

Reduction of Hypoxia-Induced Transcription through the Repression of Hypoxia-Inducible Factor-1 α /Aryl Hydrocarbon Receptor Nuclear Translocator DNA Binding by the 90-kDa Heat-Shock Protein Inhibitor Radicicol

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ABSTRACT

Under low oxygen tension, cells increase the transcription of specific genes involved in angiogenesis, erythropoiesis, and glycolysis. Hypoxia-induced gene expression depends primarily on stabilization of the α subunit of hypoxia-inducible factor-1 (HIF-1 α), which acts as a heterodimeric *trans*-activator with the nuclear protein known as the aryl hydrocarbon receptor nuclear translocator (Arnt). The resulting heterodimer (HIF-1 α /Arnt) interacts specifically with the hypoxia-responsive element (HRE), thereby increasing transcription of the genes under HRE control. Our results indicate that the 90-kDa heat-shock protein (Hsp90) inhibitor radicicol reduces the hypoxia-induced expression of both endogenous vascular endothelial growth fac-

tor (VEGF) and HRE-driven reporter plasmids. Radicicol treatment (0.5 μ g/ml) does not significantly change the stability of the HIF-1 α protein and does not inhibit the nuclear localization of HIF-1 α . However, this dose of radicicol significantly reduces HRE binding by the HIF-1 α /Arnt heterodimer. Our results, the first to show that radicicol specifically inhibits the interaction between the HIF-1 α /Arnt heterodimer and HRE, suggest that Hsp90 modulates the conformation of the HIF-1 α /Arnt heterodimer, making it suitable for interaction with HRE. Furthermore, we demonstrate that radicicol reduces hypoxia-induced VEGF expression to decrease hypoxia-induced angiogenesis.

Cells adapt to hypoxia by up-regulating the transcription of specific genes involved in angiogenesis, erythropoiesis, and glycolysis. Pathologically, tumor hypoxia contributes directly to enhanced glucose metabolism and angiogenesis, which are major features of malignant progression. The genes up-regulated during hypoxia include vascular endothelial growth factor (VEGF), erythropoietin, and several glycolytic enzymes. These diverse, targeted genes are induced by a com-

mon *trans*-activator, hypoxia-inducible factor 1 (HIF-1) (Iyer et al., 1998; Bruick and McKnight, 2001b; Semenza, 2002).

HIF-1 was first identified as a heterodimeric *trans*-activator composed of two subunits, HIF-1 α and - β , both of which belong to the growing family of basic-helix-loop-helix-PAS (bHLH-PAS) proteins, including period (Per), Arnt, and single-minded (Sim). The bHLH-PAS proteins share common characteristics: first, a bHLH-PAS protein dimerizes with a specific partner protein through the HLH-PAS domain. Second, a partner such as the aryl hydrocarbon receptor (AhR) or HIF-1 α is activated by specific stimuli (i.e., xenobiotics or low oxygen tension, respectively) before translocating to the nucleus, where it heterodimerizes with a partner protein. Alternatively, Arnt, another bHLH-PAS protein, is constitutively located in the nucleus and interacts with several

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ABBREVIATIONS: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor-1; bHLH, basic helix loop helix; Arnt, aryl hydrocarbon receptor nuclear translocator; Per, period; Sim, single-minded; AhR, aryl hydrocarbon receptor; PAS, period/aryl hydrocarbon receptor; single minded; Hsp90, 90-kDa heat-shock protein; HRE, hypoxia responsive element; VHL, von Hippel-Lindau; MEM, minimal essential medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-tubyl)pyrazolo[3,4-*d*]pyrimidine; EPO, erythropoietin; PBS, phosphate-buffered saline; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; PGK-1, phosphoglycerate kinase 1; BAEC, bovine aortic endothelial cell; bFGF, bovine fibroblast growth factor.

bHLH-PAS proteins in diverse pathways. Arnt, identical to HIF-1 β , is known to be a common partner protein of AhR, mouse Sim, and HIF-1 α . Third, interactions between HLH-PAS domains bring two basic regions of the two subunits into juxtaposition, enabling individual basic regions to contact specific corresponding DNA sequences. Thus, dimerization of bHLH-PAS proteins is a prerequisite for DNA binding (Jiang et al., 1996; Crews, 1998; Taylor and Zhulin, 1999). Arnt is capable of making a homodimer with a binding preference for the palindromic E-box sequence (CACGTG), whereas neither AhR, mouse Sim, nor HIF-1 α makes a homodimer (Swanson et al., 1995). The basic region of Arnt contacts a half-site of E-box (GTG), whereas the other partner protein determines the half-site specificity of the corresponding DNA sequence that is different from E-box.

