Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-\(\alpha\).

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Abstract

Resveratrol, a naturally occurring stilbene with antitumor properties, caused mitogen-activated protein kinase (MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2)) activation, nuclear translocation of Ser15-phosphorylated p53, and p53-dependent apoptosis in hormone-insensitive DU145 prostate cancer cells. Exposure of these cells to epidermal growth factor (EGF) for up to 4 hours resulted in brief activation of MAPK followed by inhibition of resveratrol-induced signal transduction, p53 phosphorylation, and apoptosis. Resveratrol stimulated c-fos and c-jun expression in DU145 cells, an effect also suppressed by EGF. An inhibitor of protein kinase C (PKC)-\(\alpha\), -\(\beta\), and -\(\gamma\) (CGP41251) enhanced Ser15 phosphorylation of p53 by resveratrol in the absence of EGF and blocked EGF inhibition of the resveratrol effect. EGF caused PKC-\(\alpha\)/3 phosphorylation in DU145 cells, an effect reversed by CGP41251. Activation of PKC by phorbol ester (phorbol 12-myristate 13-acetate) enhanced EGF action on ERK1/2 phosphorylation without significantly altering p53 phosphorylation by resveratrol. DU145 cells transfected with a dominant-negative PKC-\(\alpha\) construct showed resveratrol-induced ERK1/2 phosphorylation and Ser15 phosphorylation of p53 but were unresponsive to EGF. Thus, resveratrol and EGF activate MAPK by discrete mechanisms in DU145 cells. The stilbene promoted p53-dependent apoptosis, whereas EGF opposed induction of apoptosis by resveratrol via a PKC-\(\alpha\)-mediated mechanism. Resveratrol also induced p53 phosphorylation in LNCaP prostate cancer cells, an effect also inhibited by EGF. Inhibition of PKC activation in LNCaP cells, however, resulted in a reduction, rather than increase, in p53 activation and apoptosis, suggesting that resveratrol-induced apoptosis in these two cell lines occurs through different PKC-mediated and MAPK-dependent pathways. [Mol Cancer Ther 2004;3(11):1355–63]

Introduction

Resveratrol is a stilbene that inhibits experimental tumorigenesis and tumor growth (1, 2). We have shown recently that resveratrol causes apoptosis in human thyroid cancer cell lines (3) and androgen-insensitive (DU145) human prostate cancer cells (4). The mechanism of this action of resveratrol in DU145 cells involves mitogen-activated protein kinase [MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2)] phosphorylation and activation, increased cellular accumulation of p53, and phosphorylation of Ser15 of p53 (3, 4). In our prior studies of DU145 and thyroid cancer cells, it was not clear at what point in this signal transduction cascade, upstream of MAPK activation, resveratrol initiated its effect.

Epidermal growth factor (EGF) is a broad-range mitogen that we (5) and others (6) have shown to activate ERK1/2 in addition to activation of the signal transducer and activator of transcription pathway. Growth factors such as EGF, transforming growth factor-\(\alpha\) (TGF-\(\alpha\)), and fibroblast growth factor stimulate the growth of cancers (7). For example, TGF-\(\alpha\) acts via the cell surface EGF receptor (EGFR) to stimulate prostate cancer cell growth (7, 8). Both TGF-\(\alpha\) and EGF are trophic factors for prostate cancers (9, 10), promoting both growth and invasiveness. Binding to EGFR of its ligands EGF and TGF-\(\alpha\) is important with regard to tumorigenesis in various neoplasms (9, 10). The involvement of the receptor in autocrine growth stimulation is also described, as tumors expressing EGFR frequently overexpress the ligands EGF and TGF-\(\alpha\). Several human prostate cancer cell lines, including PC3, DU145, and LNCaP cells, express EGFR and secrete one or both of the ligands, EGF and TGF-\(\alpha\) (11, 12). Direct evidence suggests that autocrine activation of EGFR by EGF and/or TGF-\(\alpha\) regulates prostatic tumor cell growth (13–15).

