, which is another hallmark of apoptosis. Following agarose gel electrophoresis of HT29 cells treated with resveratrol for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed (Fig. 1B). In addition, because cells undergoing apoptosis execute the death program by activating caspases and cleavage of PARP [20], we analyzed whether treatment with resveratrol gave rise to the activation of caspase 3, a key executioner of apoptosis. As shown in Fig. 1C, treatment with resveratrol stimulated caspase 3 protease activities. Data of the Western blot in Fig. 1D showed expression levels of pro-caspase 3, cleavage of PARP, and other apoptosis related proteins. As shown in Fig. 1D, exposure to resveratrol led to a reduction of the 32-kDa precursor, accompanied by a concomitant revealed cleavage of PARP. Treatment of HT29 cells with resveratrol for 24 h resulted in a decrease in protein levels of XIAP and cIAP1, but

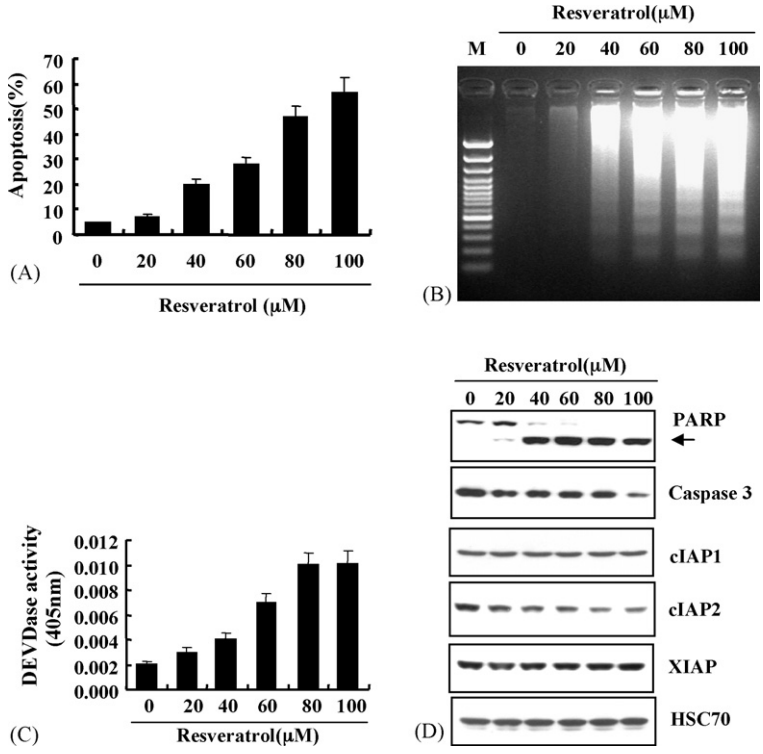

Fig. 1 – Resveratrol-induced apoptosis in HT29 cells. (A) Flow cytometric analysis of apoptotic cells. HT29 cells were treated with the indicated concentrations of resveratrol for 24 h. Apoptosis was analyzed as a sub-G1 fraction by FACS. (B) Fragmentations of genomic DNA in HT29 cells treated for 24 h with the indicated concentrations of resveratrol. Fragmented DNA was extracted and analyzed on 2% agarose gel. (C) Cells were treated with the indicated concentrations of resveratrol. Enzymatic activities of DEVDase were determined by incubation of 20 μg of total protein with 200 μM chromogenic substrate (DEVD-*p*NA) in a 100 μl assay buffer for 2 h at 37 °C. The release of chromophore *p*-nitroanilide (*p*NA) was monitored spectrophotometrically (405 nm). (D) The expression levels of apoptosis-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-cIAP1, anti-cIAP2, anti-XIAP or with anti-HSC70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results.

