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Implication of unfolded protein response in resveratrol-induced inhibition of K562 cell proliferation

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ABSTRACT

Resveratrol (RES), a natural plant polyphenol, is an effective inducer of cell cycle arrest and apoptosis in a variety of carcinoma cell types. In addition, RES has been reported to inhibit tumorigenesis in several animal models suggesting that it functions as a chemopreventive and anti-tumor agent *in vivo*. The chemopreventive and chemotherapeutic properties associated with resveratrol offer promise for the design of new chemotherapeutic agents. However, the mechanisms by which RES mediates its effects are not yet fully understood. In this study, we showed that RES caused cell cycle arrest and proliferation inhibition via induction of unfolded protein response (UPR) in human leukemia K562 cell line. Treatment of K562 cells with RES induced a number of signature UPR markers, including transcriptional induction of GRP78 and CHOP, phosphorylation of eukaryotic initiation factor 2α (eIF2α), ER stress-specific XBP-1 splicing, suggesting the induction of UPR by RES. RES inhibited proliferation of K562 in a concentration-dependent manner. Flow cytometric analyses revealed that K562 cells were arrested in G1 phase upon RES treatment. Salubrinal, an eIF2α inhibitor, or overexpression of dominant negative mutants of PERK or eIF2α, effectively restored RES-induced cell cycle arrest, underscoring the important role of PERK/eIF2α branch of UPR in RES-induced inhibition of cell proliferation.

Introduction

Resveratrol (RES) belongs to a class of defense molecules called phytoalexins and is normally present in many dietary products such as grapes, peanuts, berries and wine [1,2], which is known to affect a broad range of intracellular mediators involved in the initiation, promotion and progression of cancer [2–4]. As an anticancer agent, RES has been shown to suppress the proliferation of a variety of human cancer cell lines and to induce apoptosis [3,5–12].

Cellular stress from cytotoxic agents may result in adaptive mechanisms in several compartments, including endoplasmic reticulum (ER). A wide range of stressful situations (e.g., hypoxia, viral infection, alterations in glycosylation status, disruption of calcium homeostasis, hypoglycemia, hyperglycemia, and oxidative stress), can disrupt this maturation process, resulting in the accumulation of unfolded or misfolded proteins and causing ER stress [13]. The ER attempts to attenuate this stress by activating an adaptive set of stress response signaling pathways termed the unfolded protein response (UPR) [13,14]. UPR in cells undergoing ER stress is characterized by upregulation of ER chaperones, which can improve cell survival by facilitating correct folding or assembly of ER proteins and preventing their aggregation [13]. The primary function of the UPR is to reduce the accumulation of aberrantly folded proteins into the ER and promote cell survival through a transient decrease in protein translation coupled with increases in the ER's capacity to refold and degrade these proteins [15,16]. If this pro-survival response fails to restore homeostatic equilibrium in the ER, a secondary response, triggered in part by the same ER stress sensors that activate the UPR program, promotes apoptosis and cell death.

Although numerous studies have described intracellular changes leading to cell cycle arrest or apoptosis in response to RES treatment, the precise mechanisms by which RES confers inhibition of tumor growth have been largely unexplored. Here, we showed that RES-induced G1 phase arrest of K562 cells and UPR. We also demonstrated that the UPR inhibitor Salubrinal or overexpression of dominant negative mutants of PERK or eIF2α effectively restored RES-induced cell cycle arrest. Taken together, the present study therefore provides evidence to support an important role of ER stress response in mediating the RES-induced growth inhibition.

Materials and methods

Cell culture. Human K562 leukemia cells were grown as suspension culture in RPMI1640 medium supplemented with 10% FBS.

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Reagents. Resveratrol and Salubrinal were purchased from Sigma–Aldrich (St. Louis, MO) and Calbiochem (Darmstadt, Germany), respectively.

Cell viability assays. For cell viability assays, cells were plated in 96-well dishes (1×10^4 cells per well) and treated with different concentrations of RES for 24 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Chemicon, Bedford, MA) according to the manufacturer's instruction.

