RESEARCH ARTICLE

Resveratrol Affects Protein Kinase C Activity and Promotes Apoptosis in Human Colon Carcinoma Cells

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Abstract

Background: Resveratrol has been reported to have potential chemopreventive and apoptosis-inducing properties in a variety of tumor cell lines. Objective: In this study, to investigate the effects of resveratrol on protein kinase C (PKC) activity and apoptosis in human colon carcinoma cells, we used HT-29 cells and examined the PKCα and ERK1/2 signaling pathways. Methods: To test the effects of resveratrol on the growth of HT-29 cells, the cells were exposed to varying concentrations and assessed with the the MTT cell-viability assay. Fluorescence-activated cell sorter (FACS) analysis was applieded to determine the effects of resveratrol on cell apoptosis. Western blotting was performed to determine the protein levels of PKCα and ERK1/2. In inhibition experiments, HT-29 cells were treated with Gő6976 or PD98059 for 30 min, followed by exposure to 200 μM resveratrol for 72 h. Results: Resveratrol had a significant inhibitory effect on HT-29 cell growth. FACS revealed that resveratrol induced apoptosis. Western blotting showed that e phosphorylation of PKCα and ERK1/2 was significantly increased in response to resveratrol treatment. Pre-treatment with PKCα and ERK1/2 inhibitors (Gő6976 and PD98059) promoted apoptosis. Conclusion: Resveratrol has significant anti-proliferative effects on the colon cancer cell line HT-29. The PKC- ERK1/2 signaling pathway can partially mediate resveratrol-induced apoptosis of HT-29 cells.

Keywords: Resveratrol - HT-29 cells - apoptosis - PKC - ERK1/2 signaling pathway

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Introduction

Colon cancer is one of the most common malignancies, which ranks as the second leading cause of cancer-related deaths in developed countries, affecting men and women equally (Lynch et al., 1998; Edwards et al., 2011). Risk factors of colon cancer include age, hereditary (e.g. familial adenomatous polyposis, hereditary nonpolyposis colorectal carcinoma, and inflammatory bowel disease), history of colorectal cancer or polyps, and a family history of colorectal cancer, et al (Kambara et al., 2004; Telang et al., 2006; Brackmann et al., 2009). Potential risk factors for the development of colorectal cancer include use of alcohol and tobacco, lack of regular physical activity and vegetable intake, and obesity (Kirkegaard et al., 2010; Schütze et al., 2011).

Resveratrol (3, 4', 5-trihydroxy-trans-stilbene) is a polyphenol, which has been classified as a phytoalexin because it is produced in spermatophytes in response to injury, UV irradiation and fungal attack (De Silva et al., 1992; Jangm et al., 1997). Resveratrol has been found in 70 plant species, including grapes, mulberries, peanuts, et al (Yang et al., 2001; Endo et al., 2009). It has shown potential chemopreventive properties (Shih et al., 2004), and it has been proved to inhibit growth and induce apoptosis in a variety of tumor cells, including colon cancer, leukemia, breast cancer, and prostate cancer (Filomeni et al., 2007; Hope et al., 2008; Tian et al., 2009; Wang et al., 2010). In addition, resveratrol could maintain colon cancer cell line HT-29 in S phase of cell cycle. However, the mechanism of anti-cancer with resveratrol is still unknown (Wolter et al., 2004; Juan et al., 2008).

Protein kinase C (PKC) is a family of serine/threonine kinases which involved in many important cellular functions, including cell proliferation, migration, differentiation and apoptosis (Carter et al., 2000; Ling et al., 2009). PKC family has more than 12 isoforms, including the conventional PKCs (cPKCα, βI/βII, γ), novel PKCs (nPKC-δ, -ε, -η), and atypical PKCs (aPKCζ), which have been found to exist in various cells. PKCs play an important role in colon carcinogenesis (Atten et al., 2005).