In the present study, we show that EGF inhibits resveratrol-induced MAPK activation and p53 phosphorylation and thus opposes induction by resveratrol of apoptosis in DU145 human prostate cancer cells. Our results also suggest that activation of conventional protein
kinase C (PKC) isoforms mediates both EGF-induced signal transduction and inhibition by EGF of resveratrol-induced MAPK-dependent apoptosis in DU145 prostate cancer cells. The actions of EGF and resveratrol in this tumor cell line underscore the redundancy of signal transduction mechanisms that lead to activation of MAPK. Comparison of responses to resveratrol and EGF in DU145 cells with those in another prostate cancer cell line (LNCaP) also indicate the variability in response of signal transduction pathways leading to resveratrol-induced MAPK-dependent apoptosis in different prostate cancer cell lines.

Materials and Methods

Cell Lines

The human prostate cancer cell lines, DU145 and LNCaP (16), were kindly provided by Dr. J. Bennett (Albany Medical College, Albany, NY). Cell lines were maintained in DMEM (DU145) or RPMI 1640 (LNCaP) supplemented with 10% fetal bovine serum in a 5% CO2/95% O2 incubator at 37°C.

Reagents

Resveratrol and staurosporine were obtained from Calbiochem (San Diego, CA) and EGF was from Biosource International (Camarillo, CA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. (St. Louis, MO). CGP41251 (CGP) was obtained as a gift from Novartis Phar (Basel, Switzerland).

Cell Fractionation

Fractionation in a microfuge and preparation of nucleoproteins was done according to methods reported previously (3, 4). Nuclear extracts were prepared by rehydration of the crude nuclei in high salt buffer (420 mmol/L NaCl, 20% glycerol) at 4°C with rocking for 1 hour. The supernatants were collected after subsequent centrifugation at 4°C and 13,000 rpm for 10 minutes.

Confocal Microscopy

Exponentially growing DU145 prostate cancer cells were grown on glass microscope slides and incubated for 2 days with nonserum medium prior to being treated with resveratrol for 4 hours. The samples were fixed with 5% formaldehyde in acetone for 20 minutes. Cells were permeabilized in 100% methanol for 10 minutes and rehydrated in 90% methanol for 30 minutes. The cells on the slides were incubated with the anti-Ser15-phosphorylated p53 (pSer15-p53) antibody (Cell Signaling, Beverly, MA) for 2 hours. This was followed by incubation with a rhodamine-labeled goat anti-rabbit IgG secondary antibody (Zymed Laboratories, Inc., South San Francisco, CA) at 37°C for 1 hour. The fluorescent signals for p53 (green) were analyzed using a laser scanning confocal microscope (Zeiss LSM 510 META, Carl Zeiss, Inc., Thornwood, NY).

Immunoprecipitation and Immunoblotting

The techniques are standard and have been described previously (17–19). For immunoprecipitation, 200 μg of each nucleoprotein sample were exposed to anti-MDM2, and immunoprecipitated proteins were separated by SDS-PAGE. The proteins were electrotransferred to Immobilon membranes (Millipore, Bedford, MA) and immunoblotted with antibody to p53. Additional nucleoprotein samples were separated by electrophoresis without prior immunoprecipitation. After blocking with 5% milk in TBS containing 0.1% Tween 20, the membranes were incubated with selected antibodies overnight including polyclonal anti-phosphorylated ERK1/2 (pERK1/2), anti-p53, anti-pSer15-p53, anti-pan-PKC, and anti-phosphorylated PKC-α/β (Cell Signaling) and monoclonal anti-p53, anti-MDM2, and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies were either goat anti-rabbit IgG (1:1,000) or rabbit anti-mouse IgG (1:1,000, DAKO, Carpinteria, CA) depending on the origin of the primary antibody. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL). Results shown are representative of three or more studies.

Dominant-Negative Plasmid Transfection

Cells were transfected with a dominant-negative PKC-α (dnPKC-α) construct or with vector alone using Lipofect-AMINE Plus according to the supplier’s instructions. The plasmid was generously provided to Dr. Lennartz by Dr. Alex Toker (Beth Israel-Deaconess Medical Center and Harvard Medical School, Boston, MA).