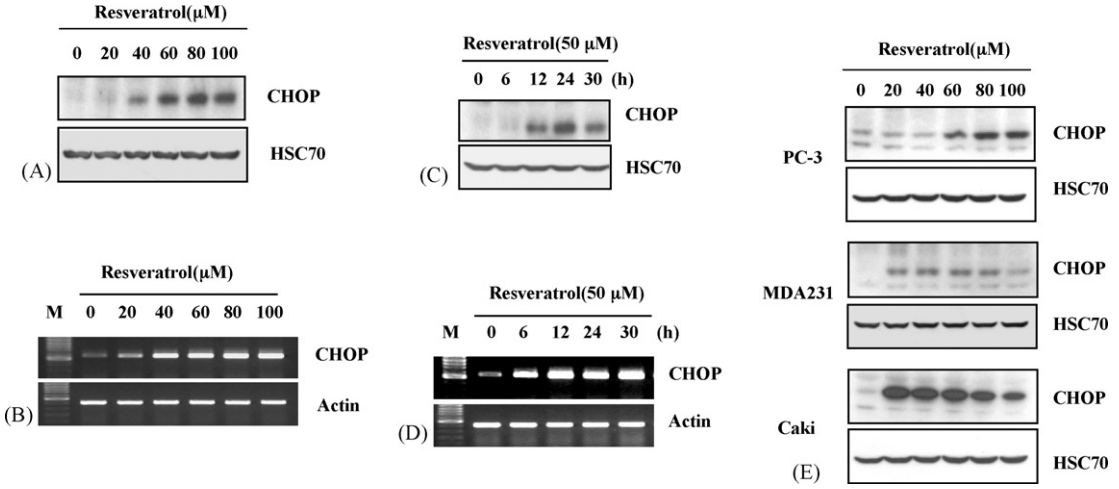

Fig. 2 – The expression levels of the CHOP mRNA and CHOP protein by treatment with resveratrol in HT29 cells. (A) HT29 cells were treated with the indicated concentrations of resveratrol. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-CHOP or with anti-HSC70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results. (B) Total RNA was isolated and RT-PCR analysis was performed as described in Section 2. A representative study is shown; two additional experiments yielded similar results. (C) HT29 cells were treated with 50 μM resveratrol for 0–30 h. Then whole cell lysates were prepared for subsequent immunoblotting analysis of CHOP and also HSC70. (D) HT29 cells were treated with 50 μM resveratrol for 0–30 h. RT-PCR analysis was performed using CHOP gene-specific primers and also the internal control gene, β-actin. The results are representational of three different experiments. (E) PC-3, MDA231, and Caki cells were treated with the indicated concentrations of resveratrol. Expression of CHOP was determined by immunoblotting analysis.

not cIAP2. Taken together, these results indicate that resveratrol induces apoptosis in HT29 cells.

3.2. *Effect of resveratrol on CHOP protein expression in HT29 cells*

A number of reports have indicated that CHOP is involved in the process of apoptosis in a variety cancer cell lines [17–19,21,22]. To further elucidate the mechanism responsible for the changes in expression levels of CHOP protein, we first examined the effect of resveratrol on the expression of CHOP by Western blotting. As shown in Fig. 2A, treatment with resveratrol induced the expression of CHOP protein in a dose-dependent manner. Next, we investigated whether resveratrol-induced CHOP induction is controlled at the transcriptional level. RT-PCR analysis demonstrated that resveratrol-induced CHOP mRNA levels in a dose-dependent manner. In further studies of the relationship between CHOP protein and CHOP mRNA in HT29 cells, we carried out time kinetic studies of resveratrol treated HT29 cells (Fig. 2C and D). Incubation with resveratrol caused a time-dependent increase in CHOP protein and CHOP mRNA. The induction of CHOP protein expression correlated with increased levels of CHOP mRNA, which became detectable as early as 6 h after treatment with resveratrol (Fig. 2D). Treatment with resveratrol significantly increased CHOP protein levels also in a variety of tumor cell types (prostate cancer cells PC-3, breast cancer cells MDA231 and renal carcinoma Caki) (Fig. 2E). These results suggest that upregulation of CHOP is a common response of cancer cell lines to resveratrol treatment.