Analysis of the cell cycle by flow cytometry. Leukemia cells were exposed to different concentrations of resveratrol for 24 h. The cells were fixed in 70% ethanol and stained with 50 μg/ml of propidium iodide (PI). The fluorescence was measured using the Becton Dickinson FACScan (Bedford, MA). Distribution of cells in distinct cell cycle phase was determined using ModFIT cell cycle analysis software.

Western blot analysis. Cells were lysed in lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Cell extract protein amounts were quantified using the BCA protein assay kit. Equivalent amounts of protein (20 μg) were separated using 12% SDS–PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA). Western immunoblotting was performed using primary antibodies against CHOP (Santa Cruz Biotechnology, Santa Cruz, CA), XBP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), GRP78 (BD Bioscience, San Diego, CA), eIF2α (Cell Signaling), phospho-eIF2α (Ser51) (Cell Signaling), caspase 3 (Cell Signaling) or GAPDH (Chemicon, Bedford, MA), horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham Biosciences, UK) and ECL solutions (Amersham Biosciences, UK).

Detection of cell death. For cell death assays, according to the manufacturer's instructions, cells were stained with Annexin V-FITC (Biovision, Mountainview, CA) and propidium iodide (PI, Sigma–Aldrich) and analyzed by fluorescence-activated cell scanner (FACScan) flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

RNA isolation and real-time RT-PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA). Real-time PCR analysis was performed in triplication on the ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA) using the SYBR Green PCR Master mix (Applied Biosystems, Warrington, UK). For CHOP, the forward primer was 5′-ATGAGGACCTGCAAGAGGTCC-3′ and the reverse was 5′-TCCTCCTCAGTCAGCCAAGC-3′. For GRP78, the forward primer was 5′-GTTCTTGCCGTTCAAGGTGG-3′ and reverse was 5′-TGGTACAGTAACAACTGCATG-3′. For β-actin, the forward primer was 5′-GAGACCTTCAACACCCCAGCC-3′ and the reverse was 5′-GGATCTTCATGAGGTAGTCAG-3′. All the reactions were performed in triplicate and normalized using β-actin as control gene.

Cell transfection. The pcDNA3-eIF2α-S51A-HA (dn-eIF2α) plasmid carries the eIF2α cDNA that codes for the unphosphorylatable,

