Resveratrol inhibits hypoxia-induced accumulation of hypoxia-inducible factor-1α and VEGF expression in human tongue squamous cell carcinoma and hepatoma cells

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Abstract

Hypoxia-inducible factor-1α (HIF-1α) is overexpressed in many human tumors and their metastases, and is closely associated with a more aggressive tumor phenotype. In this study, we investigated the effect of resveratrol, a natural product commonly found in grapes and various other fruits, on hypoxia-induced HIF-1α protein accumulation and vascular endothelial growth factor (VEGF) expression in human tongue squamous cell carcinomas and hepatoma cells. Our results showed that resveratrol significantly inhibited both basal level and hypoxia-induced HIF-1α protein accumulation in cancer cells, but did not affect HIF-1α mRNA levels. Pretreatment of cells with resveratrol significantly reduced hypoxia-induced VEGF promoter activities and VEGF expression at both mRNA and protein levels. The mechanism of resveratrol inhibition of hypoxia-induced HIF-1α accumulation seems to involve a gradually shortened half-life of HIF-1α protein caused by an enhanced protein degradation through the 26S proteasome system. In addition, resveratrol remarkably inhibited hypoxia-mediated activation of extracellular signal-regulated kinase 1/2 and Akt, leading to a marked decrease in hypoxia-induced HIF-1α protein accumulation and VEGF transcriptional activation. Functionally, we observed that resveratrol also significantly inhibited the hypoxia-stimulated invasiveness of cancer cells. These data suggested that HIF-1α/VEGF could be a promising drug target for resveratrol in the development of an effective chemopreventive and anticancer therapy in human cancers. [Mol Cancer Ther 2005;4(10):1465–74]

Introduction

Resveratrol (trans-3,4’,5-trihydroxystilbene) is a natural polyphenolic phytoalexin found in grapes, peanuts, root extracts of the weed Polygonum cuspidatum, and various other fruits (1). Recently, resveratrol has been found to be an antitumor and chemopreventive agent (2). Several in vitro studies have shown that resveratrol inhibits the proliferation of several kinds of tumors such as leukemia, prostate, breast, and colon cancers (3–6). The antiproliferative mechanism in various cancer cell lines seem to involve multiple apoptotic pathways, including the regulation of Fas expression, enhancement of p53 expression and activation, inhibition of nuclear factor-κB, as well as several signaling pathways such as mitogen-activated protein kinases (MAPK) and phosphoinositide-3-kinase (PI-3K)/Akt (6–10). At the molecular level, these effects were related to the inhibition of free radical formation and cyclooxygenase activity, blocking of ribonucleotide reductase and DNA synthesis with cellular arrest in the S phase or the S-G2 phase transition (6, 11, 12) as well as induction of differentiation (2). Studies in animals have also suggested that resveratrol exerts potent anticarcinogenic effects via affecting diverse cellular events associated with each step of carcinogenesis, i.e., tumor initiation, promotion, and progression (2, 13). Recently, resveratrol has been found to inhibit angiogenesis, but the underlying mechanism of its antiangiogenic activity remains unclear.

Hypoxia-inducible factor-1α (HIF-1α) is composed of HIF-1α and HIF-1β subunits (14). The expression of HIF-1α is tightly regulated by low oxygen tension, whereas HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator) is constitutively expressed. Under normoxic conditions, the oxygen-dependent degradation domain of HIF-1α interacts with the von Hippel-Lindau protein, a recognition component of an E3 ubiquitin-protein ligase complex. Such HIF-1α/von Hippel-Lindau protein interaction requires oxygen—and iron-dependent hydroxylation of proline residues (Pro402 and Pro564) in HIF-1α protein (15–18) and results in the ubiquitination and subsequent degradation of HIF-1α protein via the 26S proteasome (19–21). Under hypoxic conditions, the blockade of prolyl hydroxylation, ubiquitination and degradation lead to the remarkable accumulation and
translocation of HIF-1α protein to the nucleus, where it forms an active complex with HIF-1β. The formed complex initiates transcriptional activation via binding to hypoxia response element located in the promoter regions of >60 HIF-1-regulated genes (22, 23), the protein products of which are important for adaptation and survival under hypoxic stress.

