trans-3,4,5′-Trihydroxystibene Inhibits Hypoxia-Inducible Factor 1α and Vascular Endothelial Growth Factor Expression in Human Ovarian Cancer Cells

Zongxian Cao,1 Jing Fang,1 Chang Xia,1 Xianglin Shi,2 and Bing-Hua Jiang1,2
1Department of Microbiology, Immunology and Cell Biology, Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, West Virginia, and 2Institute for Nutritional Sciences, Chinese Academy of Sciences, Shanghai, China.

ABSTRACT

trans-3,4,5′-Trihydroxystibene (resveratrol) is a natural product commonly found in the human diet and has been shown recently to have anticancer effects on various human cancer cells. However, the molecular basis for its anticancer action remains to be elucidated. In this study, we investigated the effect of resveratrol on hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor (VEGF) expression in human ovarian cancer cells A2780/CP70 and OVCAR-3. We found that although resveratrol did not affect HIF-1α mRNA levels, it did dramatically inhibit both basal-level and growth factor-induced HIF-1α protein expression in the cells. Resveratrol also greatly inhibited VEGF expression. Mechanistically, we demonstrated that resveratrol inhibited HIF-1α and VEGF expression through multiple mechanisms. First, resveratrol inhibited AKT and mitogen-activated protein kinase activation, which played a partial role in the down-regulation of HIF-1α expression. Second, resveratrol inhibited insulin-like growth factor 1-induced HIF-1α expression through the inhibition of protein translational regulators, including M, 70,000 ribosomal protein S6 kinase 1, S6 ribosomal protein, eukaryotic initiation factor 4E-binding protein 1, and eukaryotic initiation factor 4E. Finally, we showed that resveratrol substantially induced HIF-1α protein degradation through the proteasome pathway. Our data suggested that resveratrol may inhibit human ovarian cancer progression and angiogenesis by inhibiting HIF-1α and VEGF expression and thus provide a novel potential mechanism for the anticancer action of resveratrol.

INTRODUCTION

trans-3,4,5′-Trihydroxystibene (resveratrol), a polyphenolic, was identified originally as a phytoalexin produced by plants in response to injury, UV irradiation, and insect or fungal attack (1). Resveratrol is present in more than 70 plant species and is especially abundant in food products such as grapes, peanuts, and mulberries (2). During the past few years, resveratrol has attracted considerable attention as one of the most promising cancer chemopreventive agents. Resveratrol was shown to affect diverse cellular events associated with each step of carcinogenesis, i.e., tumor initiation, promotion, and progression (2). At the molecular level, these effects corresponded with the inhibition of free radical formation, cyclooxygenase, and cytochrome P450 activity, as well as the inhibition of protein kinase C activity (3, 4). Additionally, resveratrol inhibited cell proliferation by decreasing DNA synthesis through its inhibitory effects on ribonucleotide reductase, DNA polymerase, and ornithine decarboxylase activities (3, 4). Resveratrol induces apoptosis in various malignant cells through multiple mechanisms, such as up-regulation of CD95L expression, enhancement of p53 expression and activity, induction of B-cell CLL/lymphoma 2-associated X protein expression, suppression of B-cell CLL/lymphoma 2 expression, and inhibition of nuclear factor κB activity (3, 4). Therefore, resveratrol possesses therapeutic potential based on its suppression of tumor cell growth by inducing cell cycle arrest and apoptosis. For example, in a rat ascitic hepatoma model, i.p. administration of resveratrol caused apoptosis in the tumor cell population and significantly decreased tumor cell numbers (5). Despite these findings, however, the molecular mechanisms by which resveratrol exerts its anticancer effects remain largely unknown.

Ovarian cancer represents the fourth leading cause of cancer-related death for women and is the most common cause of death from gynecologic cancer in the Western world (6). The overall 5-year survival rate of ovarian cancer is 50% and about 30% for advanced stage disease (7). The symptoms of the disease are observed only after it has spread to the surfaces of the peritoneal cavity. At this stage, it is impossible to remove all apparent lesions by surgical operations, and this accounts for the high rate of cancer recurrence after surgery. Consequently, the majority of ovarian cancer patients require chemotherapy. However, the major challenge in ovarian cancer treatment is the broad resistance to available chemotherapeutic drugs (6). The combination of cisplatin and paclitaxel as a chemotherapy regimen has improved the survival rate of ovarian cancer patients (8), but in the majority of cases, the cancer ultimately progresses, and the ovarian cancer patient dies from chemotherapy-refractory cancer (6).