Reverse Transcription-PCR

Total RNA was isolated as described previously (3, 4, 20). First-strand complementary DNA were synthesized from total RNA (1 μg) using oligo(dT) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). First-strand cDNA templates were amplified for c-fos, c-jun, and GAPDH mRNA by PCR using a hot start (Ampliwax, Perkin-Elmer, Foster City, CA). Primer sequences were as reported previously (3, 21). The PCR cycle was an initial step of 95°C for 3 minutes followed by 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute and 25 cycles and a final cycle of 72°C for 8 minutes. PCR products were electrophoresed through 2% agarose gels containing ethidium bromide (0.2 mg/mL). Gels were visualized under UV light and photographed with Polaroid film (Polaroid Co., Cambridge, MA). Photographs were scanned for quantitation (LabImage software, Kapelan GmbH, Halle, Germany) and illustration. Results from PCR products were normalized to the GAPDH signal.

Apoptosis/Nucleosomes

Cells were harvested and washed twice with PBS. Nucleosome ELISA assays were carried out according to the protocol provided by Oncogene Research Products (Cambridge, MA) and mean ± SE of nucleosome content in samples from three separate studies was determined. Results were normalized to a value of 1 in untreated control samples.

Survival Assay

For survival assays, cells initially seeded in 60 mm culture dishes were treated for 24 hours with resveratrol (1 μmol/L), EGF (1 ng/mL), or both and then trypsinized and subcultured in 60 mm culture dishes (100 cells/dish). After 6 days’ incubation, medium was removed
and cell colonies were stained with trypan blue. The number and percentage of viable cells were determined microscopically in three studies and the results were normalized to a value of 1 in the untreated controls in each study.

Results

Effect of EGF on Activation of p53 by Resveratrol

Treatment of DU145 prostate cancer cells with resveratrol (1 μmol/L) for 4 hours resulted in increased nuclear pSer15-p53 as shown by immunofluorescence (Fig. 1A) and immunoblotting of nuclear fractions (Fig. 1B). Total nuclear p53 and nuclear pERK1/2 also accumulated in these resveratrol-treated cells (Fig. 1B). The nuclear preparation was also blotted with anti-vimentin, a cytosolic protein marker (22), to exclude the possibility of contamination of nuclear samples with cytosolic protein. Treatment of DU145 cells with EGF (1 ng/mL) had no apparent effect alone on these variables at 4 hours in the absence of resveratrol. When EGF was applied to cells for 1 to 4 hours during treatment with resveratrol (1 μmol/L), time-dependent inhibition by EGF of the stilbene effects on ERK1/2 phosphorylation, p53 nuclear accumulation, and phosphorylation of pSer15-p53 occurred (Fig. 1B). Although EGF alone is known to activate ERK1/2, this action takes place in 10 to 30 minutes and is no longer seen in 2 to 4 hours (Fig. 1C).

Figure 1. EGF inhibits resveratrol-induced activation of p53 in human prostate cancer cells. A, DU145 cells grown on glass slides were treated with resveratrol (1 μmol/L) for 4 hours and fixed with 5% formaldehyde in acetone for 20 minutes. Cells were permeabilized and rehydrated in methanol and then incubated with anti-pSer15-p53 followed by a rhodamine-labeled secondary antibody. Slides were analyzed with a Zeiss LSM 510 META confocal microscope. Left, representative cells from control and treated samples demonstrating pSer15-p53 accumulation in nuclei in response to resveratrol; right, corresponding images of the cells by differential interference contrast microscopy (DIC). B, DU145 cells were treated with either resveratrol (RV, 1 μmol/L) or EGF (1 ng/mL) or with EGF for the last 1 to 3 hours of resveratrol treatment or during the entire 4-hour resveratrol treatment. Nuclear proteins prepared after hypotonic cell lysis as described in Materials and Methods and ref. 5 were separated by SDS-PAGE, transferred to Immobilon membranes, and immunoblotted with antibodies to p53, pSer15-p53, or pERK1/2. β-actin immunoblots reflected nuclear sample loading, and vimentin (Vim) detection indicated lack of cytosol in the nuclear preparations compared with a known positive cytosol control. Immunoblots were measured densitometrically. Representative results from three experiments are shown. C, DU145 cells were treated with EGF (1 ng/mL) for 10 to 240 minutes and nuclear samples were prepared and immunoblotted with pERK1/2 or β-actin antibody as in B. D, LNCaP cells were treated with resveratrol (10 μmol/L, 4 hours) and EGF (1 ng/mL, 1–4 hours) as in B. Resveratrol-induced nuclear accumulation of p53, pSer15-p53, and pERK1/2 was determined as in B.
Parallel studies of the actions of resveratrol and EGF were carried out in LNCaP cells and are shown in Fig. 1D. Resveratrol again caused phosphorylation of ERK1/2 in 4 hours and an increase in nuclear p53 and pSer15-p53 in these cells in the same period. EGF also inhibited these effects, particularly after 3- to 4-hour treatment.