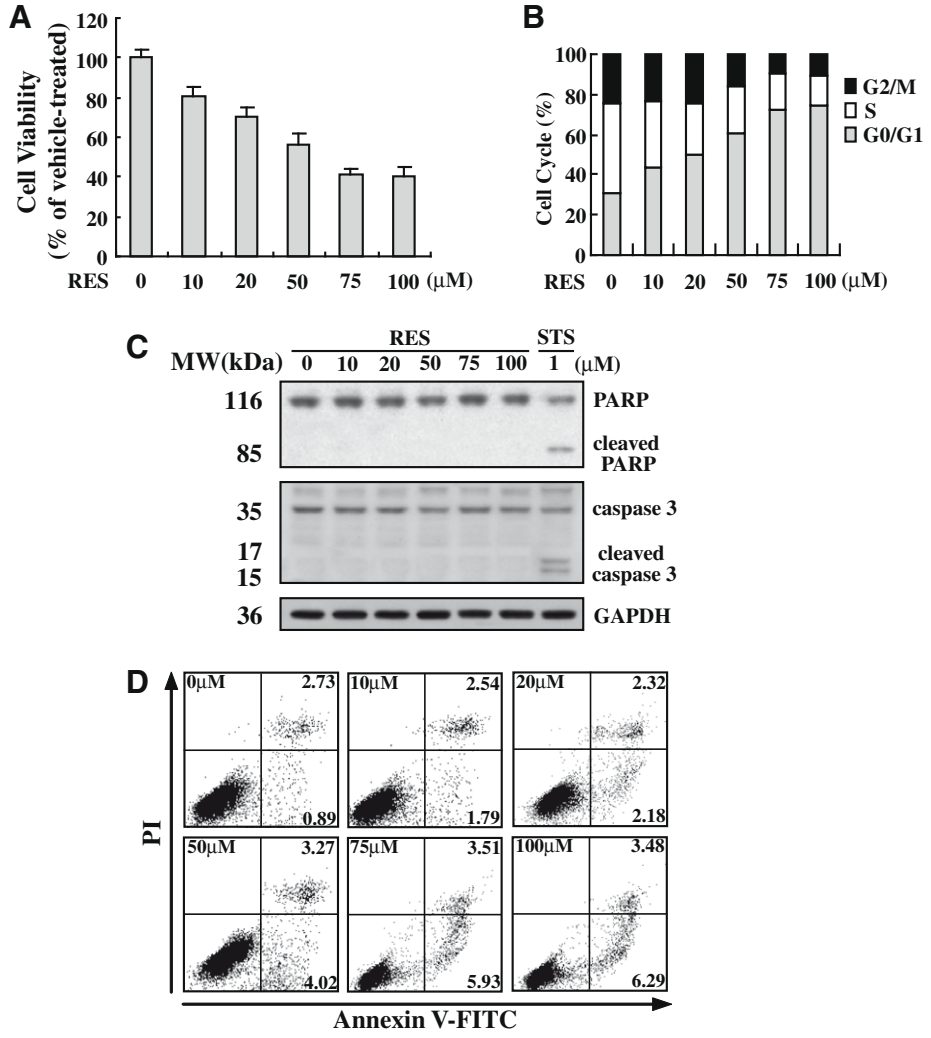

Fig. 1. RES induces loss of cell viability in K562 cells. (A) Cells (10^5/ml) were incubated for 24 h with increasing concentrations of RES. Cell viability was measured by the MTT assay. (B) Cell cycle was analyzed using flow cytometric analysis. The graph represents mean values ± SD from three independent experiments. (C) Cells were treated with RES for 24 h and Western blot was performed. (D) Cells were treated with RES for 24 h, stained with PI and annexin V, followed by flow cytometric analysis.

dominant negative form of eIF2α in which the codon for serine-51 had been changed to alanine-51 [17]. The pcDNA3-PERK-K621M-Flag (dn-PERK) carries the PERK cDNA, in which the codon for lysine-621 was altered to methionine-621, hence expressing the kinase-inactive, dominant negative PERK [18]. The cells were transfected using FuGENE 6 according to the manufacturer's instruction.

Statistics. The statistical significance of the difference was analyzed by ANOVA and post hoc Dunnett's test. Statistical significance was defined as $p < 0.05$. All experiments were repeated three times, and data were expressed as the mean ± SD (standard deviation) from a representative experiment.

Results

Inhibition of proliferation of K562 cells by RES

Exposure of exponentially growing K562 leukemia cells to RES resulted in a marked reduction of cell viability in a dose-dependent manner over the experimental period (Fig. 1A). Inhibition of the cell cycle became evident after treatment with 10 μM of RES in K562 cells (Fig. 1B). The increase of the frequency of G1 cell population by approximately 15–45% coincided with the reduction of the S phase cells when compared with the vehicle treated control (Fig. 1B). Reduction of G2/M phase cells was also demonstrated at higher concentration (75 and 100 μM) of RES (Fig. 1B). This result suggests that the growth inhibitory effect of RES was the result of a block of cell cycle primarily at G1 phase, to a lesser extent at G2/M phase.

After 24 h of exposure to RES, less than 10% of cells were dead even at 100 μM of RES treatment, while the vast majority of K562 cells remained viable, as concluded from the absence of cleavage of caspase 3 and poly-adenine-ribose polymerase (PARP), the terminal caspase target, in Western blotting analysis (Fig. 1C). As a positive control, we used 1 μM of staurosporine (STS) to induce apoptosis of K562 (Fig. 1C). Lack of marked apoptosis in RES-treated K562 cells were further confirmed by FACS evaluation (Fig. 1D).