It has been well established that solid tumor growth is angiogenesis dependent (9). Advanced solid tumors have a characteristic property of intratumoral hypoxia, which is caused...
by the structural and functional abnormalities of the tumor microvasculature, rapid expansion of tumor mass, and tumor-associated anemia (10). Hypoxia condition is a strong stimulus for angiogenesis, and this is predominately accomplished by hypoxia-inducible factor 1 (HIF-1α)-mediated up-regulation of vascular endothelial growth factor (VEGF) expression (11–13). VEGF, also known as the vascular permeability factor, is a potent and endothelial cell-specific mitogen that plays a crucial role during the process of tumor angiogenesis. VEGF expression is elevated in many human cancers, including ovarian carcinoma (14, 15). HIF-1α is a heterodimeric transcriptional factor composed of HIF-1α and HIF-1β subunits (16). HIF-1α binds to the hypoxia-responsive element in the promoter region of the VEGF gene and up-regulates VEGF expression (16). HIF-1α-deficient cells have reduced VEGF production under hypoxia (17, 18). VEGF expression levels in vivo are also much lower in HIF-1α null tumors (17). HIF-1α expression increases dramatically under hypoxia. However, under normoxic conditions, HIF-1α protein is expressed at a very low level due to rapid degradation via the ubiquitin-proteasomal pathway. Certain oncogenic proteins and growth factors have been shown to up-regulate HIF-1α expression in normoxic cells (19–23). HIF-1α was also shown to be elevated in various human tumors, including ovarian cancer (24). The effect of VEGF on vascular permeability has been implicated in the pathogenesis of ovarian cysts and malignant ascites (25, 26). In addition, increased levels of VEGF expression and the microvessel density in ovarian cancer directly correlate with poor prognosis (27, 28). Therefore, an anti-angiogenic therapy that targets the HIF-1α/VEGF system would be a rational strategy for the treatment of ovarian cancer.

In this study, we have demonstrated for the first time that resveratrol has a strong inhibitory effect on HIF-1α and VEGF expression in human ovarian cancer cells. Our data showed that resveratrol did not affect HIF-1α mRNA levels; rather, it interfered with the protein translational machinery and promoted HIF-1α protein degradation. These unique actions of resveratrol provide important clues to the molecular basis for its anticancer effects.

MATERIALS AND METHODS

Cell Culture and Reagents. A2780/CP70 and OVCAR-3 human ovarian cancer cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY), supplemented with 10% fetal bovine serum, 50 μg insulin (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained at 37°C and 5% CO2 in a humid environment. Resveratrol was purchased from ICN Biomedicals, Inc. (Aurora, OH). Cycloheximide was obtained from EMD Biosciences, Inc. (San Diego, CA). Recombinant human insulin and insulin-like growth factor 1 (IGF-1) were obtained from Sigma. LY294002, rapamycin, and PD98059 were purchased from Calbiochem (La Jolla, CA). Monoclonal HIF-1α antibody was obtained from BD Transduction Laboratories (Lexington, KY). Antibodies specific for phosphorylated (Thr-202/Tyr-204) or total p44/p42 mitogen-activated protein kinase (MAPK) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated (Ser-473) or total AKT, phosphorylated (Thr-421/Ser-424) or total M67, 70,000 ribosomal protein S6 kinase 1 (p70S6K1), phosphorylated (Ser-235/236) S6 ribosomal protein, phosphorylated (Ser-65) eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1), and phosphorylated (Ser-209) eIF4E were obtained from Cell Signaling Technology (Beverly, MA). Anti-glyceroldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was from R&D Systems (Minneapolis, MN).

Treatment of the Cells with Resveratrol. Exponentially growing cells (about 80% confluence) were treated with resveratrol at 12.5, 25, 37.5, 50, 75, 100, and 150 μM for 6 h in complete medium. For time-dependent studies, cells were treated with 50 μM resveratrol from 0 to 24 h. The control cells were incubated with the highest amount of solvent (DMSO) used for dissolving corresponding doses of resveratrol in the dose-dependent studies. For experiments in which cells received growth factor stimulation, cells were starved in serum-free and insulin-free medium overnight and then pretreated with resveratrol for 30 min, followed by incubation with growth factors for 6 h.