Intratumoral hypoxia is a common characteristic feature of solid tumors (24). The role of HIF-1 in tumor angiogenesis and cellular adaptation to the hypoxic microenvironment has been well established (25, 26). Overexpression of HIF-1α protein has been shown in many human cancers and their metastases, and is closely associated with a more aggressive tumor phenotype including an advanced tumor grade, an increased vascularity, increased resistance to chemotherapy and radiotherapy, and tumor progression (27–29). Therefore, it is likely that HIF-1α can serve as an independent marker of prognosis, and a potential predictor for mortality risk and treatment failure of tumors (30–34). Recent studies have shown that HIF-1α is involved in the up-regulation of a variety of gene products essential for cancer cell invasion and migration, including cathepsin-D, urokinase-type plasminogen activator receptor, matrix metalloproteinase 2, autocrine motility factor (35), and the met proto-oncogene (36). Collectively, these results strongly imply that the hypoxia/HIF-1 system is an appealing strategic target in cancer therapeutics (25, 37).

In the present study, we have shown for the first time that resveratrol remarkably inhibited hypoxia-induced HIF-1α accumulation and vascular endothelial growth factor (VEGF) expression in both human tongue squamous cell carcinomas (SCC-9) and hepatoma (HepG2) cells, and dramatically suppressed hypoxia-stimulated invasiveness of SCC-9 cells in vitro. Our results also supported that resveratrol exerted its inhibitory effect on hypoxia-induced HIF-1α accumulation and VEGF expression, at least in part, via p42/44 MAPK and PI-3K/Akt mechanisms. These findings have provided further evidence that resveratrol can be a potential chemopreventive and anticancer agent in human cancer by targeting the well-known tumor survival factor, HIF-1α, under hypoxic stress.

**Materials and Methods**

**Reagents**

Trans-3,4,5′-trihydroxystilbene (Resveratrol) was purchased from Sigma (St. Louis, MO) and dissolved at a concentration of 100 mmol/L in 100% DMSO as a stock solution, stored at −20°C. LY294002, PD98059, and SB203582 were from Calbiochem (San Diego, CA) and dissolved in DMSO. The final DMSO concentration did not exceed 0.1% throughout the study. The selective proteosome inhibitor, MG132 (Z-Leu-Leu-Leu-CHO), and the proteosome inhibitor, cycloheximide, were from Sigma. Primary antibody for HIF-1α and HIF-1β were from BD Transduction Laboratories (San Diego, CA). Antibody for VEGF was from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for total or phosphorylated p42/44 MAPKs (Thr202/Tyr204) or Akt (Ser473) were from New England Biolabs (Beverly, MA). Horseradish peroxidase–conjugated secondary antibodies were from Pierce (Rockford, IL).

**Cell Culture**

Human tongue squamous cell carcinoma (SCC-9) and human hepatoma (HepG2) cell lines were obtained from American Type Culture Collection (Rockville, MD). SCC-9 cells were cultured in DMEM/F-12 medium (Fisher Scientific, Tustin, CA) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL), streptomycin (100 μg/mL; Invitrogen Corp., Carlsbad, CA), and hydrocortisone (400 ng/mL; Sigma). HepG2 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS and antibiotics as described above. All cultures were maintained at 37°C in a humidified atmosphere with 5% CO2.

**Establishment of Hypoxic Culture Condition**

Cells were cultured to ~80% confluence and transferred to a hypoxic chamber with an auto-purge airlock (Coy Laboratory Products Inc., Grass Lake, MI). Environmental hypoxic conditions (1%) were achieved in an airtight humidified chamber continuously flushed with a gas mixture containing 5% CO2 and 95% N2. Maintenance of the desired oxygen concentration was constantly monitored during incubation using a microprocessor-based oxygen controller (Coy Laboratory Products, Inc.).

