Resveratrol Inhibits Phorbol Ester and UV-Induced Activator Protein 1 Activation by Interfering with Mitogen-Activated Protein Kinase Pathways

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ABSTRACT

Resveratrol, a phenolic compound found in grapes and other food products, prevents chemical-induced carcinogenesis in a number of animal models of cancers. To better understand its chemopreventive property, we examined effects of resveratrol on the activity of activator protein 1 (AP-1), a dimeric transcription factor that plays a critical role in the carcinogenesis and tumor transformation. Pretreatment of HeLa cells with resveratrol inhibited the transcription of AP-1 reporter gene by UVC and phorbol 12-myristate 13-acetate (PMA). Pretreatment with resveratrol also inhibited the activation of extracellular signal-regulated protein kinase 2 (ERK2), c-jun N-terminal kinase 1 (JNK1), and p38. Selectively blocking mitogen-activated protein kinase (MAPK) pathways by overexpression of dominant-negative mutants of kinases attenuated the AP-1 activation by PMA and UVC. Interestingly, resveratrol had little effect on the induction of AP-1 reporter gene by active Raf-1, MEKK1, or MKK6, suggesting that it inhibited MAPK pathways by targeting the signaling molecules upstream of Raf-1 or MEKK1. Indeed, incubation of resveratrol with the isolated c-Src protein tyrosine kinase and protein kinase C diminished their kinase activities. Furthermore, inhibition of protein tyrosine kinases and protein kinase C with their selective inhibitors impaired the activation of MAPKs as well as the induction of AP-1 activity by PMA and UVC. In addition, modulation of estrogen receptor activity with 17β-estradiol had no effect on the inhibition of AP-1 by resveratrol. Taken together, these results suggest that the effects of resveratrol on AP-1 and MAPK pathways may involve the inhibition of both protein tyrosine kinases and protein kinase C.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a natural phytoalexin that is found in large quantities in grapes and other food products (Jang et al., 1997; Soleas et al., 1997). The beneficial effects of wine consumption in the prevention of coronary heart disease, so called "French Paradox", have been attributed to the antioxidant and anti-inflammatory properties of resveratrol present in wines (Goldberg, 1996). Recently, resveratrol was found to have a potent anticarcinogenic activity in several animal models of cancer. Resveratrol inhibits formation of preneoplastic lesions in mouse mammary glands and blocks carcinogen-induced tumorigenesis in a two-stage model of mouse skin cancer that was promoted by treatment with phorbol ester (Jang et al., 1997). Resveratrol also strongly inhibits azoxymethane-induced aberrant colon crypts in F344 rats (Steele et al., 1998). In vitro studies show that resveratrol is able to inhibit proliferation of a variety of cancer cells (Mgbonyebi et al., 1998; Mitchell et al., 1999) and chemical-induced transformation (Jang and Pezzuto, 1998). Therefore, clinical trials have been proposed for using resveratrol, a common constituent of the human diet, as a potential cancer chemopreventive agent in humans (Steele et al., 1998). Although the chemopreventive function of resveratrol has been well appreciated, the mechanisms by which resveratrol exerts its anticarcinogenic effects remain largely unknown.

AP-1 is a dimeric transcription factor composed of members of c-Jun and c-Fos families (Angel and Karin, 1991). AP-1 binds a palindromic DNA sequence, known as 12-*O*-tetradecanoylphorbol-13-acetate-responsive element that is present within the regulatory region of a variety of genes, including *c-jun* itself (Angel et al., 1987). Several lines of evidence indicate that AP-1 plays a crucial role in the carcinogenesis and tumor promotion. First, AP-1 activity is often strongly stimulated by tumor promoters, such as 12-*O*-tetra-

ABBREVIATIONS: EGF, epidermal growth factor; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; PTK, protein tyrosine kinase; PKC, protein kinase C; E2, 17β-estradiol; MBP, myelin basic protein; TBST, Tris-buffered saline/Tween 20; AP-1, activator protein 1; ER, estrogen receptor; ATF2, activating transcription factor 2.

decanoylphorbol-13-acetate, UV irradiation, or epidermal growth factor (EGF) (Angel and Karin, 1991). Second, the increased AP-1 activity was associated with the stages of tumor promotion in JB6 cells (Dong et al., 1995). Furthermore, blocking AP-1 activity by pharmacological or biological inhibitors impaired neoplastic transformation by the tumor promoters such as UV light and PMA (Dong et al., 1997; Huang et al., 1997) or by certain oncogenes such as *v-src* and c-Ha-*ras* (Kralova et al., 1998).

AP-1 activity can be regulated by several mechanisms, one of which is the activation of mitogen-activated protein kinase (MAPK) pathways (Karin, 1995). The members of MAPK belong to the superfamily of serine/threonine kinases. To date, at least seven MAPK members have been identified in mammalian cells. Three of them have been well studied: extracellular signal-regulated protein kinases (ERK), c-jun *N*-terminal kinases [JNK; also referred to as stress-activated protein kinases (SAPK)], and p38. The ERK pathway is predominantly activated by mitogens and tumor promoters (Cobb and Goddsmith, 1995). Once activated, ERK1/2 can phosphorylate a ternary complex factor, Elk-1, that further interacts with serum response factor and induces the transcription of *c-fos* through serum-responsive element (Karin, 1995). JNK and p38 are preferentially activated by proinflammatory cytokines and various environmental stresses (Hibi et al., 1993; Kyriakis et al., 1994; Lee et al., 1994). Activation of JNK leads to the phosphorylation and activation of c-Jun and ATF2, which, in turn, activate *c-jun* transcription through the 12-*O*-tetradecanoylphorbol-13-acetate-responsive element (Karin, 1995). p38 can also phosphorylate ATF2 and activate *c-jun* through a similar mechanism (Karin, 1995). Furthermore, like ERK1/2, both JNK and p38 can activate *c-fos* gene through the phosphorylation of Elk-1 (Karin, 1995). Therefore, activation of MAPK pathways not only stimulates the transcriptional activities of AP-1 components but also increases their abundance. Among the other signaling molecules that lead to activation of AP-1 are protein tyrosine kinases (PTK) and protein kinase C (PKC) (Simonson and Herman, 1993). Although their downstream mediators remain to be elucidated, MAPKs are believed to play an essential role in PTK or PKC-mediated signaling.