Western Blotting. Cells were washed with ice-cold PBS [140 mM NaCl, 3 mM KCl, 6 mM Na2HPO4, and 1 mM KH2PO4 (pH 7.4)], scraped, and pelleted by centrifugation. Whole-cell extracts were prepared using modified radiimmune precipitation buffer [100 mM Tris, 5 mM EDTA, 1% Triton X-100, 1% deoxycytocline acid, 0.1% SDS, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 2 mM DTT, 20 μg/ml leupeptin, and 20 μg/ml pepstatin (pH 7.4)]. Protein concentrations of the lysates were assayed using a protein assay reagent (Bio-Rad). Aliquots (50 μg) of protein samples were fractionated by 8% SDS-PAGE, transferred to a nitrocellulose membrane (Schleicher & Schuell Biosciences, Keene, NH), and subjected to immunoblotting analysis. Monoclonal HIF-1α antibody was used at a dilution of 1:3,000 in blocking buffer [1× Tris-buffered saline plus Tween 20: 20 mM Tris (pH 7.4), 137 mM NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk. Anti-GAPDH antibody was used at a dilution of 1:10,000. All other polyclonal antibodies were diluted at 1:2,000 in 1× Tris-buffered saline plus Tween 20 containing 5% BSA. The blots were blocked in 1× Tris-buffered saline plus Tween 20 containing 5% BSA. The blots were blocked in 1× Tris-buffered saline plus Tween 20 containing 5% nonfat dry milk for 2 h at room temperature, followed by incubation with the appropriately diluted primary antibodies overnight at 4°C. Immunoreactivity was visualized with appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA).

Northern Blotting. Total cellular RNA was extracted from the cells using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Aliquots (15 μg) of total RNA were fractionated by electrophoresis in 1% agarose gel with 2.2 μm formaldehyde, transferred to a Nytron supercharge membrane (Schleicher & Schuell) by capillary transfer with a downward transfer system (Schleicher & Schuell), and cross-linked to the membrane by UV irradiation. The blot was prehybridized for 1 h at 42°C in 10 ml of Ultrahyb buffer (Ambion). Human VEGF, HIF-1α, and β-actin cDNA probes were labeled with [α-32P]dATP by random priming using the RadPrime DNA labeling system (Invitrogen) and purified with the ProbeQuant G-50 Micro Columns (Amersham Biosciences, Piscataway, NJ). Heat-denatured probes were added to the hybridization buffer to a final concentration of 1 × 106 cpm/ml, and hybridization was
The cells were treated with 50 or 100 \( \mu \)M resveratrol for 0–24 h as indicated. The HIF-1\( \alpha \) protein levels decreased significantly at 1 h and were almost undetectable at 6 h and thereafter (Fig. 1, C and D). It was known that prolonged treatment of resveratrol inhibited tumor cell growth and induced apoptosis (3, 4). To determine whether the effect of resveratrol on HIF-1\( \alpha \) expression might be due to the cellular cytotoxic effect, we performed cell viability assays treated with 50 and 100 \( \mu \)M resveratrol for 6 h. The resveratrol treatment did not affect cell viability (Fig. 1E), suggesting that the decrease of HIF-1\( \alpha \) expression by resveratrol was not due to the cell death.
Resveratrol Prevented HIF-1α Expression Induced by Growth Factors. Growth factors and hormones play important roles in malignant transformation and tumor cell growth (29). Furthermore, previous work by our laboratory and others showed that stimulation by serum or specific growth factors induces HIF-1α expression in several cultured cells (19, 23, 30). Here, we investigated whether certain growth factors can up-regulate HIF-1α expression in human ovarian cancer cells, and whether resveratrol can inhibit the induction. A2780/CP70 and OVCAR-3 cells were cultured in serum-free and insulin-free medium for 18 h and then exposed to serum in the absence or presence of various doses of resveratrol for 6 h. Serum markedly induced HIF-1α protein expression in both A2780/CP70 and OVCAR-3 cells (Fig. 2A). Serum-induced HIF-1α expression was inhibited by resveratrol in a dose-dependent manner (Fig. 2A). To examine the effects of specific growth factors that are reportedly relevant to ovarian cancer progression (26), the cells were exposed to 200 nM insulin or 200 ng/ml IGF-1. Both insulin and IGF-1 significantly increased HIF-1α expression in ovarian cancer cells, whereas pretreatment with resveratrol effectively inhibited the HIF-1α induction (Fig. 2, B and C). At concentrations of 50 μM and higher, resveratrol inhibited both basal-level and growth factor-induced HIF-1α expression to almost undetectable levels (Fig. 2). Overall, these results demonstrated that while specific growth factors such as insulin and IGF-1 up-regulated HIF-1α expression in human ovarian cancer cells, resveratrol strongly inhibited the growth factor-induced HIF-1α expression in the cells.