**Resveratrol Treatment of Cancer Cells**

Exponentially growing cells (70–80% confluence) in complete medium were pretreated for 1 hour with different concentrations of resveratrol, followed by continual incubation in normal culturing conditions or exposure to hypoxia (1% O2) for indicated time intervals according to the purpose of the experiment. To study the effects of resveratrol on the half-life or degradation of hypoxia-induced HIF-1α protein accumulation, cancer cells were exposed to hypoxia for 6 hours followed by treatment with 10 μg/mL of cycloheximide to inhibit further protein synthesis in the presence or absence of 100 μmol/L of resveratrol for different time periods. On the other hand, cells were pretreated with 20 μmol/L of MG132 for 30 minutes and cultured in the presence of different concentrations of resveratrol for 6 hours under normoxic conditions. HIF-1α protein levels were determined by Western blot analysis.

**RT-PCR Analysis for HIF-1α and VEGF mRNA Levels**

Total RNA was isolated from cancer cells using Trizol reagent (Invitrogen). RT-PCR analysis of HIF-1α and β-actin mRNA levels were done using the one-step RT-PCR Kit (Qiagen, Valencia, CA) with primers specific to HIF-1α (forward primer, 5′-TCACCAAGAGACGACGATGGC-3′; and reverse primer, 5′-CCAGCAAAAGTTAAGCAGCACTGGTCC-3′), VEGF (forward primer, 5′-ACGAAGTGATGAAGTGTTATGG-3′; and reverse primer, 5′-TTCCTGATATCCCTTGTTTGAG-3′), or specific to β-actin (forward primer, 5′-CTATGAGAATGGTACGTGACATCG-3′; and reverse primer, 5′-CCCTAGAATGGTACGTGACATCG-3′). All the primers were synthesized by GenoMechanix, LLC (Gainesville, FL).
Transgenic Transfection and Luciferase Reporter Assays
The luciferase reporter plasmids (pGL2-Luc) harboring human VEGF promoter region (−1,175/+336) was kindly provided by Dr. David K. Ann. SCC-9 and HepG2 cells were transiently transfected with 0.5 μg of VEGF reporter plasmids. One-tenth of a microgram of the Renilla luciferase pRL-TK plasmid was cotransfected as an indicator for normalization of transfection efficiency. After 6 hours, cells were recovered overnight and subsequently pretreated with different concentrations of resveratrol for 30 minutes, followed by exposure to normoxia or hypoxia for 24 hours before harvesting cell lysates for luciferase assays.

The relative luciferase activity from the firefly-luciferase reporter gene was determined and normalized with Renilla luciferase activity using the dual luciferase reporter assay system (Promega, Madison, WI). All fold-induction data reported were calculated after normalizing the reporter activities with the activities from the indicator plasmid. In general, there was no obvious change on the basal activity in our cotransfection and inhibitor studies. Sometimes, a particular treatment may elicit a modulating effect on the general transcriptional machinery; however, these general effects were taken into account after the normalization with the activities of the indicator plasmid.

Western Blot Analysis
Treated and untreated cells were lysed with buffer containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% Triton X-100, 10 mmol/L sodium fluoride, 20 mmol/L β-mercaptoethanol, 250 μmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and complete protease inhibitor cocktail (Sigma), and incubated at 4°C for 30 minutes. The lysates were ultrasonicated and centrifuged at 14,000 × g for 15 minutes.

The supernatants were collected and stored at −70°C. Protein concentrations were determined by bicinchoninic acid assay methods. Protein (100 μg) was separated on 10% polyacrylamide-SDS gel and electroblotted onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia, Piscataway, NJ). After blocking with TBS/5% skimmed milk, the membrane was incubated overnight at 4°C with antibodies against HIF-1α, HIF-1β, VEGF, total or phosphorylated p42/p44 MAPKs (Thr202/Tyr204) or Akt (Ser473), followed by incubation with goat anti-mouse IgG conjugated with peroxidase (1:5,000; Pierce) for 1 hour at room temperature, and signals were detected by enhanced chemiluminescence (38).