Given the important roles of AP-1 in cellular transformation, inhibition of AP-1 activity may be an important mechanism for some chemopreventive agents. Thus, in this study, we examined the effects of resveratrol on AP-1 activity induced by UV and phorbol ester and the roles of MAPKs, PTKs, and PKC.

Materials and Methods

Cell Culture and Reagents. HeLa cells (human cervical squamous carcinoma) were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured at 37°C and 5% CO_2 in minimum essential medium supplemented with 10% fetal bovine serum, 2.2 g/l sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were normally starved overnight in serum-free medium before treatment, unless otherwise indicated. Anti-phosphotyrosine monoclonal antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-ERK2 and anti-c-Src polyclonal antibodies were purchased from the Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit anti-p38 polyclonal antibody and MEK1/2 inhibitor PD98059 were purchased from New England Biolabs Inc. (Beverly, MA). EGF, the p38 inhibitor

SB230580, the PKC inhibitors GF-109203X and Go6983, and the protein tyrosine kinase inhibitor PP2, were purchased from Calbiochem (San Diego, CA). Genistein was purchased from ALEXIS (San Diego, CA). Resveratrol, PMA, and 17β-estradiol (E2), and myelin basic protein (MBP) were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from PerkinElmer Life Science Products (Boston, MA). AP-1-luciferase construct containing five copies of AP-1 consensus binding site was kindly provided by Dr. Anning Lin (University of Chicago, Chicago, IL). Rabbit anti-JNK1 antiserum (Ab101), fusion proteins GST-c-Jun (1-79) and GST-ATF2 (1-96), and plasmids encoding different dominant-negative mutants of kinases have been described previously (Chen et al., 1996; Yu et al., 2000).

Immunocomplex Kinase Assays of MAPK Activity. After treatments, cells were washed twice with ice-cold phosphate-buffered saline and harvested in a lysis buffer containing 10 mM Tris-HCl, pH 7.1, 50 mM NaCl, 50 mM NaF, 30 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 5 μM $ZnCl_2$, 2 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride, and 0.5% Triton-X-100. Cell lysates were homogenized by passing through a 23-gauge needle three times and cleared by centrifugation at 12,500g for 15 min at 4°C. Kinase activities of JNK1, p38, and ERK2 were determined by in vitro immunocomplex kinase assays as described previously (Yu et al., 2000). Briefly, endogenous JNK1, p38, or ERK2 was immunoprecipitated with their respective antibody with the aid of protein A Sepharose 4B conjugate (Zymed Laboratories, San Francisco, CA). The immunocomplex was washed twice with the lysis buffer and twice with kinase assay buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM Na_3VO_4, 50 mM β-glycerophosphate, and 10 mM ρ-nitrophenyl phosphate) and resuspended in a 30-μl kinase assay buffer with addition of 2 μCi of [γ-^{32}P]ATP, 20 μM ATP, and 5 μg of the indicated kinase substrates. Kinase reaction was performed at 30°C for 30 min in JNK1 and p38 assays, or for 15 min in ERK2 assay, and terminated by boiling at 95°C for 5 min in Laemmli buffer. The reaction products were resolved in 10% SDS-polyacrylamide gel electrophoresis, visualized by autoradiography, and quantified with the use of a Radioanalytical Imaging System (AMBIS Systems, Inc., San Diego, CA).

In Vitro Assays of Protein Tyrosine Kinase c-Src Activity. Whole-cell lysates were prepared as described under *Immunocomplex Kinase Assays of MAPK Activity*. Endogenous c-Src was immunoprecipitated by incubation of approximately 500 μg of protein with 1 μg of anti-c-Src antibody in the presence of protein A Sepharose 4B. Kinase activity of immunoprecipitated c-Src was determined by autophosphorylation. Briefly, the washed immunocomplexes were resuspended in a 30-μl kinase assay buffer as in the MAPK assay and incubated at 30°C for 15 min. The phosphorylated c-Src was resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

PKC Preparations and Activity Assays. After treatments, cells were washed twice with ice-cold phosphate buffered-saline and then harvested in a buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10 mM NaF, 100 μM Na_3VO_4, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100. Cell lysates were homogenized by passing through a 23-gauge needle four times and left on ice for 30 min before centrifugation at 12,500g for 20 min at 4°C. The total PKC activity in the resulting supernatants was isolated by DEAE-cellulose chromatography as described previously (Yu et al., 2000). Briefly, samples were applied to a 0.5-ml DEAE-cellulose column equilibrated with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, and 2 mM dithiothreitol). After washing the column with 2 ml of buffer A, PKCs were eluted with 0.2 M NaCl, and protein concentration was determined by Bradford assays (Bio-Rad, Hercules, CA). For PKC activity assay, 2 μg of isolated enzymes was incubated with 10 μg of histone H1 in a 50-μl assay buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 μCi of [γ-^{32}P]ATP, and 20 μM ATP. The kinase reaction was performed at 30°C for 10 min and terminated with Laemmli buffer. The

phosphorylated histone H1 was separated by SDS-polyacrylamide gel electrophoresis and quantified with the use of a Radioanalytical Imaging System.