Resveratrol Did Not Affect HIF-1α mRNA Levels. To examine whether inhibition of HIF-1α protein levels by resveratrol was caused by a decrease in its mRNA level, HIF-1α mRNA levels were measured by Northern blotting. As shown in Fig. 3, A and B, IGF-1 did not induce HIF-1α mRNA expression in A2780/CP70 and OVCAR-3 cells, which corresponds with previous observations that IGF-1 did not increase HIF-1α mRNA levels in other cell lines (31). Resveratrol treatment did not have any effect on HIF-1α mRNA levels in A2780/CP70 and OVCAR-3 cells (Fig. 3). These results indicated that inhibition of HIF-1α expression by resveratrol was not through the inhibition of its mRNA level, suggesting posttranscriptional mechanisms of resveratrol action on HIF-1α expression.

Resveratrol Inhibited Vascular Endothelial Growth Factor Expression in A2780/CP70 and OVCAR-3 Cells. VEGF is one of the target genes of HIF-1, which plays a crucial role in tumor angiogenesis (11–13, 16). HIF-1 regulates the expression of VEGF at the transcriptional level (11–13, 16). To determine whether resveratrol inhibits VEGF expression in human ovarian cancer cells, VEGF mRNA levels were measured...
by Northern blot analysis. A2780/CP70 and OVCAR-3 cells expressed high levels of VEGF mRNA under normal culture conditions (Fig. 4A), which correlated with high levels of HIF-1α expression in the cells (Fig. 1). Treatment of the cells with resveratrol resulted in a dose-dependent inhibition of VEGF mRNA expression (Fig. 4A). IGF-1 treatment significantly increased VEGF mRNA expression, and resveratrol inhibited IGF-1-induced VEGF mRNA expression in a dose-dependent manner (Fig. 4B). To determine whether down-regulation of VEGF mRNA levels by resveratrol subsequently leads to a decrease in VEGF protein expression, we assayed VEGF protein levels by ELISA. Treatment of the cells with resveratrol inhibited VEGF protein levels in a dose-dependent manner (Fig. 5A). Resveratrol also inhibited IGF-1-induced VEGF protein expression in the cells (Fig. 5B). No apparent cytotoxic effects were observed in the experiments when cells were treated with 50 and 100 μM resveratrol for 12 h (Fig. 5C).

Resveratrol Inhibited Vascular Endothelial Growth Factor Transcriptional Activation through HIF-1α. To determine whether resveratrol inhibited VEGF transcriptional activation, we tested the effect of resveratrol on the expression of a VEGF promoter reporter plasmid containing HIF-1 binding site, which was an indicator of VEGF transcriptional activation. Resveratrol treatment inhibited the VEGF reporter activity, and cotransfection of HIF-1α wild-type plasmid reversed resveratrol-inhibited reporter activity to an even higher level (Fig. 6A). This result suggested that resveratrol inhibited VEGF transcriptional activation through HIF-1α expression in the ovarian cancer cells. The expression of a dominant-negative HIF-1α plasmid inhibited the reporter activity in a dose-dependent manner (Fig. 6B). This result indicated that HIF-1α expression was required for VEGF transcriptional activation in the cells. Thus, these data further confirmed that HIF-1α expression was important for resveratrol-inhibited VEGF expression.