3.3. *Effect of MAPK and other signaling pathway on resveratrol-induced up-regulation of CHOP protein expression in HT29 cells*

To examine the role of the MAPK signaling pathway in the induction of CHOP by resveratrol, we investigated the induction of CHOP expression in the presence of pathway specific inhibitors. CHOP expression was examined after the cells were stimulated with 50 μM resveratrol in the presence of 20 μM SP 600125 (JNK inhibitor), 10 μM SB 203580 (p38 MAPK inhibitor) or 50 μM PD 098059 (MEK1/2 inhibitor). As shown in Fig. 3A, induction of the CHOP protein expression was dramatically reduced in the presence of JNK inhibitor SP 600125, while MEK and p38 MAPK inhibitor were ineffective to regulation of CHOP expression. Consistent with CHOP protein expression pattern, treatment of HT29 cells with JNK inhibitor attenuated resveratrol-induced CHOP mRNA up-regulation (Fig. 3B). To further confirm JNK signaling pathway involved in resveratrol-mediated CHOP protein expression, we examined the dose effect of SP 600125 on the expression of CHOP by Western blotting and RT-PCR. As shown in Fig. 3C, treatment with SP 600125 inhibited the expression levels of CHOP protein and CHOP mRNA in a dose-dependent manner. Moreover, HT29 cells were transfected with −954/CHOP-Luc and the luciferase activities were assayed 24 h after SP 600125 treatment at different doses in the presence or absence of resveratrol. We found that JNK inhibitor gradually decreased the promoter activities of −954/CHOP-Luc in a dose-dependent manner (Fig. 3D). These results suggested that signal pathway of JNK plays a role in the resveratrol-induced CHOP expression.