Transient Transfection and Reporter Gene Activity Assays. HeLa cells were plated in six-well plates at a density of 1.5×10^5 cells/well and transfected with different plasmids, as indicated in the figure legends, using the calcium-phosphate precipitation method (Jordan et al., 1996). Total amount of plasmid DNA in each well was adjusted to 5.5 μg with empty vector. Twenty-four hours after transfection, cells were harvested or further treated with different agents. β-Galactosidase activity was determined as described previously (Sambrook et al., 1989). Luciferase activity was determined according to the manufacturer's instructions (Promega, Madison, WI). Briefly, after treatment, cells were washed twice with ice-cold phosphate buffered-saline and harvested in 1× reporter lysis buffer. After brief centrifugation, a 20-μl aliquot of supernatant was assayed for luciferase activity with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The luciferase activity was normalized against β-galactosidase activity and expressed as fold induction over the control cells.

Western Blot Analysis of Tyrosine Phosphorylation. After treatment, cell lysates were prepared as described above. Fifty micrograms of total protein, as determined by the Bradford method, was resolved on 10% SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene difluoride membrane using a semidry transfer system (Fisher). Membrane was blocked with 20% bovine serum albumin in TBST buffer (20 mM Tris-HCl, pH 7.4, 8 g/l NaCl, 0.2 g/l KCl, and 0.1% Tween-20) at 4°C overnight. Membrane was then incubated with 1 μg/ml anti-phospho-tyrosine monoclonal antibody in TBST for 1 h at room temperature. Membrane was washed three times with TBST and blotted with donkey anti-mouse antibody conjugated with horseradish peroxidase for 30 min (1:10,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA). The tyrosine-phosphorylated proteins were visualized using enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Results

Resveratrol Inhibits the Induction of AP-1 Activity by PMA and UVC. PMA is a prototypical tumor promoter. UVC acts not only as a tumor promoter but also as a tumor initiator. Because the increased AP-1 activity is essential for the tumor-promoting action of PMA and UVC, we examined the effect of resveratrol on AP-1 activation by PMA and UVC. Human cervical carcinoma HeLa cells were transiently transfected with AP-1-luciferase construct. After transfection, cells were treated or untreated with different concentrations of resveratrol before challenge with PMA (100 nM) or UVC (30 J/m^2). Luciferase activity was determined 24 h after treatment. As shown in Fig. 1, both PMA and UVC strongly induced AP-1 reporter gene activity. However, in resveratrol-pretreated cells, PMA- and UVC-induced AP-1 activity was significantly reduced. This inhibitory effect of resveratrol on AP-1 activation was concentration-dependent, with an IC$_{50}$ between 25 and 50 μM. In addition, no induction of luciferase activity was observed in the cells transfected with a reporter construct lacking AP-1 binding site, after treatment with PMA or UVC, confirming the role of AP-1 in the activation of reporter gene.

Resveratrol Blocks MAPK activation by UVC and PMA. As described earlier, activation of MAPK pathways play an important role in the activation of AP-1-dependent genes by various stimuli. We next examined the effects of resveratrol on MAPK activities in PMA or UVC-treated cells.

Exposure of HeLa cells to UVC (30 J/m^2) activated three MAPK pathways, JNK, p38, and ERK, as determined by immunocomplex kinase assays using the substrates GST-c-Jun, GST-ATF2, and MBP, respectively (Fig. 2A). Pretreatment with resveratrol inhibited UVC activation of MAPKs. This inhibitory effect of resveratrol was concentration-dependent as seen in AP-1 reporter gene assays (Fig. 2A). Unlike UVC irradiation, PMA treatment had little effect on JNK and p38 activity (data not shown), but strongly stimulated ERK activity (Fig. 2B), which was also inhibited by resveratrol. Interestingly, pretreatment with resveratrol affected the activation of ERK by EGF only slightly (Fig. 2B). Furthermore, incubation of resveratrol with JNK1, p38, or ERK2 immunoprecipitated from UVC-treated HeLa cells did not affect their kinase activities (Fig. 2C), suggesting that resveratrol inhibits MAPK activation by targeting the upstream signaling molecules.

Interfering with MAPK Pathways with the Dominant-Negative Mutants of Kinases Attenuates the Induction of AP-1 by PMA and UVC. To provide evidence for the roles of MAPKs in the activation of AP-1 by PMA and UVC, we examined the effect of different dominant-negative mutants of kinases. As shown in Fig. 3A, cotransfection with a dominant-negative mutant of ERK2, JNK1 or p38 attenuated the induction of AP-1 reporter gene by UVC. Cotransfection with a dominant-negative mutant of ERK2 but not of JNK1 or p38 also inhibited AP-1 activation by PMA, consistent with the observation that PMA preferentially activated the ERK pathway. Similar to the mutants of ERK2, JNK1, and p38, the dominant-negative mutants of Raf-1 and MEKK1 also blocked the activation of AP-1 reporter gene by UVC or PMA, implicating the roles of upstream kinases. In support of this, overexpression of Raf-1, MEKK1, or MKK6 activated AP-1 reporter gene (Fig. 3A). Interestingly, the presence of resveratrol had little effect on the activation of AP-1 by the overexpressed Raf-1, MEKK1, or MKK6 (Fig. 3B). This result suggests that resveratrol may inhibit AP-1
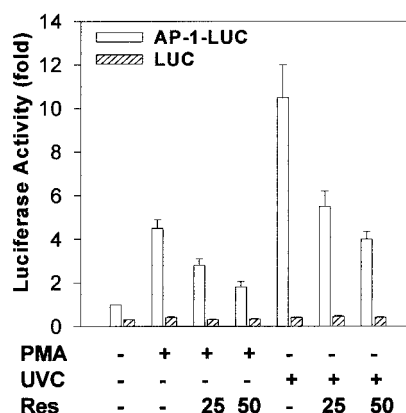