EGF dose-response effects on the actions of resveratrol are shown in Fig. 2A and B. DU145 cells treated with resveratrol showed ERK1/2 activation as well as accumulation in nuclear fractions of p53 and pSer15-p53. These effects were inhibited by EGF in a dose-dependent manner (Fig. 2A). Treatment of cells with EGF alone for 4 hours did not seem to affect p53 accumulation at the concentrations used. As indicated previously in Fig. 1B, ERK1/2 activation by EGF occurs in 10 to 30 minutes and is no longer evident after 2 hours.

Studies parallel to those shown with DU145 cells were carried out in LNCaP cells and are seen in Fig. 2B. The results are similar to those seen in DU145 cells and indicate that EGF inhibited resveratrol-induced nuclear accumulation of p53 and pSer15-p53 and caused a reduction in pERK1/2 over a 24-hour period in LNCaP prostate cancer cells.

**Effect of EGF on Resveratrol-Induced Apoptosis and Cell Survival in DU145 and LNCaP Prostate Cancer Cells**

Studies of apoptosis, determined by nucleosome ELISA, showed that DU145 cells treated with resveratrol underwent this process of programmed cell death and that EGF inhibited this effect of resveratrol (Fig. 3A). Although EGF alone had no effect on apoptosis, the growth factor caused a concentration-dependent inhibition of resveratrol-induced apoptosis; compared with resveratrol alone, the mean reductions in nucleosome content with EGF at 1, 10, and 100 ng/mL, compared with control values, were 26%, 35%, and 46%, respectively. After DU145 cell treatment with resveratrol (1 μmol/L, 24 hours) and/or EGF (1 ng/mL), cell survival assays were conducted. Results are representative of three experiments, each normalized to a value of 1 in untreated control samples. C, DU145 cells were treated with resveratrol (1 μmol/L) with or without EGF (1 ng/mL) for 24 hours. Cytosols from each sample were immunoprecipitated with antibody to MDM2 and the precipitated proteins were separated and immunoblotted with antibody to p53. Western blotting of the MDM2 in aliquots from each immunoprecipitate reflects equality of sample loading.

**Figure 2.** Inhibitory effect of EGF on resveratrol action is EGF concentration dependent in DU145 and LNCaP prostate cancer cells. A, DU145 cells were treated with EGF alone (1–100 ng/mL) for 4 hours or with resveratrol (1 μmol/L, 4 hours). Nuclear fractions were prepared and nucleoproteins were separated by SDS-PAGE and transferred to membranes for immunoblotting with antibodies to p53, pSer15-p53, or pERK1/2. B, LNCaP cells were similarly treated and nucleoproteins were prepared and examined for the presence of p53, pSer15-p53, or pERK1/2.

**Figure 3.** EGF inhibits resveratrol-induced apoptosis in DU145 cells. A, DU145 cells were treated with EGF (1–100 ng/mL) and/or resveratrol (1 μmol/L) for 24 hours. Apoptosis was measured by nucleosome ELISA. Results of three experiments, each normalized to a value of 1 in control samples. Columns, mean; bars, SE. B, DU145 cells were treated for 24 hours with resveratrol (1 μmol/L) and/or EGF (1 ng/mL) and cell survival assays were conducted. Results are representative of three experiments, each normalized to a value of 1 in untreated control samples. C, DU145 cells were treated with resveratrol (1 μmol/L) with or without EGF (1 ng/mL) for 24 hours. Cytosols from each sample were immunoprecipitated with antibody to MDM2 and the precipitated proteins were separated and immunoblotted with antibody to p53. Western blotting of the MDM2 in aliquots from each immunoprecipitate reflects equality of sample loading.
Because MDM2 is involved in p53 turnover (23), it was of interest to determine whether resveratrol and/or EGF altered the complexing of MDM2 and p53 in DU145 cells. The cells were treated with resveratrol (1 μmol/L) with or without EGF (1 ng/mL) for 24 hours. Cytosols were immunoprecipitated with antibody to MDM2, and the precipitated proteins separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with antibody to p53. In untreated cell cytosols, there was some immunocomplex formation of MDM2 and p53 (Fig. 3C). Resveratrol reduced the complexing of MDM2 and p53 in DU145 cells, thus favoring nuclear retention of p53; this effect was partially reversed by the addition of EGF to resveratrol. In contrast, EGF alone enhanced coimmunoprecipitation of p53 and MDM2.