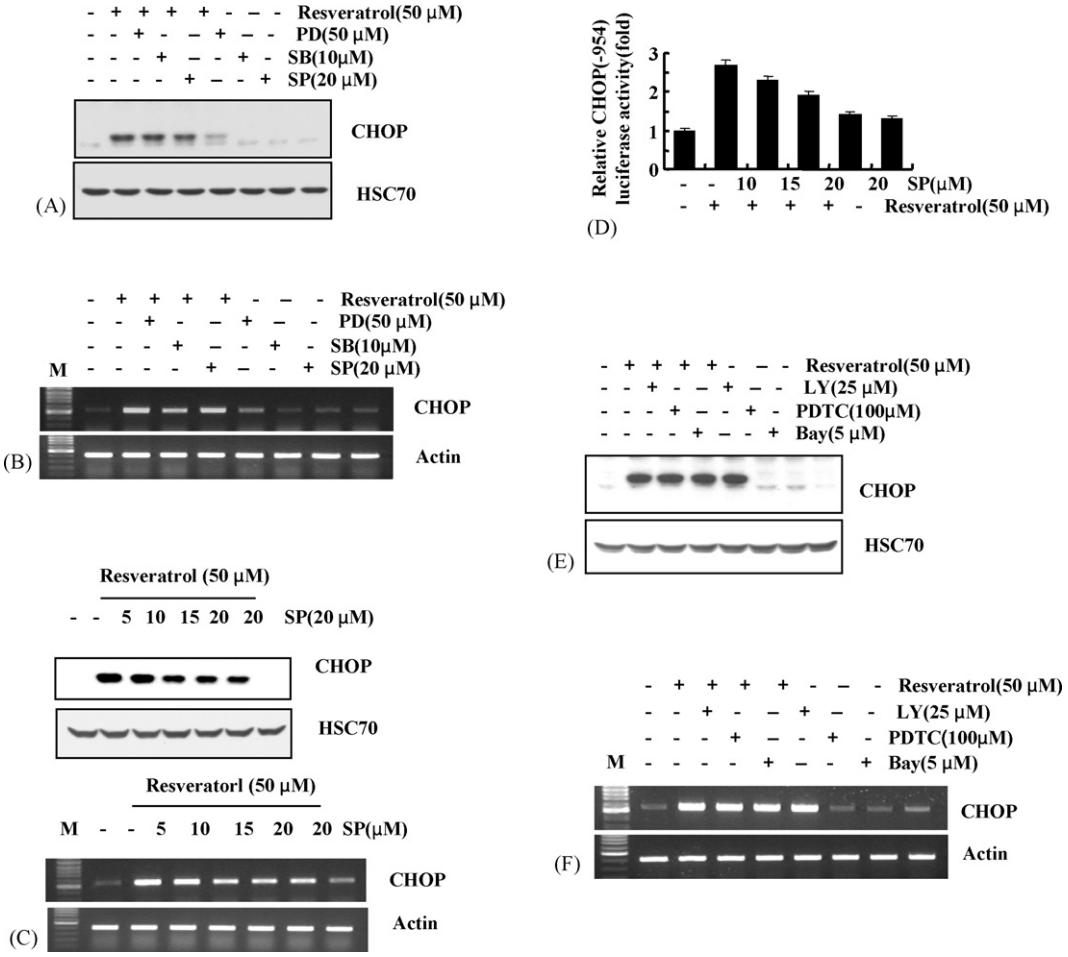

Fig. 3 – Effect of MAPK and other signaling pathway on resveratrol-induced up-regulation of CHOP protein expression in HT29 cells. (A) HT29 cells were pretreated with various inhibitors (50 μM PD 98059, 10 μM SB 203580, 20 μM SP 600125) for 30 min followed by treatment with resveratrol (50 μM) for 24 h. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against CHOP. The equal loading in each lane was demonstrated by the similar intensities of HSC70. (B) Total RNA was isolated and RT-PCR analysis was performed. The values below the figure represent the change in mRNA expression of the bands normalized to actin. (C) HT29 cells were treated with indicated concentrations of SP 600125 in the presence of resveratrol (50 μM) for 12 h. The cells were lysed, and the lysates were analyzed by immunoblotting using anti-CHOP. The blot was stripped of the bound antibody and reprobed with anti-HSC70 antibody to confirm equal loading. Total RNA was isolated and RT-PCR analysis was performed. (D) HT29 cells were transfected with CHOP promoter plasmid (−954/CHOP-Luc) and further cultured with resveratrol (50 μM) in the presence or absence of SP 600125. The cells were lysed and luciferase activity measured. Data represent the mean ± S.D. of at least three independent experiments. (E) HT29 cells were pretreated with PI3K inhibitor (LY 294002) or NF-κB inhibitor (PDTC and Bay 11-7082) for 30 min followed by treatment with resveratrol (50 μM) for 24 h. Equal amounts of soluble lysates (50 μg) were subjected to electrophoresis. The blots were analyzed using a specific antibody against CHOP. (F) Total RNA was isolated and RT-PCR analysis was performed. The values below the figure represent the change in mRNA expression of the bands normalized to actin.

To determine if phosphatidylinositol 3-kinase (PI3K) and NF-κB are important in resveratrol-induced up-regulation of CHOP protein expression, cells were pretreated with PI3K inhibitor (LY 294002) and NF-κB inhibitor (PDTC and Bay 11-7082) before exposing them to resveratrol. As shown in Fig. 3E and F, the PI3K inhibitor and the NF-κB inhibitor failed to prevent resveratrol-induced upregulation of CHOP protein and CHOP mRNA. Thus, it is clear that signal pathways of PI3K and NF-κB are not associated with resveratrol-induced CHOP expression.

3.4. *Localization of an upstream positive response element involved in activation of CHOP transcription by resveratrol*

To investigate whether or not resveratrol-induced CHOP induction is due to promoter activity, transient transfection of CHOP reporter gene constructs was performed. Used series of deletion CHOP promoter constructs were generated by PCR and fused to the coding region of the luciferase (LUC) reporter gene (Fig. 4A) [16]. These constructs were transiently transfected into HT29 cells, and the response to resveratrol