Immunofluorescence Studies
Cancer cells were seeded on four-well Lab-Tek II chamber slide system (Nalge Nunc Int., Naperville, IL; 1 × 10⁴) and cultured for 24 hours under normal conditions. Cells were pretreated with 100 μmol/L of resveratrol for 30 minutes followed by incubation under normoxic or hypoxic conditions for 6 hours. Cells were then fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature. After two washes, cells were permeabilized with a solution of PBS containing 1% Triton X-100, preincubated with PBS containing 5% bovine serum album, and incubated at 4°C overnight in a humidified chamber with a mouse monoclonal anti-human HIF-1α antibody (1:100; BD Transduction Laboratories). After washing, cells were incubated for 1 hour at room temperature with Alexa Fluor 488 conjugated goat anti-mouse IgG (1:2,000; 0.5 μg/mL; Molecular Probes, Eugene, OR). After three washes, cover glasses were applied with mounting solution (ImmunoMount, Shandon, Pittsburgh, PA) and image analysis was made by using a fluorescence microscope, whereas HIF-1α expression appeared green. Cells incubated with fluorescein-conjugated secondary antibodies in the absence of primary antibodies served as negative controls.

Cell Invasion Assay
Cell invasion kit (Chemicon, Temecula, CA) was used for the cell invasion assay according to the manufacturer’s protocol. Confluent cells were added to the inner chamber of the insert in 300 μL of serum-free medium. Medium (500 μL) with or without 10% FBS was added to the lower chamber. To determine the effect of resveratrol on cell invasion, resveratrol with a final concentration of 100 μmol/L was added to the lower chamber and DMSO was used as a control. The invasion kit was incubated under normoxia or hypoxia for 48 hours at 37°C. The non–invading cells and the extracellular matrix gel from the interior side of the inserts were gently removed using a cotton-tipped swab. The invasive cells that migrated through the gel insert to the lower surface of the membrane were stained and photographed using a computer imaging system. Afterwards, the stained cells were extracted with the extract solution, and the absorbance was determined at 562 nm.

Cell Viability Assay
SCC-9 or HepG2 cells were plated in 96-well plates at 10⁴ cells per well. After pretreatment with different concentrations of resveratrol for 30 minutes under normal conditions, cells were incubated in normoxia or hypoxia for the indicated time intervals and viable cells were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay kit (Chemicon) according to the manufacturer’s protocol. Each experiment was done in triplicate.

Data Analysis
Data are presented as the mean ± SD for three separate experiments. A paired Student’s test was employed for statistical analysis, with significant differences determined as P < 0.05.

Results
Resveratrol Inhibited Hypoxia-Induced HIF-1α Protein Accumulation in SCC-9 and HepG2 Cells
Our previous study has shown that a steady-state basal level of HIF-1α protein was expressed in SCC-9 cells (38), which was confirmed in the present study using immunofluorescence microscopy (Fig. 1B, top left). However, the basal level of HIF-1α protein was low to undetectable in HepG2 cells cultured under normal conditions (38–40). To explore the temporal effects of resveratrol treatment on...
baseline HIF-1α protein, we exposed SCC-9 cells to 50 μmol/L of resveratrol under normal conditions and found that the steady-state basal level of HIF-1α protein was reduced in a time-dependent manner (Fig. 1A). Immunofluorescent staining further illustrated that treatment of SCC-9 cells with 100 μmol/L resveratrol for 6 hours significantly suppressed HIF-1α protein present in their nuclei (Fig. 1B, top right). We next asked whether resveratrol inhibits HIF-1α induced by tumor cells upon exposure to hypoxic stress, a common condition in aggressive solid tumor. We observed that pretreatment of both cell types, SCC-9 and HepG2, with resveratrol abrogated hypoxia-induced HIF-1α protein accumulation in a dose-dependent manner (Fig. 1C), which was also confirmed by immunofluorescence studies in SCC-9 cells (Fig. 1B, bottom right versus bottom left).