Expression of c-fos and c-jun Induced by Resveratrol and EGF

Resveratrol stimulated expression of the immediate-early genes c-fos and c-jun in DU145 cells (Fig. 4), as we have reported previously in thyroid cancer cells (3). In contrast, there was no significant c-fos or c-jun induced by EGF in DU145 cells (Fig. 4). However, resveratrol-induced expression of these immediate-early genes was inhibited by EGF treatment. Resveratrol stimulated expression of both immediate-early genes (lane 2), whereas EGF had only a slight effect on c-fos expression at the concentration used. The effect of resveratrol was substantially blocked by EGF treatment (lane 4). EGF caused 22.5% and 48.4% reductions, respectively, in the expression of c-fos and c-jun by resveratrol (comparing lanes 2 and 4).

Role of PKC in Transduction of Resveratrol and EGF Signals

DU145 prostate cancer cells were treated with resveratrol (1 μmol/L) for 4 hours in the absence or presence of 1 to 100 nmol/L CGP, a specific PKC-α, -β, and -γ inhibitor (24). In DU145 cells, CGP did not inhibit MAPK activation by resveratrol as shown in Fig. 5A in the immunoblots. The nuclear abundance of p53 and pSer15-p53 induced by resveratrol was not reduced by CGP in these cells. Rather, there was CGP concentration-dependent enhancement of nuclear p53 and Ser15-p53 phosphorylation by resveratrol in DU145 cells as shown in the immunoblots and the accompanying graph. In parallel studies of nucleosome formation (Fig. 5A, bottom graph), resveratrol-induced apoptosis in DU145 cells increased in a manner that was CGP concentration-dependent and paralleled the increase in Ser15 phosphorylation of p53 seen in the graph above. These results suggest that PKC mediates partial suppression of the effect of resveratrol in cells treated with the stilbene.

We also studied the effect of CGP on resveratrol-induced MAPK and p53 activation in the hormone-sensitive prostate cancer cell line (LNCaP). In contrast to the findings in DU145 cells, resveratrol-induced accumulation of nuclear pERK1/2 as well as p53 accumulation and phosphorylation of Ser15-p53 were all inhibited by CGP, again in a concentration-dependent manner (Fig. 5B, immunoblots and top graph). Apoptosis, indicated by nucleosome ELISA in resveratrol-treated LNCaP cells, was also inhibited by CGP (Fig. 5B, bottom graph). This suggests that in LNCaP cells the action of resveratrol requires traditional PKC activity. Thus, PKC has cell line–specific actions in the setting of resveratrol treatment.

When DU145 cells were treated with resveratrol in the presence of the less specific PKC inhibitor, staurosporine (25), there were minimal increases in resveratrol-induced MAPK activation and Ser15 phosphorylation of p53 (Fig. 6A). EGF inhibited these effects of resveratrol, but with the further addition of staurosporine the effects of resveratrol on MAPK activation and p53 phosphorylation were again seen. Furthermore, resveratrol-induced dissociation of p53 and MDM2 in DU145 cells was reversed by EGF treatment and this effect of EGF was blocked by staurosporine (Fig. 6B).