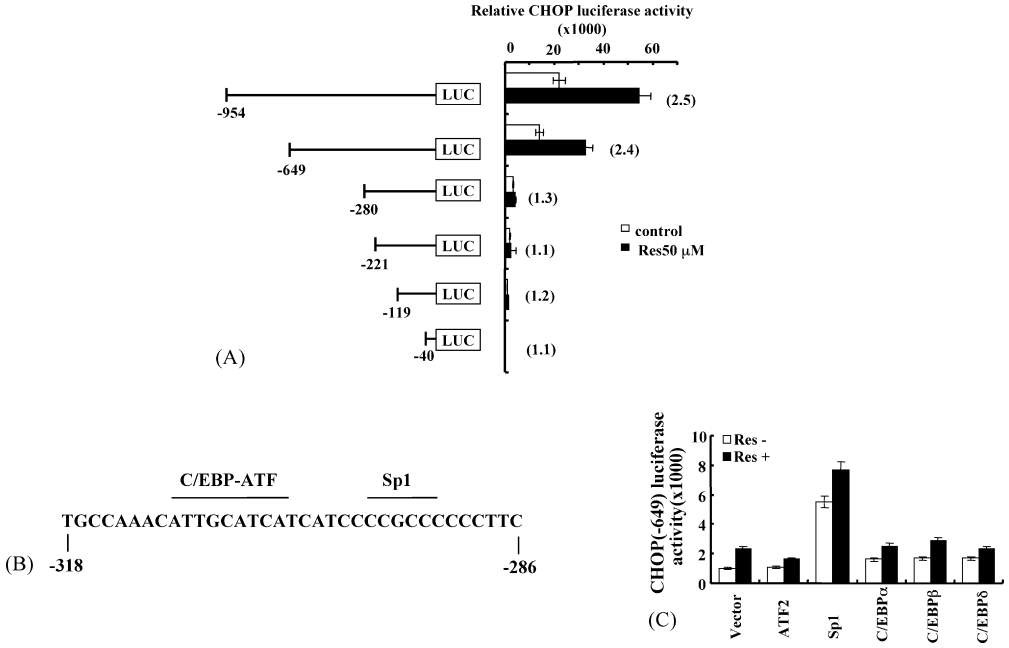

Fig. 4 – Effect of resveratrol on 5′ deletions of the CHOP promoter. (A) HT29 cells were transiently transfected with progressive 5′ deletion mutants of CHOP promoter. Twenty-four hours after transfection, cells were treated with resveratrol (50 μM) for 16 h. The cells were lysed and luciferase activity measured. Data represent the mean ± S.D. of at least three independent experiments. The relative fold induction, defined as the ratio of the relative luciferase activity of resveratrol-treated cells to untreated cells, is indicated in parentheses to the right of the bars. (B) Nucleotide sequence from −318 to −286 relative to the start site of CHOP promoter. The position of the C/EBP and Sp1 sites are indicated. (C) Effect of ectopic expression of transcriptional factors on NAG-1 expression. HT29 cells were cotransfected with Sp1, ATF2, C/EBPα, C/EBPβ and C/EBPδ cDNA and −649/CHOP-Luc promoter plasmids. The cells were treated with or without resveratrol, and lysed and luciferase activity measured. Data represent the mean ± S.D. of at least three independent experiments.

was determined by luciferase assay, in the presence or absence of resveratrol. Deletion to −649/CHOP-Luc had no significant effect on the activation of the CHOP promoter by resveratrol. By contrast, deletion from −280/CHOP-Luc decreased resveratrol inducibility, suggesting that the region between −649 and −280 contains *cis*-positive elements involved in the transcriptional activation of CHOP by resveratrol (Fig. 4A). To identify potential transcription factor binding sites in the region between −649 and −280 of the CHOP promoter, the genomic nucleotide sequence was analyzed for known transcription factor binding consensus with a computer-based program (MacVector sequence analysis software). The CHOP promoter region from −318 to −286 reveals the presence of a site that is similar to both the C/EBP consensus and the ATF/CRE-like sequence and the presence of a putative Sp1 binding site (Fig. 4B). To determine the role of each site in the resveratrol responsiveness of the CHOP promoter, we transfected HT29 cells with each transcription factor cDNA (ATF2, Sp1, C/EBPα, C/EBPβ, and C/EBPδ) and then treated with resveratrol. Ectopic expression of Sp1 significantly increased the basal promoter activity and resveratrol responsive promoter activity in −649/CHOP-Luc (Fig. 4C). However, ectopic expression of ATF2 or C/EBP transcription factor families did not affected the induction of the basal promoter activity and resveratrol responsive promoter activity in −649/CHOP-Luc. These results suggest that Sp1 site is implicated in the transcriptional control of CHOP gene expression by treatment with resveratrol.