Fig. 1. Inhibition of PMA- and UVC-induced AP-1-reporter gene activity by resveratrol. HeLa cells were plated in six-well plates and transfected with 4 μg of AP-1 reporter construct AP-1-LUC) or the construct lacking AP-1 binding sites (LUC), together with 1 μg plasmid encoding β-galactosidase using calcium-phosphate precipitation method. After transfection, cells were pretreated with different concentrations of resveratrol (micromolar) for 1 h as indicated, and then challenged with PMA (100 nM) or UVC (30 J/m^2) or with the vehicle (0.1% Me$_2$SO) for 24 h. Luciferase activity was measured and normalized as described under *Materials and Methods*. Data shown are means ± S.D. of four independent experiments.

and MAPK pathways by targeting the molecules upstream of Raf-1 or MEKK1.

Resveratrol Blocks Protein Tyrosine Phosphorylation Induced by PMA and UVC. To search for the upstream targets of resveratrol, we examined the involvement of tyrosine kinases, because piceatannol, an analog of resveratrol, has been shown to effectively inhibit protein tyrosine kinases (Geahlen and McLaughlin, 1989). As shown in Fig. 4, treatment of HeLa cells with PMA or UVC stimulated tyrosine phosphorylation of several proteins compared with the control cells. Preincubation of cells with resveratrol (50 μM) abolished the induced tyrosine phosphorylation by both PMA and UVC. However, resveratrol had little effect on

EGF-induced protein tyrosine phosphorylation, consistent with the observation that resveratrol showed little effect on EGF-induced ERK activation (Fig. 2B).

Inhibition of Protein Tyrosine Kinases Attenuates MAPK Activation by UVC and PMA. Inhibition of tyrosine phosphorylation suggests that resveratrol may affect MAPK pathways by acting on protein tyrosine kinases. To test this hypothesis, we examined the effects of protein tyrosine kinase inhibitors on MAPK activation by PMA and UVC. As shown in Fig. 5, pretreatment of HeLa cells with genistein inhibited JNK1 and p38 activation by UVC, and also impaired ERK2 activation by UVC and PMA. Similar result was obtained when cells were treated with another potent protein tyrosine kinase inhibitor PP2 (data not shown). These data suggest the activation of MAPKs by UVC or PMA involves a protein tyrosine kinase-dependent mechanism.

Resveratrol Inhibits PMA- and UVC-Induced c-Src Tyrosine Kinase Activity. As demonstrated above, PMA
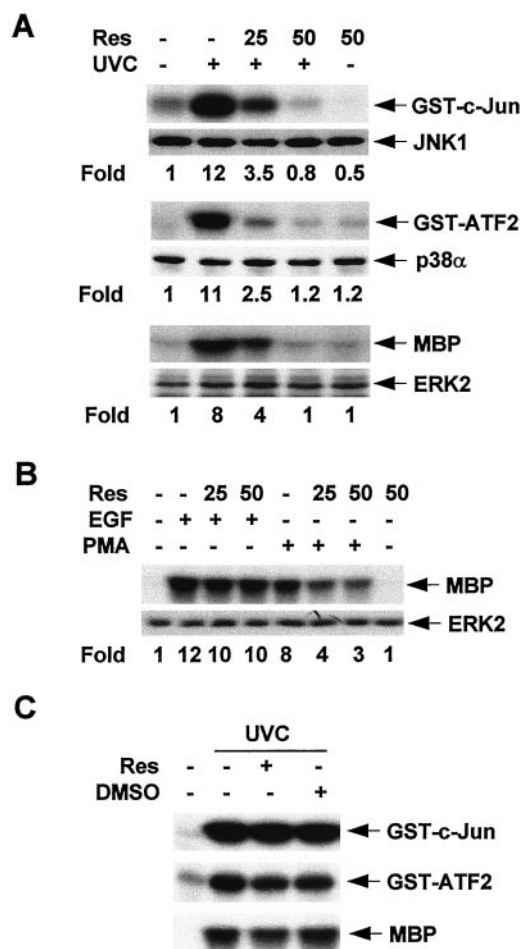

Fig. 2. Effects of resveratrol on MAPK activation by UVC and PMA. A, inhibition of UVC-induced JNK, p38, and ERK2 activities by resveratrol. HeLa cells were untreated or treated with different concentrations of resveratrol (micromolar) for 1 h, and then irradiated with UVC (30 J/m^2). After incubation for additional 30 min, cells were harvested and assayed for JNK, p38, and ERK2 activities by immunocomplex kinase assays with the substrates, GST-c-Jun, GST-ATF2, and MBP, respectively. The protein levels of JNK1, p38, and ERK2 were determined by Western blotting. B, inhibition of PMA-induced ERK2 activation by resveratrol. HeLa cells were pretreated with resveratrol as in A, and then challenged with EGF (10 ng/ml) or PMA (100 nM) for 30 min. ERK2 activity was determined as in A. C, effect of resveratrol on the kinase activity of ERK2, JNK1, and p38 in vitro. HeLa cells were irradiated with UVC (30 J/m^2) or untreated as control. Endogenous JNK1, p38, and ERK2 were immunoprecipitated from the treated or untreated cells with their specific antibody. The immunoprecipitated MAPKs were incubated with kinase assay buffer alone or in the presence of resveratrol (50 μM) or Me$_2$SO (0.1%) for 30 min, and then assayed for kinase activity with their respective substrates as described above. The blots shown are samples of three separate experiments.
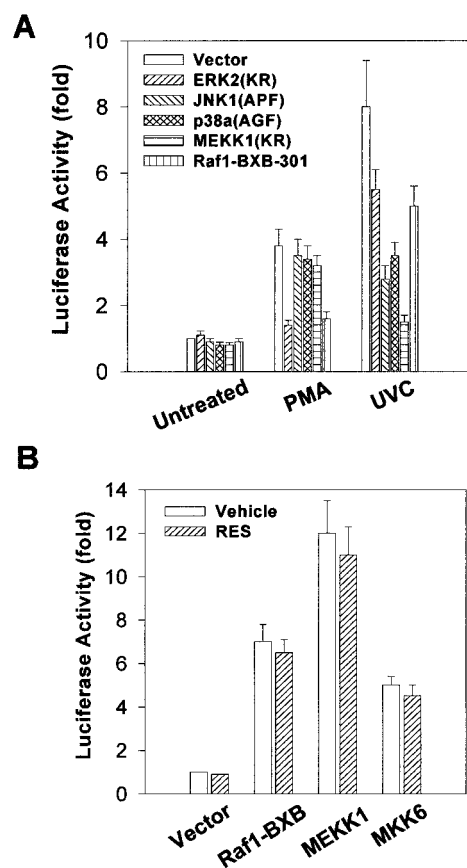