DU145 cells were treated with EGF, resveratrol, or both in the presence or absence of CGP (10-100 nmol/L). As shown above with staurosporine, inhibition by EGF of the action of resveratrol on p53 serine phosphorylation was blocked by CGP (Fig. 7A, lanes 6-8). Cytosolic extracts of DU145 cells treated in a similar manner were immunoblotted with antibodies to pan-phosphorylated PKC or phosphorylated PKC-α/β (Fig. 7B). EGF enhanced PKC-α/β phosphorylation (activation), an effect partially inhibited by CGP (lanes 2 and 3). Resveratrol did not affect PKC-α/β phosphorylation (lane 4); however, in the presence of both resveratrol and EGF, phosphorylation increased (lane 6); this effect was inhibited by CGP (lane 7). These results support our hypothesis that the EGF-induced signal is PKC-α/β dependent in DU145 cells, whereas the resveratrol-induced signal is not dependent on PKC-α/β and in fact seems to be inhibited by EGF-induced PKC activation as shown indirectly in Fig. 7A by the action of CGP.

Figure 4. Effects of EGF and resveratrol on c-fos and c-jun expression in DU145 cells. DU145 cells were treated with EGF (1 ng/mL) or resveratrol (1 μmol/L) or both agents for 24 hours and measurements of c-fos, c-jun, and GAPDH RNA were carried out by reverse transcription-PCR. Results of three experiments, normalized to levels of GAPDH. Columns, mean; bars, SE.
Results of measurement of apoptosis in DU145 cells by nucleosome ELISA (Fig. 7C) also indicated that CGP did not affect resveratrol-induced apoptosis, again demonstrating that the stilbene effect was not principally PKC-α/β dependent. However, the suppression of the effect of resveratrol by EGF was PKC-α/β dependent, as it was restored by increasing concentrations of CGP, which inhibited the action of the growth factor.

When DU145 cells were treated with PMA for 24 hours to deplete cellular PKC activity and then treated with resveratrol for another 4 hours, MAPK and p53 phosphorylation were inhibited (Fig. 8A, compare lanes 3 and 6), although there remained some residual PKC in these cells (lane 2). Activation of PKC-α/β by EGF (lane 4) was inhibited by PKC depletion (lane 7). The inhibitory effect of EGF on resveratrol-induced p53 phosphorylation (lane 5) was not reversed by PMA-induced PKC depletion (lane 8), suggesting that PKC depletion was not complete (Fig. 8A).

When DU145 cells were treated instead with resveratrol in the presence of PMA for 4 hours, resveratrol-induced phosphorylation of p53 and ERK1/2 was reduced by PMA (Fig. 8B). There was less pSer15-p53 in cells treated with both EGF and resveratrol in presence of PMA (lane 8) compared with cells without PMA treatment (lane 5). PKC-α/β was activated in EGF-treated cells (lane 4) and in cells exposed to PMA for 4 hours (Fig. 8B, lane 2). DU145 cells were transfected with dnPKC-α plasmid or plasmid control, resulting in a reduction in cellular PKC-α (Fig. 8C, lanes 5-8). The inhibitory effects of EGF on actions of resveratrol in cells with wild-type PKC-α (lane 3 versus lane 4) were not seen in dnPKC-α-transfected cells (comparing lanes 7 and 8) and indicated a clear role for PKC-α in the effect of EGF on resveratrol-induced signaling and apoptosis in DU145 cells.

LNCaP cells were also treated with resveratrol and/or EGF for 4 hours in the presence or absence of PMA. Without PMA exposure, EGF inhibited resveratrol-induced serine phosphorylation of p53 (compare lanes 5 and 3) as also shown in Fig. 2B. Short-term PMA treatment did not suppress serine phosphorylation of p53 by resveratrol in LNCaP cells. The addition of EGF to PMA and resveratrol, however, did suppress serine phosphorylation of p53, suggesting that the signal transduction pathways used by EGF might involve kinases other than traditional PKC in these particular cells.

**Discussion**

In the studies presented above, the induction by resveratrol of apoptosis in androgen-independent (DU145) and androgen-dependent (LNCaP) prostate cancer cells is shown. In both cell lines, this effect of the stilbene is associated with activation and nuclear translocation of ERK1/2, nuclear accumulation of p53 (which is Ser15 phosphorylated), and apoptosis as shown by nucleosome ELISA and survival.