3.5. *The Sp1 site is required for resveratrol-induced transactivation of CHOP in HT29 cells*

Resveratrol-induced activation of Sp1 transcription factor was further confirmed from the experiments using the Sp1 reporter vector, an artificially constructed plasmid containing three Sp1 binding sites and luciferase-coding gene. Transcriptional activity of this promoter was a ∼2.5-fold increased by treatment with 50 μM resveratrol (Fig. 5A). We next tested whether inhibition of Sp1 actually affects the resveratrol-induced CHOP promoter activity. HT29 cells were transfected with −649/CHOP-Luc and treated with mithramycin A, which interferes with the binding of the Sp family of transcription factors to GC-rich promoter regions [23]. Mithramycin A significantly decreased −649/CHOP-Luc promoter activity in a dose-dependent manner (Fig. 5B). Interestingly, Western blotting analysis using these cell extracts demonstrates that resveratrol-induced up-regulation of CHOP protein were significantly reduced by mithramycin treatment (Fig. 5C). Taken together, these results suggest that Sp1 binding site in the proximal promoter region is critical not only for the basal transcription of CHOP but also for resveratrol-mediated transactivation of CHOP promoter.

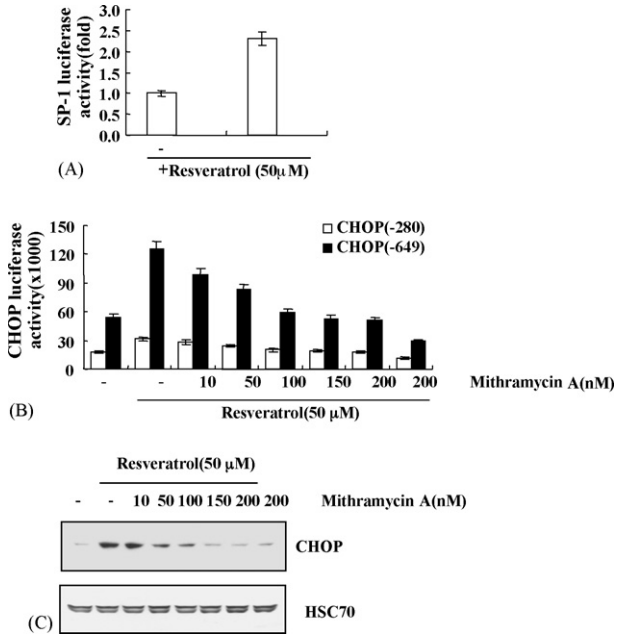

Fig. 5 – Effect of mithramycin A on resveratrol-induced up-regulation of CHOP protein expression in HT29 cells. (A) Effects of resveratrol on the activities of Sp1. To elucidate the effects of resveratrol on Sp1 activities, a reporter vector (pSp1-luc) that has three Sp1-binding sites was transfected. The cells were treated with or without resveratrol (50 μM), and lysed and luciferase activity measured. (B) HT29 cells were transfected with −649/CHOP-Luc promoter plasmid and further cultured with resveratrol (50 μM) in the presence or absence of mithramycin A. The cells were lysed and luciferase activity measured. Data represent the mean ± S.D. of at least three independent experiments. (C) The expression levels of CHOP protein in transfected cells were determined by Western blot analysis. A representative study is shown; two additional experiments yielded similar results.

3.6. *Suppression of CHOP expression attenuates resveratrol-induced apoptosis*

To investigate whether CHOP expression is associated with resveratrol-induced apoptosis, we assessed the CHOP expression levels in cells treated with mithramycin A in addition to 50 μM resveratrol. As shown in Fig. 6A, the treatment with resveratrol significantly increased CHOP protein levels, whereas cotreatment with mithramycin A markedly inhibited this resveratrol-induced CHOP upregulation. In addition, treatment of HT29 cells with mithramycin A prevented reduction of the protein levels of 32 kDa precursor and cleavage of PARP (Fig. 6A). Next, we attempted to clarify the significance of CHOP up-regulation in resveratrol-induced apoptosis. We examined the effects of mithramycin A on resveratrol-induced apoptosis in HT29 cells. Flow cytometric analysis to determine the sub-G1 population after mithramycin A cotreatment with resveratrol demonstrates that accumulation of the sub-G1 phase was markedly decreased (Fig. 6B).