Fig. 3. Role of MAPK pathways in AP-1 activation by PMA and UVC. A, inhibition of PMA or UVC-induced AP-1 activity by dominant-negative mutants of kinases. HeLa cells were transfected with 1 μg of AP-1 reporter construct and 0.5 μg of expression vector of galactosidase, together with 4 μg of plasmid encoding different dominant-negative mutants of kinases. Twenty-four hours after transfection, cells were treated with PMA (100 nM), exposed to UVC (30 J/m^2), or left untreated as control cells. After incubation for additional 24 h, cells were harvested and assayed for luciferase activity. The amount of luciferase activity in empty vector-transfected control cells was arbitrarily set to 1. B, lack of effect of resveratrol on the induction of AP-1 activity by MEKK1, Raf-1, and MKK6. HeLa cells were cotransfected as in A with the plasmid encoding wild-type MEKK1, MKK6, or active form of Raf-1 (Raf1-BXB) in the absence or presence of resveratrol (25 μM). Twenty-four hours after transfection, cells were assayed for luciferase activity as in A. Data presented are means ± S.D. of three separate experiments.

and UVC induced tyrosine phosphorylation of several proteins (Fig. 4). To show one of these tyrosine kinases, we examined the role of c-Src, a nonreceptor protein tyrosine kinase that has been implicated in MAPK activation by several agents including PMA and UVC (Devary et al., 1992; Renee and Kahn, 1997). Treatment of HeLa cells with PMA or UVC stimulated kinase activity of c-Src, as determined by autophosphorylation (Fig. 6A). Pretreatment with resveratrol severely impaired c-Src activity induced by PMA and UVC (Fig. 6A). Furthermore, incubation of resveratrol with activated c-Src immunoprecipitated from UVC-treated cells resulted in loss of kinase activity (Fig. 6B), suggesting that resveratrol acts as an inhibitor of c-Src tyrosine kinase. As a positive control, genistein also abolished the induced c-Src kinase activity when incubated directly with the immunoprecipitated kinase (Fig. 6B).

Involvement of PKC in Action of Resveratrol. Another established signaling molecule involved in the activation of MAPK pathways by PMA and UVC is PKC. We next asked whether resveratrol also acted on PKC. Total PKC (membrane-bound and cytosolic) was isolated from UVC-treated HeLa cells. As illustrated in Fig. 7A, incubation of resveratrol with the isolated PKC resulted in a dose-dependent decrease of kinase activity as determined by the phosphorylation of histone H1. The IC_{50} value for resveratrol to inhibit PKC was around 50 μM, similar to that seen in the inhibition of MAPKs. Furthermore, inhibition of PKC activity with the known inhibitors GF-109203X or Gö6983 diminished the induction of AP-1 reporter gene expression by PMA and UVC (Fig. 7B). Therefore, PKC seems to be one of the targets that leads to the inhibition of MAPK pathways and AP-1 by resveratrol.

The Role of Estrogen Receptors in the Inhibition of AP-1 Activity by Resveratrol. Previous studies suggested that resveratrol may have a potential effect on estrogen receptors (Gehm et al., 1997; Bowers et al., 2000), the intracellular receptors that have been shown to be able to modulate AP-1-dependent gene expression (Paech et al., 1997; Webb et al., 1999). Therefore, inhibition of AP-1 by resveratrol may also involve interaction with estrogen receptors. To test this possibility, we first measured the effects of estradiol, a potent estrogen, on the induction of AP-1 by PMA and UVC. As shown in Fig. 8A, although E2 alone slightly enhanced the induction of AP-1 activity (approximately 1.5-fold over the control), it had no effect on the fold induction of AP-1 activity by PMA or UVC. Furthermore, cotreatment with E2 did not affect the inhibition of PMA and UVC-induced AP-1 activity by resveratrol (Fig. 8B).