Induction of unfolded protein response by RES in K562 leukemia cells

To assess the effect of RES on the UPR, we analyzed the induction of UPR targets GRP78 and CHOP. RES-induced GRP78 expression in a dose-dependent manner (Fig. 2A). A significant increase in the

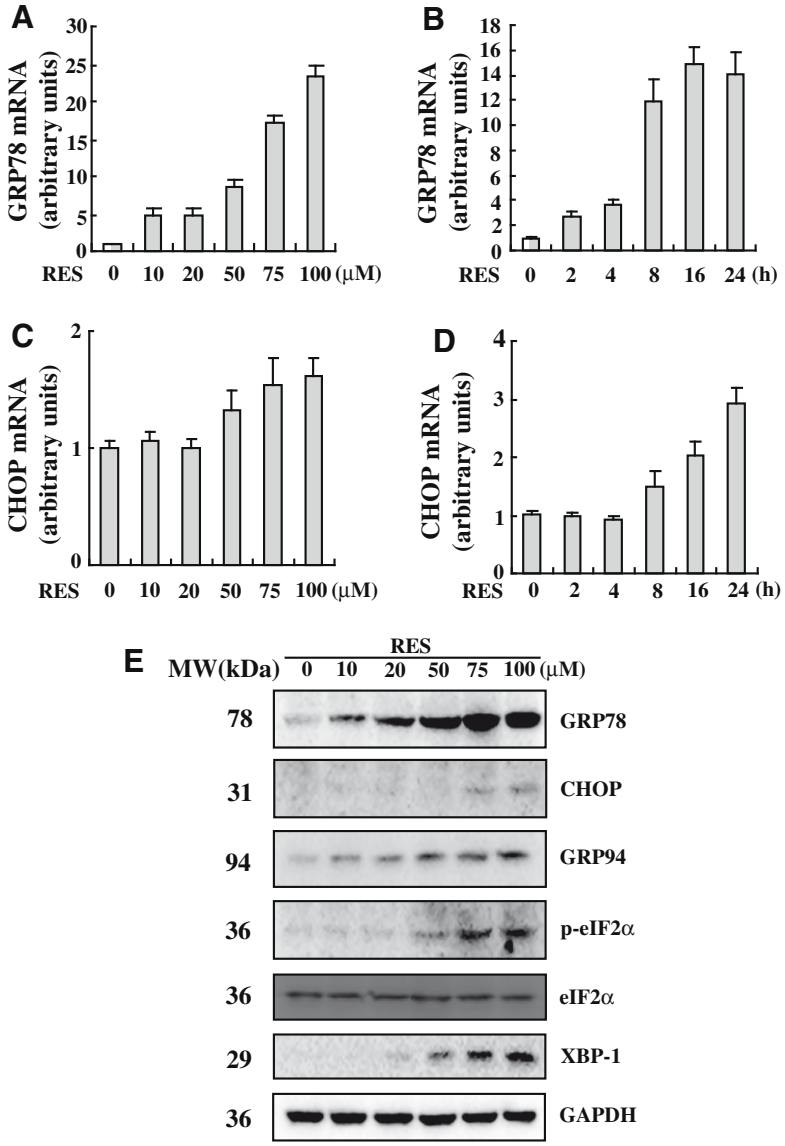

Fig. 2. RES induces UPR like response in K562 cells. (A) Cells were with RES for 8 h, GRP78mRNA levels were analyzed. (B) K562 cells were treated with 100 μM of RES for the indicated period and GRP78 mRNA levels were analyzed. (C) Cells were treated as A, and CHOP mRNA levels were analyzed. (D) Cells were treated as B, and CHOP mRNA levels were analyzed. (E) Cells were treated with various concentrations of RES for 24 h and Western blotting was performed.