Mitogen-activated protein kinases (MAPK) are serine–threonine protein kinases activated by diverse stimuli,

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such as, cytokines, growth factors, neurotransmitters, hormones, cellular stress and cell adherence. MAPKs play critical roles in cell survival and apoptosis (Peyssonnaux et al., 2001; Wu et al., 2007). The MAPK family has four members, including extracellular regulated kinases (ERKs), c-jun N-terminal or stress-activated protein kinases (JNK/SAPK), ERK 5/big MAP kinase 1 (BMK1) and the p38 group of protein kinases, which have been involved in distinct cellular processes. Activation of ERK has been considered to be involved in cell proliferation (McCubrey et al., 2007; Abrams et al., 2010); while inhibition of ERK activation with a MEK inhibitor, PD98059, induced apoptosis in a dose-dependent manner in breast cancer cells. The ERK pathway was up-regulated in human cancers, such as colon cancer (Davies et al., 2010), and blockage of the ERK pathway would enhance the induction of apoptosis in cancer cells (Kim et al., 2010).

There were no reports which have studied resveratrol-induced apoptosis via the PKCα-ERK1/2 signaling pathway in HT-29 cells. In the study, we evaluated the effects of resveratrol-induced apoptosis on HT-29 cells and investigated the phosphorylation of PKCα and ERK1/2. Inhibition of PKCα and ERK1/2 could significantly enhance HT-29 cell apoptosis which were induced by resveratrol via the decreased phosphorylation of ERK1/2.

Materials and Methods

Reagents

Resveratrol (Sigma-Aldrich, MO, USA) was solubilized in dimethyl sulfoxide (DMSO) at 10 mM and stored at -20ºC. The reagents were diluted to the indicated concentration according to the experimental design. Gö6976 (Calbiochem, CA, USA) was solubilized in DMSO at 1 mM and stored at -20ºC. Rabbit polyclonal antibodies phospho-PKCα, PKCα, ERK, and β-actin and a mouse monoclonal antibody phospho-ERK were obtained from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from QED, Biovision, Inc (Hercules, CA, USA). The enhanced chemiluminescence (ECL) kit was purchased from GE Healthcare (USA).

Cell culture

The HT-29 cell line was obtained from ATCC. The cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin under standard incubator conditions (humidified atmosphere, 95% air, 5% CO_2, 37°C). Cells were passaged every 3 days with 1:3 ratio using 0.125% trypsin-0.02% EDTA. Passage three to four were used for all experiments, and either 5×10^5 cells in 90-mm dish or 105 cells in 60-mm dish were grown in RPMI 1640 supplemented with 10% dextran-coated charcoal (DCC)-FBS. After culture for 24 h, the cells were starved overnight with RPMI 1640 containing 1% DCC-FBS. After washing with phosphate-buffered saline (PBS), the cells were collected at the indicated time points. To test the effects of resveratrol on the growth of HT-29 cells, the cells were exposed to varying concentrations of resveratrol. In inhibition experiments, quiescent HT-29 cells were treated with 100 nM Gő6976 or 20 μM PD98059 for 30 min, followed by treatment with 200 μM resveratrol for 72 h. After incubation, the cells were harvested for further experiments.

Cell death analysis

To evaluate the effects of resveratrol on cell death, HT-29 cells were seeded with a density of 5×10^5 cells in 60-mm plate. After starvation with RPMI 1640 containing 1% DCC-FCS for 24 h, cells were treated with different concentration resveratrol (50 μM, 100 μM , 200 μM, 300 μM and 400 μM) for 24 h, 48 h, and 72 h. Next the cells were detached with trypsin–EDTA, stained with trypan blue solution (0.04% w/v) and counted using a hemocytometer. The number of dead and live cells was calculated in each group, and vehicle controls (0.1% DMSO) were included in each experiment. Each condition was repeated three times.