Effects of p38, MEK, and Tyrosine Kinase Inhibitors on AP-1-Dependent Gene Induction by UVC and PMA. To substantiate the roles of MAPKs and tyrosine kinases in the activation of AP-1 by UVC and PMA, we employed the known inhibitor of p38, MEK1/2, and protein tyrosine kinases. As shown in Fig. 6, treatment with PD98059 (25 μM), a potent inhibitor of MEK1/2, decreased AP-1 induction by both PMA and UVC. Incubation with SB203580 (5 μM), a specific inhibitor of p38, also reduced the AP-1 activation by UVC but had little effect on AP-1 activation by PMA. Compared with PD98059 and SB203580, genistein showed a more
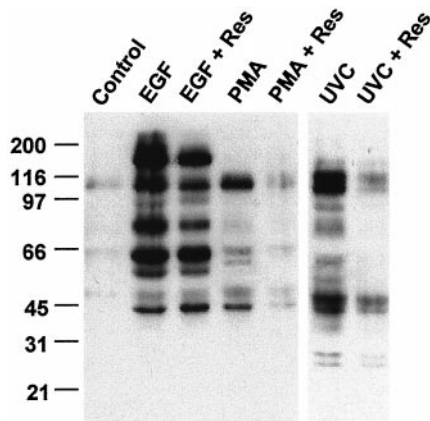

Fig. 4. Inhibition of UVC- and PMA-induced protein tyrosine phosphorylation by resveratrol. HeLa cells were untreated or treated with resveratrol (50 μM) for 1 h, stimulated with EGF (10 ng/ml), PMA (100 nM), or UVC (30 J/m^2) for 30 min, or left untreated as control. The phosphorylation of protein tyrosine was determined by Western blotting with antiphosphorylated tyrosine antibody (4G10). Data shown are examples of three independent experiments.
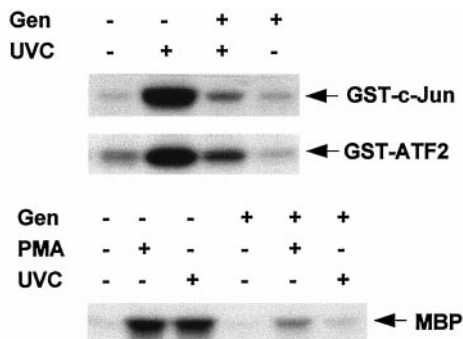

Fig. 5. Inhibition of PMA- and UVC-induced MAPK activation by tyrosine kinase inhibitor genistein. HeLa cells were untreated or treated with genistein (50 μM) for 1 h, and then treated with UVC (30 J/m^2) or PMA (100 nM) for 30 min. MAPK activities were assayed as described under *Materials and Methods*. Blots shown are samples of two independent experiments.
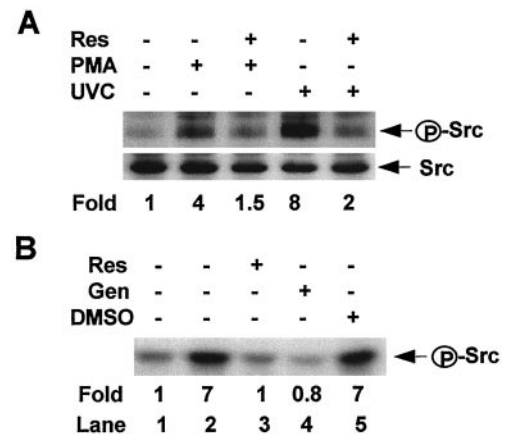

Fig. 6. Effect of resveratrol on c-Src tyrosine kinase activity. A, inhibition of PMA- and UVC-stimulated autophosphorylation of c-Src. HeLa cells were treated with resveratrol (50 μM) for 1 h, followed by challenge with PMA (100 nM) or UVC (30 J/m^2) for 30 min. Autophosphorylation of c-Src was determined as described under *Materials and Methods*. B, in vitro inhibition of Src autophosphorylation by resveratrol. HeLa cells were treated with UVC (30 J/m^2) (lane 2 to 5), or left untreated as control (lane 1). Endogenous c-Src molecules were immunoprecipitated with anti-c-Src antibody and incubated with kinase assay buffer alone (lane 1 and 2) for 30 min, or together with resveratrol (50 μM, lane 3), genistein (50 μM, lane 4), or Me$_2$SO (0.1%, lane 5). Autophosphorylation of c-Src was determined as in A. Data shown are examples of two independent experiments.

pronounced inhibitory effect on both UVC- and PMA-induced AP-1 activity. Similar to genistein, PP2, a potent and selective inhibitor of the Src family of protein tyrosine kinases, also strongly inhibited the induction of AP-1 activity by PMA and UVC.

Discussion

In this study, we demonstrated that resveratrol inhibited UVC and PMA-induced AP-1 activity by interfering with PTK, PKC, and MAPK pathways. Given the important roles of AP-1, MAPKs, PTKs, and PKC in carcinogenesis induced by UV and phorbol esters as well as by other carcinogens, the data obtained in this study may provide a molecular mechanism for the cancer chemopreventive actions of resveratrol.

Resveratrol inhibited activation of ERK, JNK, and p38 by UVC and PMA. However, direct incubation of resveratrol with the activated molecules of ERK2, JNK1, and p38 did not affect their kinase activity (Fig. 2C). Furthermore, resveratrol had no effect on the induction of AP-1 activity by active Raf-1, MEKK1, and MKK6, suggesting that resveratrol inhibits MAPK pathways by targeting the signaling molecules