To further confirm the functional significance of resveratrol-induced CHOP upregulation, we employed the siRNA duplex against CHOP mRNA. HT29 cells were transfected with the indicated siRNA were treated with or without resveratrol for 24 h. Immunoblot analysis demonstrated that transfection of siRNA against CHOP resulted in a suppression of CHOP expression induced by resveratrol in HT29 cells, compared with cells transfected with control GFP siRNA (Fig. 7A). Under these conditions, accumulation of sub-G1 cell population induced by resveratrol was attenuated in cells transfected with CHOP siRNA when compared with GFP siRNA-transfected cells (Fig. 7B). Taken together, resveratrol-induced apoptosis may be involved, at least in part, through the CHOP upregulation.

4. Discussion

The central novel finding in the present study is the upregulation of CHOP gene expression by resveratrol that also induced apoptosis in colon carcinoma cells. The CHOP gene is typically induced in response to cellular stress [14]. CHOP belongs to the C/EBP family of transcription factors. CHOP forms heterodimers with other members of the C/EBP family, resulting in inhibition of transcriptional activities [13,14].

In our present study, the upregulation of CHOP gene expression by resveratrol was verified both by RT-PCR and promoter assay. These data suggest that resveratrol induced CHOP expression is due to primarily to regulated transcription. However, amino acid starvation-induced CHOP mRNA expression that was attributed to both transcriptional and post-transcriptional components. Abcouwer et al. reported that glutamine deprivation induces the expression of CHOP primarily by mRNA stabilization [24].

Regarding the CHOP gene, it has been reported that there is a regulatory element for AP-1 besides regulatory elements for interleukin-6 response element, C/EBP and Sp1 [25,26]. Deletion analysis of the CHOP promoter region suggested that multiple elements within −778 to −250 regions are likely to contribute to regulation of CHOP activity in response to arsenite, H_2O_2 and UVC [25]. Using a combination of the luciferase reporter assay and transfection of the transcription factors, we demonstrated that the *cis* DNA sequence located upstream from the transcription start site (−318 to −286) is essential for resveratrol activation of the CHOP promoter. In this report, we have further found that Sp1 transcription factor may be an important target protein to regulate CHOP expression by resveratrol. As shown in Fig. 5A, transcriptional activity of Sp1 was increased by treatment with resveratrol. Mithramycin A is known to inhibit tumors by cross-linking GC-rich DNA, thus blocking binding of Sp1-family transcription factors to gene regulatory elements [23]. Mithramycin A strongly suppressed resveratrol-induced promoter activity and protein expression of CHOP, possibly through inhibition of Sp1-mediated transcriptional activities.

Recently, it was reported that CHOP expression can be regulated through different MAP kinase signaling pathways and that the particular signaling pathway involved is dependent on the type of stimuli [27–30]. The induction of