Detection of apoptotic cells

HT-29 cells were grown in 6-well plates with a density of 10,000 cells per well. After treatment with 100 μM of resveratrol for 24 h, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature, then permeabilized with 0.1% Triton-X-100 (Sigma-Aldrich) for 30 min, rinsed with PBS, and incubated for 5 min at room temperature with 0.01% DAPI stain (Sigma-Aldrich). Excess stain was removed by washing with PBS. Stained nuclei were observed under a Leica fluorescent microscope. The cells with condensed and/or fragmented nuclei were scored as apoptotic cells.

Quantification of apoptosis by flow cytometry

HT-29 cells were treated with 50 μM, 100 μM, 200 μM, 300 μM resveratrol, or 0.1% DMSO for 72 h. Adherent cells were harvested and fixed in 70% cold ethanol overnight. The cells were subsequently treated with propidium iodide (50 mg/ml) and RNase (20 mg/ml) for 15 min and protected from light, until analysis using a flow cytometer (Beckman) equipped with an air-cooled argon ion laser emitting at a wavelength of 488 nm at 15 mw. DNA histograms were assessed using the Beckman Coulter cytometry software from a minimum of 10,000 events per sample. In addition, the cells were pre-treated with 100 nM Gö6976 or 20 μM PD98059 for 1 h, then treated with or without 100, 200, or 300 μM resveratrol for 72 h and harvested for flow cytometry. All of the experiments were performed in triplicate.

Western blot of PKCα and ERK

In our study, PKCα and ERK expression and phosphorylation were detected by Western blot. HT-29 cells were incubated with resveratrol (200 μM, 300 μM, or 400 μM) for 24 h. PKCα and ERK expression was detected after incubating with 200 μM resveratrol for 5 to 120 min. The treated cells were lysed and western blot was performed using a standard protocol. Briefly, protein were extracted by lysing 5×10^6 cells in buffer A (20 mM Hepes, pH 7.4, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM dithiotheitol, 1 mM Na_3VO_4,

1% Triton, 10% glycerol) supplemented with protease inhibitors (4%) for 15 min on ice followed by sonication for 10 seconds. The protein concentration was determined with the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). 60 μg proteins were boiled for 10 min and electrophoresed on 12% SDS–polyacrylamide gel, then transferred onto nitrocellulose membranes (Pall Corporation, Ann Arbor, MI, USA) by electroblotting. Membranes were probed with antibodies as indicated (non-specific binding was blocked in TBS with 5% non-fat dry milk), then incubated with HRP-conjugated rabbit or mouse secondary antibodies. Antibody binding was detected using enhanced chemiluminescence (Amersham) on Hyperfilm (Fuji, Japan) according to the manufacturer's instructions. The same blots were subsequently stripped and re-blotted with corresponding pan antibodies, and the membranes were re-probed with a β-actin antibody as a loading control. Band intensities were quantified using GeneSnap software and normalized to the corresponding total ERK or β-actin levels.

Statistical analysis

Each experiment was performed in triplicate and the data were expressed as mean values ± standard errors (mean ± SEM). Statistical analysis was performed with the SPSS 11.5 software for one-way analysis of variance (ANOVA) and Student's t-tests at each time or concentration point. Differences were considered significant when $p<0.05$.

Results

Resveratrol inducing HT-29 cell death

To explore the possibility of colon cancer cell death occurrence after resveratrol treatment, HT-29 cells were incubated with 50 μM, 100 μM, 200 μM, 300 μM, or 400 μM resveratrol for 24 h, 48 h, or 72 h. Trypan blue exclusion assay showed both inhibition of cell growth and the induction of cell death occurred in a dose and time-dependent manner (Figure 1).

Resveratrol inducing HT-29 cell apoptosis

It has been shown that resveratrol included cell necrosis and apoptosis. To determine whether resveratrol induces HT-29 cell death via the apoptosis pathway, FACS was used. Typical apoptotic bodies were observed in cells with DAPI staining after treated with 100 μM resveratrol for 24 h. FACS showed that resveratrol (100 μM, 200 μM, or 300 μM) induced significant apoptosis at 72 h ($p<0.05$; Figure 2), suggesting HT-29 cell death induced by resveratrol was via apoptosis pathway.