To determine whether inhibition of hypoxia-induced HIF-1α protein accumulation by resveratrol was due to a decrease in its mRNA level, HIF-1α messages were evaluated by RT-PCR. As shown in Fig. 1D, no apparent changes in HIF-1α mRNA were observed in SCC-9 cells after exposure to hypoxia for 6 hours (Fig. 1D), and treatment of SCC-9 cells with different concentrations of resveratrol did not have any effects on HIF-1α mRNA expression (Fig. 1D). Similar results were obtained in HepG2 cells under the same experimental conditions (data not shown). These results suggest that resveratrol inhibits hypoxia-induced HIF-1α protein accumulation through a posttranscriptional mechanism.

It was reported that prolonged treatment with resveratrol inhibited cell growth and triggered apoptosis in a variety of cancer cells (6–10). To rule out the possibility of a cytotoxic effect on HIF-1α protein suppression by resveratrol, cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was done. No obvious changes in cell viability were observed in both SCC-9 and HepG2 cells after being challenged with different concentrations of resveratrol up to 100 μmol/L under both normoxic and hypoxic conditions for 6 hours (Fig. 1E and F), indicating that the inhibition of HIF-1α protein by resveratrol was not ascribed to nonspecific tumor cell toxicity.

**Resveratrol Inhibited Hypoxia-Induced VEGF Expression and Transcriptional Activation**

VEGF, an immediate downstream target gene of HIF-1α, plays a pivotal role in tumor angiogenesis (25, 41), especially under conditions of intratumoral hypoxia. To determine whether resveratrol could inhibit hypoxia-induced VEGF expression, we examine VEGF expression by RT-PCR and Western blot. Our results showed that treatment of SCC-9 cells with resveratrol resulted in a dose-dependent decrease of hypoxia-induced VEGF expression, SCC-9 and HepG2 cells after being challenged with different concentrations of resveratrol for 6 h. Western blot analysis for HIF-1α protein level, hypoxia for 6 h under both normoxic and hypoxic conditions. C, SCC-9 and HepG2 cells were pretreated with different concentrations of resveratrol for 1 h followed by exposure to normoxia or hypoxia for 6 h. Western blot was done to confirm the effects of resveratrol on the expression of HIF-1α messages in SCC-9 cells as determined by RT-PCR. E and F, SCC-9 (E) and HepG2 (F) cells were treated with various concentrations of resveratrol for 6 h under both normoxic and hypoxic conditions and cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. During the experiment, cells treated with the solvent DMSO alone served as controls. Columns, mean of viable cells; bars, ± SD.

*Figure 1. Resveratrol inhibits hypoxia-induced HIF-1α protein accumulation in SCC-9 and HepG2 cells. A, SCC-9 cells cultured under normal conditions were treated with 50 μmol/L of resveratrol for different time intervals, and cell lysates were collected for Western blot analysis for HIF-1α protein level. B, immunofluorescence studies of HIF-1α protein expression in SCC-9 cells after being treated with 100 μmol/L of resveratrol (Res) for 6 h under both normoxic and hypoxic conditions. C, SCC-9 and HepG2 cells were pretreated with different concentrations of resveratrol for 1 h followed by exposure to normoxia or hypoxia for 6 h. Western blot was done to confirm the effects of resveratrol on the expression of HIF-1α messages in SCC-9 cells as determined by RT-PCR. E and F, SCC-9 (E) and HepG2 (F) cells were treated with various concentrations of resveratrol for 6 h under both normoxic and hypoxic conditions and cell viability was assayed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. During the experiment, cells treated with the solvent DMSO alone served as controls. Columns, mean of viable cells; bars, ± SD.*
Resveratrol Inhibited Hypoxia-Induced HIF-1α Protein Accumulation by Promoting Protein Degradation in SCC-9 and HepG2 Cells