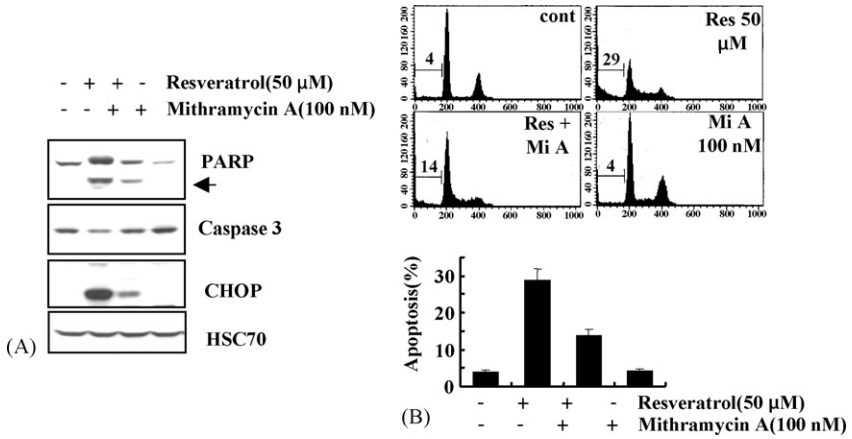

Fig. 6 – Effect of mithramycin A on resveratrol-induced apoptosis. (A) HT29 cells were treated with resveratrol in the presence or absence of mithramycin A (100 nM). Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-caspase 3, anti-CHOP, anti-PARP or with anti-HSC70 antibody to serve as control for the loading of protein level. The proteolytic cleavage of PARP is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results. (B) HT29 cells were treated with resveratrol in the presence or absence of mithramycin A (100 nM). Apoptosis was analyzed as a sub-G1 fraction by FACS.

CHOP by anisomycin has been demonstrated to require activation of p38 MAPK signal pathway [31]. The suppression of resveratrol-induced CHOP expression by JNK specific inhibitor, SP 600125, suggested that resveratrol induces CHOP expression via activation of JNK. However, other MAP kinase inhibitors, PI3K inhibitor and NF-κB inhibitor did not prevent or noticeably attenuate the increased CHOP mRNA expression caused by resveratrol. The anti-tumor activity of resveratrol occurs through JNK-mediated p53 activation and induction of apoptosis [32]. Resveratrol activated JNK dose-dependently within a dose range of 10–40 μM in JB6 mouse epidermal cell line. These dosages are responsible for the inhibition of tumor promoter-induced cell transformation [32].

Although the full significance of induction of CHOP gene expression in resveratrol-treated cells is not known, the effect of resveratrol on CHOP in particular might contribute to the capacity of resveratrol to induce apoptosis. There is substantial recognition that CHOP has a role in triggering apoptosis [17,19]. Therefore, in this study, we suggested that CHOP is a regulator for resveratrol-induced apoptosis. CHOP was induced by resveratrol in a dose-dependent manner (Fig. 1), and down-regulation of CHOP by mithramycin A and siRNA attenuated resveratrol-induced apoptosis (Figs 6 and 7).

The association between the ability of resveratrol to induce ER stress and apoptosis suggests that ER stress may be of importance for the cytotoxic activity of resveratrol. The linkage between CHOP induction and resveratrol revealed in this study provides a new molecular mechanism that may contribute to the anti-tumorigenic activities of resveratrol. Although resveratrol engages certain components of the apoptotic machinery, a lot of further study on resveratrol is required to delineate exactly its reactions inside the cell, to explain its biological effects.

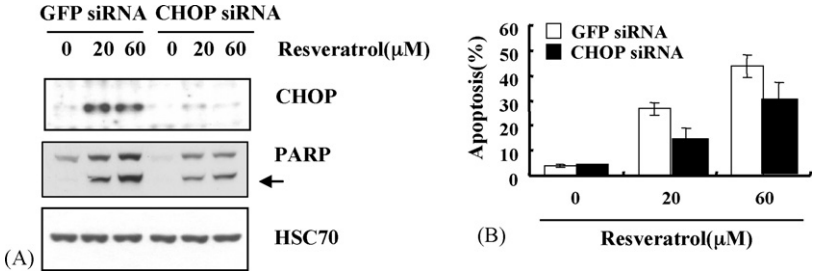

Fig. 7 – Down-regulation of CHOP reduces resveratrol-induced apoptosis in HT29 cells. (A) HT29 cells were transfected with CHOP siRNA or GFP siRNA. Twenty-fours hours after transfection, cells were treated with resveratrol for 24 h. Equal amounts of cell lysates (40 μg) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-CHOP, anti-PARP, or with anti-HSC70 antibody to serve as control for the loading of protein level. The proteolytic cleavage of PARP is indicated by an arrow. A representative study is shown; two additional experiments yielded similar results. (B) HT29 cells were transfected with CHOP siRNA or GFP siRNA. Twenty-fours after transfection, cells were treated with resveratrol for 24 h. Apoptosis was analyzed as a sub-G1 fraction by FACS.

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