PKCα and ERK levels and phosphorylation

Western blot results showed that PKCα phosphorylation was significantly increased in a time- and dose- dependent manner with resveratrol treatment. The increasing phosphorylation of PKCα was initially detected in HT-29 cells treated with 200 μM resveratrol for 5 min, and up to 120 min (Figure 3). This result suggests that PKCα activation was involved in resveratrol-induced apoptosis.

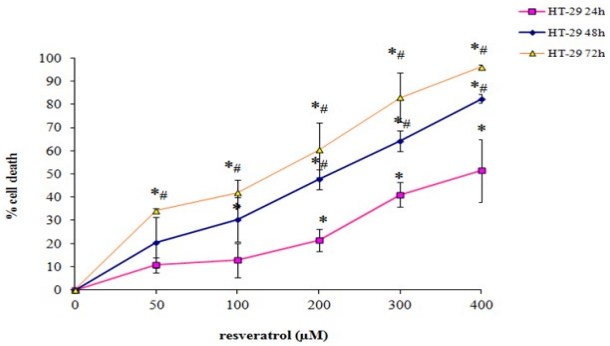

Figure 1. Effects of Resveratrol on HT-29 Cell Death. Cells were treated with the indicated concentrations of resveratrol for 24 h, 48 h, or 72 h. Cellular viability was assessed using MTT assay. Data represent mean±SD from three independent experiments. *$p<0.05$ as compared with control, #$p<0.05$ as compared with treatment of resveratrol for 24h

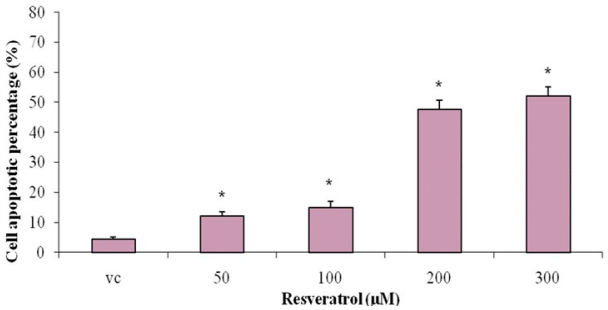

Figure 2. Resveratrol Induced Significant Apoptosis after 72 h. The percentage of cell apoptosis was measured by FACS. vc = vehicle control. All experiments were performed three times and provided the same results. Data represent mean±SD of three values. *$p<0.05$ compared to VC

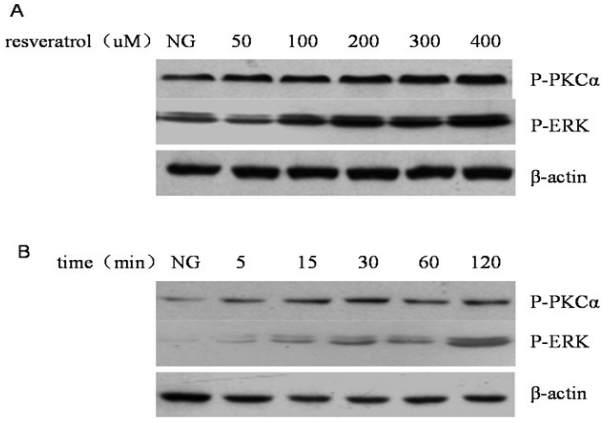

Figure 3. Western Blot of PKC and ERK Expression in HT-29 Cells. A. HT-29 cells treated with 50μM, 100 μM ,200 μM ,300 μM or 400 μM resveratrol for 15min. B. HT-29 cells treated with 200 μM resveratrol for 5min,15min, 30min,60min or 120min. Every experiment was repeated three times independently

Inhibition of PKCα enhancing resveratrol-induced apoptosis

To explore the relationship between resveratrol-induced apoptosis and PKCα activation, HT-29 cells were treated with PKCα inhibitor Gő6976 before incubation with resveratrol. Gő6976 significantly enhanced resveratrol-induced apoptosis. The percentage of apoptosis was increased from 15.0% to 24.9% (100 μM resveratrol,