upstream of Raf-1 or MEKK1. Indeed, resveratrol inhibited UVC and PMA-induced protein tyrosine phosphorylation, and incubation of resveratrol with the immunoprecipitated c-Src proteins resulted in loss of kinase activity, indicating that resveratrol may block MAPK pathways by directly inhibiting protein tyrosine kinases. In support of this notion, inhibition of protein tyrosine kinases by genistein or PP2 attenuated induction of MAPK and AP-1 activities by UVC or PMA. However, it should be noted that although both PMA and UVC activated c-Src, they exerted differential effects on MAPK pathways. Therefore, it is unlikely that inhibition of c-Src alone can account for the inhibitory effect of resveratrol on all three MAPK pathways. Given that resveratrol blocks the tyrosine phosphorylation of several proteins (Fig. 4), one possible explanation for such a multiple effect of resveratrol is inhibition of other c-Src-related tyrosine kinases that are able to differentially activate MAPK pathways. Alternatively, resveratrol may act on distinct kinases, leading to the inhibition of MAPK and AP-1 activities. To resolve this possibility, we examined the involvement of PKC, a family of serine/threonine kinases that consists of at least 11 isozymes and has been implicated in the activation of different MAPK
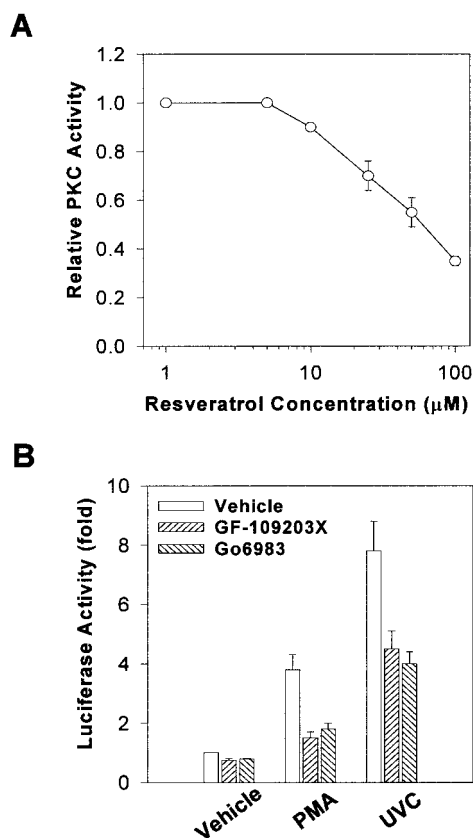

Fig. 7. Involvement of PKC in the inhibition of PMA or UVC-induced AP-1 activity by resveratrol. A, inhibition of PKC activity by resveratrol. HeLa cells were treated with UVC (30 J/m^2 for 30 min). Total PKC was isolated as described under *Materials and Methods*. The isolated PKC was incubated with different concentrations of resveratrol and assayed for kinase activity by using histone H1 as substrate. Amount of PKC activity in control reaction (incubated with 0.1% Me_2SO) was arbitrarily set to 1. B, effects of PKC inhibitors on AP-1 activity induced PMA and UVC. HeLa cells were transfected as in Fig. 1. Twenty-four hours after transfection, cells were pretreated with 5 μM PKC inhibitor GF-109203X or Gö6983 for 1 h, and then challenged with PMA (100 nM), or UVC (30 J/m^2), or vehicle (0.1% Me_2SO) for 24 h. Luciferase activity was determined and normalized as described under *Materials and Methods*.
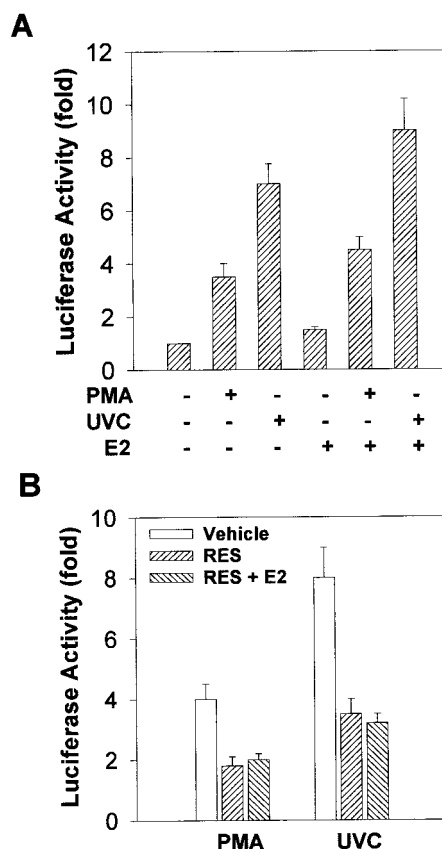

Fig. 8. Role of estrogen receptors in AP-1 activation by PMA and UVC. A, effect of estradiol on PMA and UVC-induced AP-1 activity. HeLa cells were transfected with AP-1 reporter construct as described as above. After transfection, cells were challenged with PMA (100 nM) or UVC (30 J/m^2) for 24 h in the absence or presence of 17-β-estradiol (E2, 20 nM). Cells were then harvested for luciferase activity assays. B, effect of estradiol on inhibition of PMA and UVC-induced AP-1 activity by resveratrol. HeLa cells were transfected as in A. After transfection, cells were treated with resveratrol (50 M) along or together with E2 (20 nM) before challenge with PMA (100 nM) or UVC (30 J/m^2). Cells treated with vehicle alone (0.1% Me_2SO) were used as control. Data presented are means ± S.D. of three independent experiments.

pathways by various stimuli including PMA and UVC (Schultz et al., 1997; Chen et al., 1999). Interestingly, incubation with resveratrol reduced the PKC activity. Furthermore, selective inhibition of PKC attenuated the induction of AP-1 activity by PMA and UVC. Consistent with this result, a recent study has also shown that resveratrol is able to inhibit redistribution of PKC activity induced by PMA (Subbaramaiah et al., 1998). Thus, the effect of resveratrol on MAPK and AP-1 may involve the inhibition of both protein tyrosine kinases and PKC, although the mechanisms by which resveratrol interacts with these two distinct groups of kinases remain to be elucidated.