Resveratrol Inhibited AKT and Mitogen-Activated Protein Kinase Activation. Next, we investigated the signaling pathways involved in resveratrol-induced inhibition of HIF-1α protein expression in the cells. As shown in Fig. 3, resveratrol did not affect HIF-1α mRNA levels, suggesting that resveratrol may either decrease HIF-1α protein synthesis, and/or increase HIF-1α protein degradation. Previously, we and others have shown that specific growth factors, such as insulin and IGF-1, induce HIF-1α protein expression via the phosphatidylinositol 3'-kinase (PI3K)/AKT and MAPK signaling pathways in several cell types (23, 31, 32). Previous studies further showed that growth factor-induced activation of PI3K/AKT and MAPK signaling mediates HIF-1α expression by increasing HIF-1α protein synthesis but not decreasing HIF-1α protein degradation (19, 31, 33). In this study, we found that serum and specific growth factors such as insulin and IGF-1 induce high levels of HIF-1α protein expression in human ovarian cancer cells (Fig. 2). Thus, we examined whether resveratrol treatment affected IGF-1-induced activation of the PI3K/AKT and MAPK signaling pathways. Phosphorylation levels of AKT and MAPK, which correspond with their activation, were analyzed by immunoblotting. IGF-1 greatly increased AKT and MAPK phosphorylation in both cell lines (Fig. 7). Treatment of the cells with PI3K-specific inhibitor LY294002 completely blocked IGF-1-induced AKT phosphorylation and inhibited IGF-1-induced HIF-1α expression (data not shown), which was consistent with the effect of LY294002 on HIF-1α expression in other cell lines (23, 31, 32). MAPK/extracellular signal-regulated kinase kinase 1 inhibitor PD98059 inhibited MAPK activation and prevented HIF-1α expression induced by IGF-1 (Fig. 7, C and D). Although resveratrol could inhibit HIF-1α expression completely, it only partly reduced both AKT and MAPK phosphorylation induced by IGF-1 in the cells (Fig. 7). These results indicated that inhibition of AKT and MAPK activation by resveratrol played a partial role in its down-regulation of HIF-1α expression.

Resveratrol Interfered with Protein Translational Machinery. Growth factor-induced activation of PI3K/AKT and MAPK pathways has been shown to regulate HIF-1α protein synthesis via phosphorylation of protein translational regulators, including p70S6K1 and 4E-BP1 (31–35). We next examined whether resveratrol affected the protein translational machinery.

![Fig 4](image-url) Effect of resveratrol (RES) on VEGF mRNA expression in the cells. A, A2780/CP70 and OVCAR-3 cells cultured in complete medium were treated with solvent alone (Lane 1) or various doses of resveratrol for 6 h. Total RNA was extracted and analyzed by Northern blotting using a VEGF cDNA probe. The blot was stripped off and reprobed with β-actin cDNA probe as an internal control. B, serum-starved A2780/CP70 and OVCAR-3 cells were pretreated with solvent alone (Lanes 1 and 2) or various doses of resveratrol for 30 min, followed by incubation with 200 ng/ml IGF-1 for 6 h. Total RNA was extracted and analyzed by Northern blotting using VEGF or β-actin cDNA probe.
We investigated the effect of resveratrol on the phosphorylation of the components of protein translational apparatus, including p70S6K1, S6 ribosomal protein, 4E-BP1, and eIF4E. In A2780/CP70 and OVCAR-3 cells, the phosphorylation of p70S6K1 induced by IGF-1 was blocked by LY294002, rapamycin, and PD98059 (Fig. 8A). Similarly, resveratrol inhibited IGF-1-induced p70S6K1 phosphorylation at 50 and 100 μM (Fig. 8A). Strikingly, treatment of the cells with resveratrol at as low as 10 μM dramatically inhibited IGF-1-induced phosphorylation of S6 ribosomal protein, comparable with the effect of LY294002, rapamycin, and PD98059 (Fig. 8A). Similarly, resveratrol inhibited IGF-1-induced phosphorylation of S6 ribosomal protein at 50 and 100 μM (Fig. 8A). Strikingly, treatment of the cells with resveratrol at as low as 10 μM dramatically inhibited IGF-1-induced phosphorylation of S6 ribosomal protein, comparable with the effect of LY294002, rapamycin, and PD98059 (Fig. 8A). Phosphorylation of the S6 ribosomal protein by p70S6K1 and other kinases is involved in the initiation of protein synthesis (36). Resveratrol also reduced phosphorylation of 4E-BP1 and, to a much greater extent, the mRNA cap-binding protein eIF4E (Fig. 8C), which is the key enzyme for cap-dependent initiation of protein translation. Expression of eIF4E was shown recently to be sufficient to elevate HIF-1α protein levels (21). In this study, we found that treatment of the cells with rapamycin also inhibited IGF-1-induced HIF-1α expression in the cells (Fig. 8D). Rapamycin is a specific inhibitor of mammalian target of rapamycin (mTOR), which is the upstream kinase for 4E-BP1 and p70S6K1. These results indicated that resveratrol interfered with protein translational regulation, which contributed to its inhibitory effect on HIF-1α protein expression.