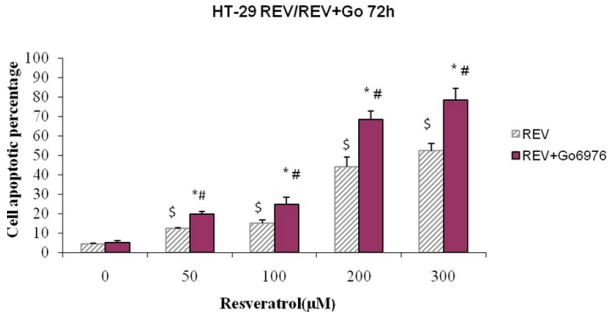

Figure 4. Effects of PKC Inhibitor Gő6976 on Resveratrol (REV) Induced Apoptosis. HT-29 cells were pre-treated with Go6976 (Go 100 nM) for 1 h followed by resveratrol incubation for 72 h. $, p<0.05 compared to vehicle control, *p<0.05 compared to the corresponding resveratrol only value, #p<0.05 compared to the Go-treated only value

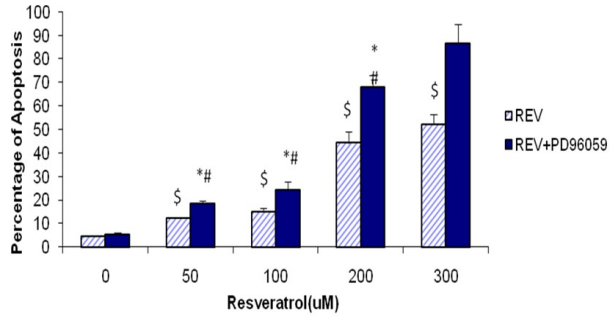

Figure 5. Effects of ERK1/2 Inhibitor PD 96059 on Resveratrol (REV) Induced Apoptosis. HT-29 cells were pre-treated with PD 96059 (100 nM) for 1 h followed by resveratrol incubation for 72 h. $ p<0.05 compared to vehicle control, *p<0.05 compared to the corresponding resveratrol only value, #p<0.05 compared to Go-treated only value

72 h), 44.1% to 68.4% (200 μM resveratrol), and 52.2% to 78.3% (300 μM resveratrol), respectively (Figure 4). However, no apoptotic cells were detected in the vehicle control or in the presence of Gő6976 alone. These results imply that the activation of PKCα by resveratrol plays a negative regulatory role in resveratrol-induced apoptosis.

Resveratrol-induced increasing ERK1/2 phosphorylation

MAPKs are located in downstream of PKC signaling pathways, and it could trigger apoptosis and chemosensitivity in tumor cells. To investigate whether resveratrol-induced apoptosis is associated with the activation of MAPKs, HT-29 cell lysates were treated with 200 μM resveratrol for varying durations or with different doses for 24 h, then performed western blot with antiphospho-ERK1/2 antibody. The phosphorylation of ERK1/2 increased rapidly after 30 min of resveratrol treatment and increased gradually to 120 min (Figure 3). A time- and dose- dependent increase of ERK1/2 also appeared with resveratrol treatment. These results suggest that ERK1/2 might be involved in resveratrol-induced signaling pathway in HT-29 cells.

Inhibition of ERK1/2 enhancing resveratrol-induced apoptosis

To understand the role of ERK1/2 in resveratrol-induced HT-29 cell apoptosis, we investigated the relationship between ERK1/2 activity and resveratrol–induced apoptosis. HT-29 cells were treated with PD98059, an inhibitor of ERK1/2, prior to resveratrol incubation, and then apoptosis was analyzed by FACS. The inhibition of ERK1/2 significantly enhanced resveratrol-induced cell apoptosis compared to resveratrol alone (Figure 5), indicating that ERK1/2 plays a negative regulatory role in resveratrol–induced cell apoptosis. Inhibition of PKCα or ERK1/2 reduced the phosphorylation of ERK1/2.