**Figure 5.** Effect of CGP on resveratrol-induced changes in ERK1/2 and p53 and apoptosis in two prostate cancer cell lines. A, DU145 cells were treated with resveratrol (1 μmol/L) in the presence or absence of CGP (1–100 nmol/L) for 4 hours. Nucleoproteins from each sample were separated by SDS-PAGE, transferred to Immobilon membranes, and immunoblotted with antibodies to pERK1/2, p53, or pSer15-p53. Upper graph, results from three experiments showing an increase in resveratrol-induced pSer15-p53 with addition of CGP. Changes in total nuclear p53 and pERK1/2. Lower graph, apoptosis was also measured by nucleosome ELISA. Results from three similar experiments, normalized to a value of 1 in control samples. Columns, mean; bars, SE. B, LNCaP cells were similarly treated with resveratrol (10 μmol/L) in the presence or absence of CGP. Nuclear samples were immunoblotted as in A. Upper graph, results of pSer15-p53 immunoblots in three similar experiments. Lower graph, studies of apoptosis by nucleosome ELISA (n = 3). Columns, mean; bars, SE.
assays. Resveratrol action is also associated with cellular accumulation of c-fos and c-jun, and of p21, as we have shown previously with resveratrol treatment of thyroid cancer cells (3) and prostate cancer cells (4).

Endogenous peptide growth factors, including EGF, insulin-like growth factor, and fibroblast growth factor, have been implicated in the development and progression of androgen-independent prostate cancer (26). EGF is capable of rapidly activating MAPK in several cell lines including DU145 prostate cancer cells, although the effect is short-lived with a maximal effect in 10 to 30 minutes. The present observation that EGF inhibits the action of resveratrol on both MAPK activation and induction of apoptosis in DU145 cells suggests that increased ambient EGF levels would oppose any clinical actions that resveratrol may have.

There are several ways in which p53-dependent transcriptional activity may be regulated. For example, EGF increases the complexing of MDM2 and p53 in cytosol, thus blocking the function of p53 and increasing p53 degradation (27). Studies by others have shown that glucocorticoids prevent p53-induced apoptosis through interaction of the glucocorticoid receptor with p53 and enhanced p53 degradation by Hdm2 (28). On the other hand, thyroid hormone is known to regulate mdm2 oncogene expression independently of p53 (29).

Resveratrol induces expression of c-fos and c-jun in DU145 cells as shown previously by others (1). EGF has been shown to induce c-fos expression in LNCaP cells (30), but we did not observe EGF-induced expression of c-fos and c-jun in the time frames in which our present studies were carried out. It is possible that EGF-induced c-fos and c-jun expression occurs early in onset and is of very limited duration, whereas p53-dependent induction of c-fos and c-jun expression is gradual and prolonged (31). EGF-induced immediate-early genes are involved in cell proliferation (32, 33) and this process may interfere with resveratrol-induced c-fos and c-jun expression. c-fos and c-jun participate in a plethora of signaling pathways (34). Growth factor–induced c-fos and c-jun expression occurs very rapidly and transiently (35, 36). This induction does not involve p53 (37). However, c-fos and c-jun induction can

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Effect of staurosporine on the actions of resveratrol and EGF in DU145 cells. A, DU145 cells were treated with EGF (1 ng/mL), resveratrol (1 µmol/L), or both agents for 4 hours with or without staurosporine (1 or 100 nmol/L), a more general protein kinase inhibitor than CGP. Resveratrol-induced phosphorylation of ERK1/2 and Ser15 phosphorylation of p53 were examined by immunoblotting of nuclear fractions. B, association of MDM2 and p53 was also studied in DU145 cells in the presence or absence of resveratrol (1 µmol/L), EGF (1 ng/mL), and staurosporine (Stauro, 1 or 100 nmol/L). MDM2 was immunoprecipitated from cytosolic fractions and the precipitates separated by SDS-PAGE and immunoblotted with antibody to p53. Immunoprecipitates were also immunoblotted with anti-MDM2 to assure equal loading of samples. Representative of three experiments.