AP-1 is a dimeric transcription factor that consists of the members of basic leucine zipper protein (bZIP) family, such as c-Jun and c-Fos. Recent studies suggest that the transcriptional activity of AP-1 can be modulated by nuclear receptors, among which are estrogen receptors (ER) (Paech et al., 1997; Webb et al., 1999). The biological outcomes of interaction between estrogen receptors and AP-1 vary, depending on the subtype of estrogen receptors and the nature of ligands. For example, 17β-estradiol activates AP-1-dependent transcription when binding to ERα, whereas the binding of 17β-estradiol to ERβ result in suppression of AP-1 transcriptional activity (Paech et al., 1997). Because of the structural characteristics, resveratrol was considered a phytoestrogen. Consistent with this view, resveratrol has been shown to bind to the estrogen receptors in a competitive manner with 17β-estradiol and to activate transcription of estrogen-responsive reporter genes (Gehm et al., 1997). Further studies show that resveratrol may act as a mixed agonist/antagonist for ERα and β, depending on the sequence of estrogen-responsive element and the subtypes of ER (Bowers et al., 2000). Considering these reported effects of resveratrol on ER, in this study, we investigated the roles of ER in the inhibition of AP-1 activity by resveratrol. Our data show that modulation of ER with 17β-estradiol has little effect on the induction of AP-1 activity by PMA and UVC. Furthermore, pretreatment with 17β-estradiol does not affect the inhibition of AP-1 ac-

tivity. Instead, blocking MAPK pathways by overexpresson of dominant-negative mutants of kinases or by pharmacological inhibitors impairs PMA or UVC-induced AP-1 activity. Thus, interaction with ER does not seem to have any impact on the inhibition of AP-1 by resveratrol. However, our results can not completely rule out the roles of ER, because it is possible that resveratrol may interact with ER via an estradiol-independent way. Activation of MAPK pathways may also lead to phosphorylation of ER, which, in turn, affects the transcriptional activity of AP-1. Further studies are needed to resolve these possibilities.

In search of cancer chemopreventive agents from natural sources, resveratrol was identified as an inhibitor of cyclooxygenases (Jang et al., 1997). Later studies showed that resveratrol not only inhibited cyclooxygenase activity but also inhibited transactivation of cyclooxygenase genes (Subbaramaiah et al., 1998). Because the induction of cyclooxygenases by PMA has been shown to require AP-1 activity (Subbaramaiah et al., 2000), the inhibitory effects of resveratrol on AP-1 activity as demonstrated in this study may provide a molecular basis for its negative role in cyclooxygenase gene activation. Recently, resveratrol has also been shown to inhibit induction of cytochrome P-450 1A1/1A2 by benzo-[*a*]pyrene, dimethylbenz[*a*]anthracene, and dioxin (Casper et al., 1999; Ciolino and Yeh, 1999). Although the exact mechanisms are not clear, it may involve inhibition of aryl hydrocarbon receptor-mediated signaling pathways. Because the activity of aryl hydrocarbon receptor can be regulated by the protein kinases such as protein kinase C (Long et al., 1998), it will be interesting to test whether inhibition of PKC contributes to the effect of resveratrol on aryl hydrocarbon receptor-mediated gene expression.

In addition to the anticarcinogenic properties, resveratrol also prevents coronary heart disease (Goldberg, 1996). This cardiovascular benefit of resveratrol is associated with inhibition of muscle cell proliferation and contraction, blockade of platelet aggregation, and perturbation of prostanoid synthesis (Goldberg, 1996). AP-1, MAPKs, and protein tyrosine kinases are known to play important roles in cell proliferation. Furthermore, activation of such MAPKs as p38 has been implicated in platelet function and aggregation (Saklatvala et al., 1996). Therefore, inhibition of AP-1, MAPKs, and protein tyrosine kinases may provide a plausible mechanism for the protective effect of resveratrol against cardiovascular diseases. In support of this notion, a recent study shows that resveratrol remarkably attenuates the activation of ERK2, JNK1, and p38 in porcine coronary arteries by endothelin-1, a primary endogenous mediator of cardiovascular disorders (El-Mowafy and White, 1999).

In summary, this study demonstrates that resveratrol blocks UVC and PMA-induced AP-1 activation by inhibiting protein tyrosine kinases, PKC, and subsequently down-regulating MAPK activity. However, inhibition of tyrosine kinases, PKC and MAPKs may have the consequences other than blocking AP-1-mediated gene expression. Therefore, one of the future challenges is to investigate whether the biochemical effects shown in this study also regulate other biological activities of resveratrol.
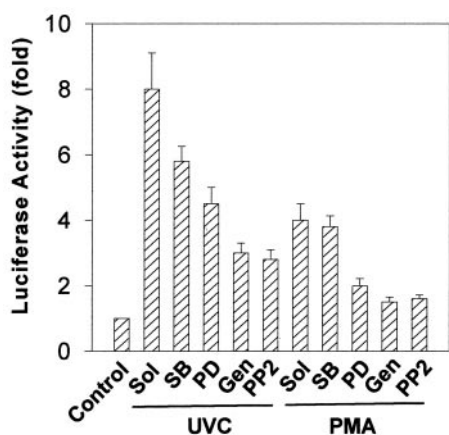

Fig. 9. Effects of p38, MEK1/2, and tyrosine kinase inhibitors on AP-1-dependent gene induction by UVC and PMA. HeLa cells were plated in six-well plates and transfected with 4 μg of AP-1 reporter construct together with 1 μg of plasmid encoding β-galactosidase using calcium-phosphate precipitation method. After transfection, cells were treated with p38 inhibitor (SB203580, 5 μM), MEK inhibitor (PD98059, 25 μM), tyrosine kinase inhibitors (genistein, 50 μM; or PP2, 1 μM), or Me$_2$SO (0.1%) for 1 h, and then stimulated with UVC (30 J/m^2) or PMA (100 nM) for additional 24 h. Luciferase activity was determined as in Fig. 1. Data shown are means ± S.D. of four independent experiments.

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