HT-29 cells were treated with Gő6976 or PD98059 prior to resveratrol and the cell lysates were analyzed by FACS to reveal the relationship between resveratrol-induced apoptosis and PKCα/ERK1/2 activation. ERK1/2 phosphorylation was significantly inhibited by either inhibitor, though it was increased during long-term resveratrol treatment, even in the presence of the inhibitors, suggesting a partial effect of the two inhibitors on ERK1/2. The results suggest that ERK1/2 may be one of the downstream intermediates in the PKCα signaling pathway.

Discussion

In the study, resveratrol was shown to have significant anti-proliferative effects on the colon cancer cell line HT-29. The PKC- ERK1/2 signaling pathway could partially mediate resveratrol-induced HT-29 cell apoptosis. Resveratrol induced the apoptosis and anti-apoptosis pathways simultaneously. Both PKC inhibitor Gő6976 and ERK1/2 inhibitor PD98059 could promote resveratrol-induced apoptosis. These findings may offer a therapeutic benefit in resveratrol treatment.

In recent years, many studies have been carried out to investigate the inhibitory effects of resveratrol on growth of cancer cells. Resveratrol induced apoptosis in a number of cell types, including human prostate cancer cells (Hsieh et al., 2011), colon cancer cells (Marel et al., 2008), gastric cancer cells, and breast cancer cells (Atten et al., 2005). Resveratrol has been proposed not only as a cancer chemopreventive reagent, but also an adjuvant therapy in the treatment of cancer (Fukui et al., 2010). In previous studies, PKCα was considered as a PKC isoform which was associated with proliferation in many cell types, and many reports have indicated that the inhibition of PKCα was sufficient for apoptosis induction, suggesting that PKCα could suppress apoptosis in some cells (McMillan et al., 2003;\Wen-Sheng et al., 2006). We also found that PKCα was stimulated by resveratrol.

Although extensive studies have been conducted on the apoptosis–inducing in several cancer cell lines with resveratrol, however, the mechanism of phosphorylation has not been adequately investigated in HT-29 cells. To the best of our knowledge, we provide the first evidence that resveratrol induces remarkable PKCα phosphorylation in a time- and dose-dependent manner. The PKCα-ERK1/2 signaling pathway could partially mediate the inhibition of resveratrol-induced HT-29 cell apoptosis.

Signal transduction pathways play an important role in cancer cell proliferation, apoptosis, oncogenic transformation, and tumor progression (MurrayNR et al., 2011). These pathways involved protein kinase at multiple levels. In this study, ERK1/2 levels were changed substantially when cells were treated with

resveratrol; treatment with ERK inhibitor PD98059 further promoted cell apoptosis, which are consistent with other studies (Komatsu et al., 2006; Keller et al., 2007). The results suggest that resveratrol interfered with the phosphorylation of ERK.

We also showed for the first time that resveratrol simultaneously evoked the apoptosis and anti-apoptosis pathways. By activating the downstream pathway, PKCα played a role in drug resistance in HT-29 cells. Both Gő6976 and PD98059 promoted resveratrol-induced apoptosis through inhibiting ERK phosphorylation.

Activation of PKCα-ERK1/2 signal pathway might be one of mechanisms of resveratrol-induced HT-29 cells apoptosis. However, the molecular mechanisms involved in resveratrol-induced apoptosis have been reported to be different, Fas/Fas ligand mediated apoptosis, p53 and cyclins A, B1 and cyclin-dependent kinases cdk 1 and 2. In addition, resveratrol also possesses antioxidant and anti-angiogenic properties.

Resveratrol selectively antagonizes PKCα activation in association with the suppression of ERK1/2 activation in HT-29 cells. Our results provide evidence that resveratrol may have potential value as an adjuvant therapy in the treatment of colon cancer.

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The author(s) declare that they have no competing interests.

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