**Resveratrol Promoted HIF-1α Protein Degradation via the Proteasomal Pathway.** HIF-1α protein levels are regulated by a balance between HIF-1α protein synthesis and degradation in the cells. Hypoxia or hypoxia-mimetic agent CoCl2 can rapidly induce HIF-1α protein accumulation due to a marked decrease in HIF-1α protein degradation (37). Based on the results presented above, we found that resveratrol could inhibit completely both growth factor-induced and basal-level HIF-1α expression (Fig. 1; Fig. 2). These observations suggested that, in addition to inhibiting the HIF-1α protein synthesis, resveratrol may also promote HIF-1α protein degradation. Thus, we examined the effect of resveratrol on the stability of HIF-1α protein in the cells. A2780/CP70 cells were cultured in...
compared with the control (H11021 P/H9251 MG132 increased HIF-1 protein levels. As shown in Fig. 10, treatment of the cells with proteasome inhibitor MG132 in the presence or absence of resveratrol, and analyzed HIF-1 way, we treated the cells with solvent alone (7.5 min; Fig. 9). This result demonstrated that resveratrol increased HIF-1 that in cells treated with solvent alone (7.5 min; Fig. 9). This result demonstrated that resveratrol increased HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1 protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1 expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1 target gene VEGF expression in human ovarian cancer cells. Therefore, we investigated the mechanism of resveratrol on HIF-1α expression and VEGF transcript in human ovarian cancer cells. We demonstrated for the first time that resveratrol, a natural product with cancer chemopreventive effects, markedly inhibited HIF-1α and VEGF expression in human ovarian cancer cells. Resveratrol was shown to affect PI3K/AKT and MAPK-signaling pathways, to interfere with protein translational machinery, and to enhance HIF-1α protein degradation. The data presented here suggested that resveratrol may inhibit human ovarian cancer progression by interfering with tumor angiogenesis mediated by HIF-1α and VEGF, thereby providing a novel mechanism for the anticancer action of resveratrol.

Numerous studies have implicated the role of IGFs in the development and progression of human malignancies such as ovarian carcinoma (29). In addition, IGF-1 has been shown to induce HIF-1α expression in several cultured cells (30, 31). IGF-1 is a growth factor commonly used to up-regulate the PI3K/AKT and MAPK signaling pathways in various cell types. In this study, we used IGF-1 as a stimulus to investigate the mechanism of resveratrol on HIF-1α expression in human ovarian cancer cells. IGF-1 treatment significantly increased HIF-1α protein levels in human ovarian cancer cells, which is consistent with previous findings that IGF-1 stimulates HIF-1α expression in other cell lines (30, 31). Moreover, IGF-1 also markedly up-regulated HIF-1α target gene VEGF expression in human ovarian cancer cells. These results correspond with previous reports that IGF-1 is involved in tumor-induced angiogenesis associated with the up-regulation of VEGF expression in human colon cancer and pancreatic cancer (39, 40). Several studies have shown that growth factors including IGF-1 do not induce HIF-1α mRNA expression but increase HIF-1α protein synthesis (31, 32). Similarly, we did not observe the induction of HIF-1α mRNA expression by IGF-1 in the ovarian cancer cells. Resveratrol treatment did not have any effect on HIF-1α mRNA levels in the cells, suggesting that resveratrol inhibited HIF-1α protein expression through post-transcriptional mechanisms, for example, by influencing HIF-1α protein synthesis and/or degradation.