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Effect of CGP on resveratrol-induced p53 and PKC activation and apoptosis in the presence or absence of EGF. A, DU145 cells were treated with EGF (1 ng/mL), resveratrol (1 µmol/L), or both in the presence or absence of CGP (10 or 100 nmol/L) for 4 hours. Activation of MAPK and p53 phosphorylation were determined by immunoblotting of nuclear aliquots with antibodies to pERK1/2 and pSer15-p53. Representative of three experiments. B, DU145 cells were treated for 4 hours with EGF (1 ng/mL), resveratrol (1 µmol/L), or both in the presence or absence of CGP (100 nmol/L). Cytosolic extracts were immunoblotted with either anti-phosphorylated PKC-α/β (pPKC-α/β) or anti-pan-phosphorylated PKC (pan-pPKC) antibody. Representative of three experiments. C, DU145 cells were treated for 4 hours with resveratrol (10 µmol/L), EGF (1 ng/mL), or both agents with or without CGP (10 or 100 nmol/L). The degree of apoptosis determined by nucleosome ELISA was measured in three separate experiments, normalized to a value of 1 in control samples. Columns, mean; bars, SE.
also follow a slower course in hours rather than in minutes and persist for an extended period. In such situations, a role for p53 might be envisioned. In some models, persistent c-fos and c-jun induction has been reported to be associated with an apoptotic outcome (38, 39), but we do not as yet know whether these immediate-early genes are required for resveratrol-induced apoptosis.

In some reports, resveratrol inhibits phorbol ester-induced PKC activation (40, 41). The concentrations of resveratrol in those studies were >10 μmol/L. We observed no increase in phosphorylation of PKC-α/β in cells treated for 4 hours with resveratrol (1 μmol/L), and resveratrol-induced MAPK activation was not inhibited in DU145 cells by either the traditional PKC inhibitor, CGP (10 nmol/L), or the more broad-spectrum kinase inhibitor, staurosporine (100 nmol/L). These findings suggested that conventional PKC isoforms do not play a role in the action of resveratrol on MAPK activation in DU145 cells and were confirmed in our experiments with anti-pan-phosphorylated PKC and anti-phosphorylated PKC-α/β immunoblotting of cells treated with resveratrol.

In contrast, conventional PKC activity was involved in resveratrol-induced action in androgen-sensitive LNCaP cells. Both the conventional PKC-α and the novel PKC-δ have been reported to mediate the apoptotic responses of PKC activators in LNCaP cells (42, 43). LNCaP cells contain p53 wild-type, whereas DU145 cells contain two point mutations in the DNA binding domain of p53 (44). Whether these differences between LNCaP and DU145 cells are relevant to the different actions of PKC and of agents affecting PKC in these two cell lines is not yet known.

PKC-α is involved in EGF action (45, 46). The PKC inhibitors CGP and staurosporine inhibit EGF action as shown above. Immunoblots with anti-pan-phosphorylated PKC and anti-phosphorylated PKC-α/β showed that EGF increased PKC-α/β phosphorylation, an effect inhibited by CGP. Depletion of PKC by 24-hour PMA treatment reduced the EGF effect on resveratrol action (Fig. 8A), and activation of PKC by short-term PMA treatment (4 hours) enhanced the inhibitory effect of EGF on resveratrol-induced Ser15 phosphorylation of p53 (Fig. 8B). dnPKC-α transfection blocked EGF effects but not the action of resveratrol (Fig. 8C). Because EGF-induced inhibition of MAPK activation, p53 phosphorylation, and apoptosis caused by resveratrol were blocked by the inhibitors CGP and staurosporine and by depletion of PKC-α, we conclude that EGF induces conventional PKC-α activation in DU145 prostate cancer cells.

In summary, resveratrol causes activation of ERK1/2 and serine phosphorylation and nuclear accumulation of p53, leading to apoptosis in two prostate cancer cell lines, DU145 and LNCaP. This effect of resveratrol is inhibited by EGF in both cell lines. The action of EGF in DU145 cells is blocked by inhibitors of PKC-α and does not occur in dnPKC-α-transfected DU145 cells. Traditional PKC activity is required for resveratrol-induced effects in LNCaP cells but is inhibitory in resveratrol-treated DU145 cells, suggesting that activation of MAPK and serine phosphorylation of p53 by resveratrol occur by different pathways in these two cell lines. Despite these differences, the stillbene causes apoptosis in both cell lines, although higher concentrations of resveratrol are required to induce apoptosis in LNCaP than in DU145 cells. Our studies suggest that naturally occurring EGF may oppose induction of MAPK-dependent apoptosis by selected chemotherapeutic agents in selected cell populations.
References


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Inhibitory effect of epidermal growth factor on resveratrol-induced apoptosis in prostate cancer cells is mediated by protein kinase C-α

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