It has been well documented that hypoxia induces HIF-1α protein accumulation mainly by promoting its stability instead of increasing its synthesis (20, 25, 42). Based on our observation that resveratrol had no inhibitory effects on HIF-1α mRNA levels, we propose that resveratrol inhibited hypoxia-induced HIF-1α protein accumulation mainly by promoting its degradation. To this end, we explored the effects of resveratrol treatment on the stability of HIF-1α protein. SCC-9 cells at 70% to 80% confluence were exposed to hypoxia for 6 hours, followed by treatment with cycloheximide to block ongoing protein synthesis, in the presence or absence of resveratrol for different time periods. Our results showed that in SCC-9 cells treated with resveratrol, the hypoxia-induced HIF-1α protein accumulation gradually degraded to an undetectable level at 120 minutes following exposure to cycloheximide (Fig. 3A); whereas in the absence of resveratrol, the HIF-1α protein level remained stable under hypoxic conditions (Fig. 3B). These findings suggest that resveratrol exerts its inhibitory effect by promoting the degradation of hypoxia-induced HIF-1α protein. To further explore whether the degradation of hypoxia-induced HIF-1α protein promoted by resveratrol is mediated by the proteasome degradation pathway, SCC-9 and HepG2 cells were treated with a potent and specific 26S proteasome inhibitor, MG132, in the presence or absence of resveratrol. Our results indicated that treatment with MG132 led to a significant increase in the ubiquitinated fractions, as well as total HIF-1α protein levels, whereas the resveratrol-mediated inhibition of HIF-1α protein expression was significantly blocked in the presence of MG132 (Fig. 3C and D). Taken together, these results suggested that the degradation of hypoxia-induced HIF-1α protein mediated by resveratrol is dependent on the proteasome degradation pathway in SCC-9 and HepG2 cells.

Resveratrol Inhibited Hypoxia-Induced HIF-1α Protein Accumulation and Transcriptional Activation via Blocking of PI-3K/Akt and p42/p44 MAPK Signaling Pathways

Previous studies have shown that multiple signaling pathways, particularly PI-3K/Akt and MAPK, are involved in hypoxia-induced HIF-1α protein accumulation and its downstream target gene expressions (43, 44). In this study, we also showed that exposure to hypoxia resulted in a transient increase in the phosphorylated Akt and p42/p44 MAPK levels in SCC-9 cells (Fig. 4A). To explore whether resveratrol can inhibit hypoxia-mediated activation of Akt and p42/p44 MAPK, SCC-9 cells were pretreated with various concentrations of resveratrol for 30 minutes followed by incubation under hypoxia for 1 hour, whereas cells pretreated with 50 μmol/L PD98059, a specific inhibitor of p42/p44 MAPK, or 50 μmol/L LY294002, a specific inhibitor of PI-3K, serve as positive controls. Our results showed that resveratrol attenuated the phosphorylated p42/p44 MAPK and Akt levels in a dose-dependent manner (Fig. 4B and C). To further confirm whether the activated p42/p44 MAPK and PI-3K/Akt are involved in hypoxia-induced HIF-1α protein accumulation, SCC-9 cells were pretreated with 50 μmol/L of resveratrol or various specific protein kinase inhibitors for 30 minutes followed by incubation under hypoxia for 6 hours. As shown in Fig. 4D, pretreatment of SCC-9 cells with LY294002 significantly inhibited hypoxia-induced HIF-1α protein accumulation. Pretreatment with PD98059 led to a moderate decrease in hypoxia-induced HIF-1α protein accumulation, but only a slight inhibitory effect was observed after pretreatment with 10 μmol/L SB203580, a specific inhibitor of p38 MAPK. Similar results were observed in HepG2 cells (data not shown).
To evaluate whether the activated p42/p44 MAPKs and PI-3K/Akt are also involved in hypoxia-induced transcriptional activation, SCC-9 and HepG2 cells were transiently transfected with VEGF promoter reporter followed by pretreatment with specific protein kinase inhibitors, LY294002, PD98059, or SB203528, and then exposed to normoxia or hypoxia for 16 hours. Our data indicated that pretreatment with LY294002 or PD98059, but not with SB203528, significantly abrogated hypoxia-induced VEGF promoter activity (Fig. 4E). Taken together, these results suggest that resveratrol inhibits hypoxia-induced HIF-1α protein accumulation and VEGF expression through the inhibition of PI-3K/Akt and p42/p44 MAPK activation in SCC-9 and HepG2 cells.