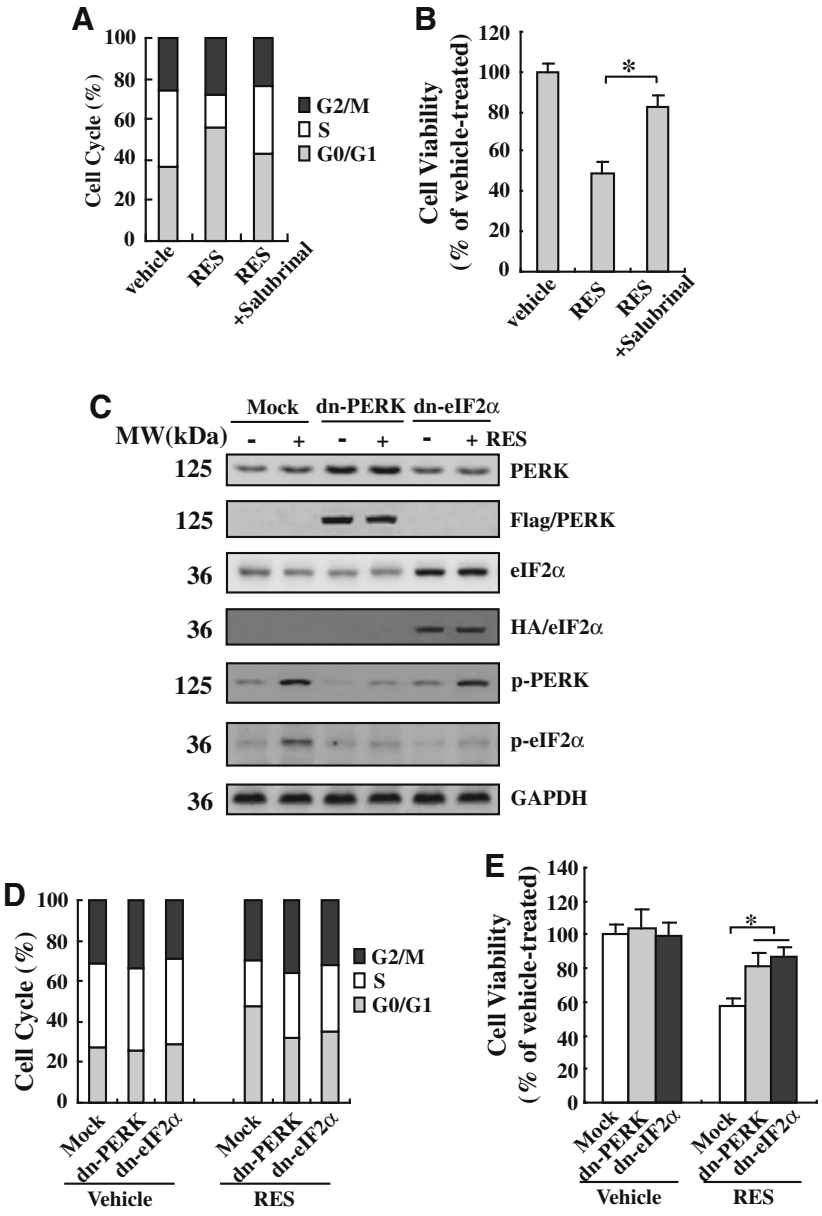

Fig. 3. Ameliorating ER stress restores RES-induced G1 arrest and growth inhibition of K562 cell. (A) K562 cells (10^6/ml) were incubated for 24 h with RES (100 μM) alone or in combination with Salubrinal (5 μM). Cells were harvested and the cell cycle distribution was determined by fluorescence-activated cell sorting. The percentage of cells in each phase of the cell cycle (G0/G1, S, and G2/M) was calculated. The graph represents mean values ± SD from three independent experiments. (B) Cells were treated as A, cell viability was measured by the MTT assay. (C) After transfection with dn-PERK or dn-eIF2a, cells were treated with 100 mM of RES and Western blot was performed. (D,E), Cells were treated as C, and cell cycle distribution (D) and cell viability (E) was investigated using FACS and MTT assay, respectively. $^*P < 0.05$.

expression of GRP78 mRNAs was observed within 2 h after the addition of RES, and the expression reached a maximum at 8–16 h of RES treatment (Fig. 2B). After longer period of RES exposure, CHOP mRNA levels was also upregulated, but with rather minor degree (Fig. 2C and D). Consistent with the data of real-time PCR, Western blotting analysis demonstrated marked increase in GRP78 proteins (Fig. 2E). Several other targets of UPR including GRP94, phosphorylated eIF2α and splicing of XBP-1 were also demonstrated to be increased (Fig. 2E). These results suggest that RES causes activation of the ER stress signaling pathway in K562 cells.