To further define the molecular mechanisms by which resveratrol inhibited HIF-1α expression, we next examined whether resveratrol affected HIF-1α protein synthesis. Recently, it was reported that IGF-1-induced expression of HIF-1α and VEGF in HCT116 colon cancer cells can be blocked by the PI3K inhibitor wortmannin, MAPK/extracellular signal-regulated kinase inhibitor PD98095, or mTOR inhibitor rapamycin and that these inhibitors also blocked the phosphorylation of the translational regulatory protein 4E-BP1, p70S6K1, and elf4E (31). Epidermal growth factor and HER neu were also shown to induce HIF-1α expression through similar signaling pathways in breast cancer and prostate cancer cells, respectively (19, 33). Induction of HIF-1α expression by these growth fac-

**Fig. 6** HIF-1α-mediated VEGF transcriptional activation in human ovarian cancer cells. A, A2780/CP70 cells were cultured in normal growth medium. HIF-1α-expressing plasmid was cotransfected into the cells with the reporter plasmid and the pCMV-β-gal plasmid. An empty vector plasmid was used to adjust the same amounts of plasmids used in each transfection. After overnight recovery, the cells were treated with solvent or 50 μM resveratrol (RES) for 12 h. Luciferase activity was measured and normalized to β-galactosidase activity. Assays were performed in triplicate, and data were presented as mean ± SD. *p < 0.01 versus solvent control; #p < 0.01 versus vector control. B, cells were cotransfected with the indicated amount of a dominant-negative HIF-1α plasmid, pCMV-β-gal plasmid, and a VEGF promoter reporter plasmid. Luciferase activity was measured 24 h after transfection and normalized to β-galactosidase activity. Assays were performed in triplicate, and data were presented as mean ± SD. *p < 0.01 versus vector control.
tors and oncogenes was due to an increase in HIF-1α protein synthesis but was not due to a decrease in HIF-1α protein degradation (19, 31, 33). In this study, we found that IGF-1 treatment induced HIF-1α and VEGF expression and activation of AKT and MAPK in human ovarian cancer cells. Resveratrol treatment partly reduced AKT and MAPK activation; however, resveratrol could dramatically inhibit HIF-1α protein expression to undetectable levels. Thus, inhibition of AKT and MAPK activation by resveratrol only played a partial role in its down-regulation of HIF-1α expression.

We next examined the effect of resveratrol on protein translational machinery, which has been shown to regulate HIF-1α protein synthesis induced by growth factors. Regulation of protein synthesis allows for a more rapid response to diverse stimuli in the absence of transcription. Eukaryotic initiation factors (eIFs) and p70S6K1 play critical roles in protein translational regulation. p70S6K1 phosphorylates the S6 ribosomal protein of the 40S subunit of the ribosome, and stimulates the translation of mRNAs with a 5′ oligopyrimidine tract that encodes major components of the protein synthesis apparatus (41). p70S6K1 is a downstream effector of PI3K; full activation of p70S6K1 also requires mTOR activity (36, 41, 42). The activity of p70S6K1 is controlled by multiple phosphorylation events. Phosphorylation of Thr-421 and Ser-424 on the COOH-terminal autoinhibitory domain is mediated by MAPK (41). In this study, we found that IGF-1-induced phosphorylation of p70S6K1 was inhibited by resveratrol at 50–100 μM. This effect was consistent with its inhibitory effects on AKT and MAPK activation as shown in Fig. 9. Remarkably, phosphorylation of S6 ribosomal protein, a downstream effector of p70S6K1, was dramatically inhibited by resveratrol treatment at as low as 10 μM. Because other kinases including cAMP-dependent protein kinase and protein kinase C are also known to phosphorylate S6 ribosomal protein (43), it is possible that resveratrol may also inhibit some of these kinases in addition to p70S6K1. 4E-BP1 functions in the PI3K/AKT pathway and is phosphorylated by mTOR and other unidentified kinases (36). 4E-BP1 binds to eIF4E and inhibits eIF4E function. Hyperphosphorylation of 4E-BP1 functions in the PI3K/AKT pathway and is phosphorylated by mTOR and other unidentified kinases (36). 4E-BP1 binds to eIF4E and inhibits eIF4E function. Hyperphosphorylation of 4E-BP1 disrupts this binding, releasing eIF4E to be phosphorylated at Ser-209 by Mnk1 and to associate with eIF4G to initiate cap-dependent translation (36). eIF4E is the key enzyme for cap-dependent initiation of protein translation. Expression of eIF4E was shown recently to be sufficient to elevate HIF-1α protein levels (21). In the present study, we found that resveratrol inhibited phosphorylation of 4E-BP1 at Ser-65 and greatly inhibited phosphorylation of eIF4E at Ser-209. We showed that treatment of the cells with mTOR inhibitor rapamycin completely inhibited IGF-1-induced HIF-1α expression. Thus, these data indicated that resveratrol interfered with protein transla-
tional regulation, which contributed to its inhibitory effect on HIF-1α protein expression.