Effects of Resveratrol on Cell Invasion of SCC-9 Cells
Recent studies have shown the possible role of HIF-1α in the regulation of colon carcinoma cell invasion (35). To investigate whether resveratrol can inhibit cancer cell invasiveness, an in vitro cell invasion assay was done. As shown in Fig. 5, under normoxic conditions, an increase in
the baseline invasiveness of SCC-9 cells was observed under culture conditions of 10% FBS as compared with 0% FBS (Fig. 5A and C, bottom left versus top left). Pretreatment with 50 μmol/L of resveratrol suppressed the 10% FBS–stimulated invasiveness of SCC-9 cells (Fig. 5A and C, bottom right). We next examine whether hypoxic condition enhances the invasiveness of SCC-9 cells and whether resveratrol can suppress tumor migration. Our results showed that exposure to hypoxia for 48 h at 37°C under normoxia (A) or hypoxia (B) in the presence or absence of 50 μmol/L resveratrol. The invasive cells that migrated from the upper to the lower surface of the membrane were stained and photographed using a computer imaging system. C, the stained invasive cells were extracted with 200 μL extract solution and the absorbance was determined at 562 nm. Columns, mean; bars, ± SD.

**Discussion**

Invasive solid tumors suffer a characteristic condition of intratumoral hypoxia secondary to an abnormality in microvasculature and a rapid expansion of tumor mass. Hypoxia is the driving force for tumor angiogenesis, and this process is predominantly accomplished by HIF-1α-mediated overexpression of VEGF (26, 27, 41). In recent years, resveratrol has been found to inhibit tumor angiogenesis (45), but the mechanism of its antiangiogenic activity remains unclear. Recently, Cao et al. reported that
resveratrol inhibits the basal and insulin-like growth factor-1-induced HIF-1α and VEGF expression in human ovarian cancer cells (46). However, the mechanisms of growth factors and hypoxia-induced up-regulation of HIF-1α and VEGF expression are not the same. In most circumstances, growth factors and cytokines are found to up-regulate HIF-1α expression mainly by enhancing the protein translation as well as by inhibiting its degradation (40), whereas hypoxia-mediated HIF-1α protein accumulation occurs mainly by inhibiting its degradation through the ubiquitin-proteasomal pathway (17–21). Meanwhile, an autocrine loop between hypoxia and growth factors may exist in tumors, thus enhancing their angiogenic capability, tumor growth and metastases (47). In the present study, we have shown for the first time that resveratrol directly inhibits hypoxia-mediated HIF-1α protein accumulation by inhibiting its degradation via the proteasomal pathway in both SCC-9 and HepG2 cells. These findings provided the first evidence supporting the antiangiogenic effects of resveratrol in the setting of in vitro hypoxia or the equivalent of in vivo intratumoral hypoxia.

HIF-1α protein level is tightly regulated by oxygen tension via the ubiquitination and 26S proteasomal degradation system (19–21). Under hypoxic conditions, the ubiquitination and degradation of HIF-1α protein is inhibited, thus leading to the stabilization and accumulation of HIF-1α protein (15–18). In this study, we found that resveratrol inhibited the basal level of HIF-1α protein expression in SCC-9 cells (Fig. 1A) and significantly shortened the half-life of hypoxia-induced HIF-1α protein (Fig. 3A and B). Moreover, we further showed that the inhibition of hypoxia-induced HIF-1α protein accumulation by resveratrol was abolished in the presence of MG132, a potent inhibitor of the 26S proteasome (Fig. 3C and D). Taken together, these results are consistent with previous findings reported by Cao et al. in human ovarian cancer cells (46), thus supporting the notion that resveratrol inhibits HIF-1α protein expression via regulating both protein translation and HIF-1α protein degradation.