Inhibition of PERK/eIF2α pathway prevents growth arrest induced by RES in K562

It has been shown that UPR induces phosphorylated eIF2α and causes cell cycle arrest (G1 phase) [19]. Therefore, we hypothesized that RES might induce G1 phase arrest via UPR signal. Treatment of cells with 5 μM Salubrinal, which can regulate eIF2α phosphorylation [20], significantly prevented cell cycle arrest mediated by RES (Fig. 3A). In addition, Salubrinal effectively restored the growth inhibition induced by RES (Fig. 3B).

To confirm the role of PERK/eIF2α branch of UPR, we perform transfection assays in which the cDNA encoding either dn-PERK or dn-eIF2α was transfected into K562 cells. Dn-PERK or dn-eIF2α markedly reduced RES-induced phosphorylation of PERK or eIF2α (Fig. 3C), importantly, the inhibition of the PERK/eIF2α pathway by expression of dn-PERK or dn-eIF2α significantly protected cells from RES-induced cell cycle arrest (Fig. 3D) and growth inhibition (Fig. 3E).

Discussion

The use of nontoxic chemical substances is considered a promising alternative strategy for the treatment of human cancer. Over

the past decade, RES has emerged as one of the most promising naturally occurring compound with immense therapeutic potential. Several reports demonstrate the anti-proliferative effect of resveratrol in leukemic cell lines [21–23]. However, unlike other commonly occurring natural or synthetic drugs, the precise effect and mode of action of RES has remained enigmatic. RES has been shown to reduce cell proliferation, cause cell cycle arrest in the G0/G1 phase, the G2/M phase or S phase at concentrations from 5 to 100 μM in various cell models [24–26]. In the current study, we found that RES caused G1 arrest of K562 at 24 h. The occurrence of cell cycle arrest in different phases across various cell lines is likely due to the specific cell type and/or the concentration of RES and/or duration of exposure. However, the mechanisms by which RES induces cell cycle arrest have not been fully established. It has been shown that a substantial overlap between RES-induced genes and genes induced by tunicamycin was observed in *Caenorhabditis* elegans [27]. Moreover, it has been reported that ER stress inhibits the cell cycle through a partial block in the cell transition from G1 to S phase by down-regulating cyclin D [28]. The current study showed that in the experimental conditions employed RES-induced dose-dependent K562 cell G1 arrest and growth inhibition. We also demonstrated that RES induced some markers of ER stress including GRP78, CHOP, GRP94, indicating that RES-induced ER stress in K562 cells. Furthermore, We confirmed that ER stress inhibitor Salubrinal or overexpression of dominant PERK eIF2α prevented RES-induced cell cycle arrest and growth inhibition, indicating that this anti-proliferative effects of RES appeared to be due to its ability to induce ER stress in K562 cells.

Induction of the mammalian UPR involves, in part, enhanced transcription of genes that encode ER chaperone molecules, such as BiP/GRP78, which serves to correct protein misfolding. This response is concomitant with a marked decrease in the rate of overall protein synthesis and with arrest in the G1 phase of the cell division cycle [28]. UPR is activated to restore cellular homeostasis and induces transcription of genes encoding both antiapoptotic and proapoptotic proteins. Thus, severe or prolonged ER stress may cause induction of apoptosis. We found that GRP78 (antiapoptotic) were significantly increased, whereas CHOP (proapoptotic) were only slightly induced by RES exposure. This may suggest that RES preferentially induce survival branches of UPR in K562 cells. In consistent with these results, our data suggested that RES did not significantly induce apoptosis of K562 cells, indicating that the survival threshold is not exceeded in these cells within the time period assayed and concentration used.

Collectively, the current study suggests that RES possesses the property of induction of UPR, which causes cell cycle arrest in K562 cells.

Conflict of interest

There is no competing financial interest in relation to this work.

Acknowledgments

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