HIF-1α protein levels are also subject to posttranslational regulation. Under normoxic conditions, HIF-1α protein is expressed at very low levels due to rapid degradation via the ubiquitin-proteasomal pathway. Conversely, under hypoxic conditions, HIF-1α protein levels are increased dramatically due to a marked decrease in HIF-1α protein degradation (37). In this study, we found that resveratrol could eliminate both growth factor-induced and basal-level HIF-1α expression. These observations suggested that, in addition to inhibiting HIF-1α protein synthesis, resveratrol may also promote HIF-1α protein degradation. Indeed, the half-life of HIF-1α protein was shortened significantly in the presence of resveratrol, demonstrating that resveratrol induced HIF-1α protein degradation (Fig. 9). We further showed that resveratrol-induced HIF-1α protein degradation was through the proteasome pathway. HIF-1α protein degradation is mediated by the oxygen-dependent HIF-prolyl hydroxylases. Prolyl hydroxylation of HIF-1α by HIF-prolyl hydroxylase is required for the binding of HIF-1α to the von Hippel Lindau tumor suppressor protein, which serves as the E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation (37). HIF-prolyl hydroxylases are hydroxygenases requiring oxygen and 2-oxoglutarate as cosubstrates. The binding of oxygen to the iron-containing central moiety of HIF-prolyl hydroxylase requires the vitamin C-dependent maintenance of iron in its ferrous state (37). Recently, vitamin C was shown to abrogate efficiently HIF-1α protein levels in several human cancer cell lines by increasing HIF-prolyl hydroxylase activity (44). Both vitamin C and resveratrol have multiple hydroxyl groups, which are essential for their antioxidant activities. In addition, resveratrol was shown to be a much more potent antioxidant than vitamin C.
and can enhance the activity of vitamin C when used together (45). We speculate that the effect of resveratrol on HIF-1α degradation could possibly result from its interference with HIF-prolyl hydroxylase activity, which requires additional investigation.

The distinct ability of resveratrol to inhibit HIF-1α and VEGF expression observed in this study raises the possibility of its usefulness in the therapy of human ovarian cancer. Tumor angiogenesis triggered by HIF-1α and VEGF is a vital process for tumor progression because it nourishes tumor cell growth and facilitates metastases (38). In addition, tumor cell-derived VEGF was also shown to have an autocrine stimulatory effect on tumor cell growth because various human tumor cells, including ovarian cancer cells, express VEGF receptors (14, 38, 46). Therefore, resveratrol may potentially inhibit ovarian cancer progression based on its remarkable inhibitory effect on HIF-1α and VEGF expression. Furthermore, the A2780/CP70 human ovarian cancer cell line used in this study is cisplatin resistant. A2780/CP70 was developed by chronic exposure of the parent cisplatin-sensitive A2780 cell line to increasing concentrations of cisplatin in culture (47). OVCAR-3 was derived from the malignant ascites of a patient with progressive ovarian cancer resistant to clinically relevant concentrations of cisplatin (48). Therefore, our data suggest that resveratrol may exert anticancer effects even in cisplatin-resistant ovarian cancer patients. Indeed, resveratrol recently has been shown to have synergistic cytotoxic activity when used in combination with chemotherapeutic drugs or cytotoxic factors in the treatment of drug refractory tumor cells (49). Finally, based on its effects on protein translational regulation and HIF-1α protein degradation, two processes that are not cell-type specific, resveratrol may inhibit expression of HIF-1α and VEGF in other human malignancies with high levels of HIF-1α expression. This may also explain, at least in part, the broad spectrum of anticancer effects of resveratrol observed previously in various human cancer cell lines.

REFERENCES


trans-3,4,5′-Trihydroxystibene Inhibits Hypoxia-Inducible Factor 1 α and Vascular Endothelial Growth Factor Expression in Human Ovarian Cancer Cells

Zongxian Cao, Jing Fang, Chang Xia, et al.