Previous studies have shown that hypoxia stimulates the activation of several signaling pathways, particularly PI-3K/Akt and p42/p44 MAPKs (43, 44). In this study, we showed that exposure of SCC-9 to hypoxia resulted in the activation of Akt and p42/p44 MAPKs (Fig. 4A). Treatment with LY294002, a specific inhibitor of PI-3K/Akt, and PD98059, a specific inhibitor of p42/p44 MAPKs, supports the notion that p42/p44 MAPKs play an important role in regulating hypoxia-mediated HIF-1α trans-activation, but not its stabilization (48–50), whereas PI-3K/Akt pathway is important for hypoxia-mediated HIF-1α stabilization (39, 43, 44). Consistent with its inhibitory effects on hypoxia-induced HIF-1α protein accumulation and VEGF expression (Fig. 2), resveratrol inhibited hypoxia-stimulated activation of PI-3K/Akt and p42/p44 MAPK in a dose-dependent manner (Fig. 4B and C), suggesting the involvement of these signaling pathways.

HIF-1α overexpression was observed in human brain and colon cancer biopsies at the invading tumor margin (27, 29). HIF-1α protein is also constitutively expressed in in vitro cultured glioma cells (27), pancreatic cancer cells (51), prostate carcinoma cells (29), as well as in human tongue squamous cell carcinomas as recently reported by us (38), and further confirmed in the current study (Fig. 1A and B). Most recently, studies have shown that HIF-1α overexpression, either as a result of intratumoral hypoxia or genetic alterations, activates the transcription of genes, the protein products of which contribute to the basement membrane invasion of colon cancer cells (35). These findings provided a molecular basis for clinical and experimental evidence associating tumor invasion and patient mortality with hypoxia and/or HIF-1α overexpression (35). In the present study, we have shown that resveratrol dramatically inhibited the stimulatory effects of hypoxia on the invasive ability of SCC-9 cells (Fig. 5), which could be attributed to its potent inhibitory effects on hypoxia-induced HIF-1α protein accumulation and VEGF expression. However, additional studies are needed to identify the associated genes that are directly or indirectly involved in resveratrol-regulated cancer cell invasion in response to hypoxia and/or HIF-1α overexpression.

One of the major concerns of using resveratrol is the potential toxicity at high dosages. In most in vitro studies, resveratrol was used at concentrations ranging from 5 to 100 μmol/L (6). Generally, in most systems tested, the dose at which an apoptotic effect was seen is relatively higher (100–300 μmol/L) than the dose used to induce cell cycle arrest or cell proliferation inhibition (10–30 μmol/L; refs. 52, 53). Recently, Tseng et al. showed that resveratrol elicited a concentration- and time-dependent inhibition of glioma cell proliferation with an IC50 of 164.7, 46.8, and 12.8 μmol/L after treatment with resveratrol for 6, 24, and 48 hours, respectively. This IC50 was comparable to those for other tumors such as leukemia, prostate, breast, and colon cancers (45). On the other hand, the dosages of resveratrol used in in vitro studies ranged from 1 to 500 mg/kg/d. Tseng et al. reported that administration of 40 and 100 mg/kg/d achieved significant antitumor effects and prolonged the survival in s.c. and intracerebral glioma models, respectively (45). When resveratrol was given orally to rats for 28 days at 20 mg/kg/d, which is 1,000 times the amount consumed by a 70-kg person taking 1.4 g of trans-resveratrol per day (54), no differences in body weight, water consumption, hematologic, or biochemical measurements were observed between resveratrol-treated and control groups. Recently, Goldberg et al. tested the absorption of resveratrol in healthy human subjects and found that nanomolar concentrations of resveratrol were achieved in serum (55). Taken together, there is no evidence of cytotoxicity or toxic effect reported in vitro or in preclinical animal studies. In the present study, the dosages of resveratrol are from 10 to 100 μmol/L, comparable to those used in ovarian cancer cells (46). At these concentrations, we reported a concentration-dependent inhibition of hypoxia-induced HIF-1α protein accumulation and VEGF expression in both SCC-9 and HepG2 cells with no obvious cytotoxicity.
In summary, our present study has provided evidence that resveratrol, as a potent naturally occurring antioxidant, exerts its broad spectrum of anticancer effects through its potent inhibition of HIF-1α and its downstream target gene, VEGF, in the context of tumor hypoxia, a common feature of most invasive cancers.

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