Analysis of AhR function led to the established paradigm for bHLH-PAS protein function. A lipophilic inducer, such as dioxin, passively diffuses into cells and binds to AhR, which is maintained in a receptive configuration by the 90-kDa heat-shock protein (Hsp90). Liganded AhR then separates from Hsp90 and appears in the nucleus, where it heterodimerizes with Arnt, generating a functional transcription factor that makes contact with the dioxin-responsive element. Antonsson et al. (1995) demonstrated that at least two separate domains of AhR interact with Hsp90: the ligand-binding domain within the PAS region and the bHLH domain. The PAS-Hsp90 interaction corrects the conformation of the ligand-binding domain, whereas the bHLH-Hsp90 interaction seems to be important for DNA-binding activity but not for dimerization between AhR and Arnt. Association between the AhR and cytosolic Hsp90 complex holds AhR in the cytosol in the absence of ligand, thereby inhibiting dimerization between unliganded AhR and the nuclear protein Arnt. Ligand binding to AhR triggers conformational changes in AhR, which is followed by nuclear translocation and dimerization with Arnt. Therefore, interaction with Hsp90 reduces dimerization of unliganded AhR with Arnt and simultaneously induces the conformation of AhR appropriate for both ligand binding and DNA interaction. Thus, the dioxin receptor system provides a complex and interesting model of the regulation of the bHLH-PAS transcription factor by Hsp90 (Coumailleau et al., 1995).

McGuire et al. (1995) demonstrated that Sim is also stably associated with Hsp90 and that dimerization with either Arnt or another PAS protein, Per, efficiently disrupts the Sim-Hsp90 interaction. Several studies have shown that Hsp90 interacts with HIF-1 α and that the Hsp90 inhibitor geldanamycin reduces the hypoxia-induced expression of the HRE reporter gene, suggesting that Hsp90 regulates HIF-1 α -dependent gene expression (Gradin et al., 1996; Minet et al., 1999). AhR is primarily activated by the small lipophilic ligand dioxin, whereas HIF-1 α is primarily regulated by hypoxia-induced accumulation of the HIF-1 α protein, which is otherwise rapidly degraded by the ubiquitin-proteasome pathway in normoxic cells (Kallio et al., 1999; Maxwell et al., 1999). Recent studies have demonstrated that hydroxylation of the proline residue at position 564 of HIF-1 α is catalyzed by proline hydroxylase in the presence of molecular oxygen and iron. The tumor suppressor von Hippel-Lindau (VHL) protein specifically interacts with hydroxylated HIF-1 α and mediates the assembly of a complex that activates the ubiquitin-E3 ligase. Ubiquitinated HIF-1 α is degraded by the

proteasome. When cells are hypoxic, the proline residue is not hydroxylated and HIF-1 α protein accumulates (Bruick and McKnight, 2001a; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Although the activation processes of AhR and HIF-1 α are initiated by different mechanisms, they share many functional properties. Specifically, both are associated with Hsp90 and translocate to the nucleus upon stimulation and make heterodimers with the same partner protein Arnt (Gradin et al., 1996). Because results from studies with AhR and MyoD suggest that interaction with Hsp90 corrects the conformation of the bHLH protein for DNA binding, we expect that Hsp90 is also important for DNA-binding of the HIF-1 α /Arnt heterodimer (Shaknovich et al., 1992; Antonsson et al., 1995).

Both radicicol and geldanamycin were first identified as transformation suppressors of diverse oncogenes such as Src, Ras, and Mos. Interestingly, the specific cellular target that binds radicicol is Hsp90 which is not considered to be a signaling molecule (Kwon et al., 1992; Sharma et al., 1998). Both radicicol and geldanamycin bind the ATP-binding site of Hsp90 and block the assembly of Hsp90 heterocomplexes, including accessory cochaperone, p23, and immunophilin (Pratt, 1997; Schulte et al., 1998). These inhibitors, which do not inhibit kinase activity, prevent Src-Hsp90 or Raf-1-Hsp90 heterocomplex formation, thereby decreasing the stability and functionality of signaling molecules. Immunoblotting analysis demonstrated that Raf-1, p53, and p185erbB2 protein levels were substantially decreased by treatment with radicicol and geldanamycin in SKBR3 cells, a breast cancer cell line (Schulte et al., 1998). Radicicol and geldanamycin exhibit similar antiproliferative activities, probably because of modulation of Hsp90; therefore, Hsp90 may become an important novel target in anticancer drug development (Neckers et al., 1999).

Here, we investigated whether radicicol inhibits the activation process of HIF-1 α in response to hypoxia. We dissected the HIF-1 α activation process into several steps, including stabilization, nuclear localization, DNA binding, and the start of transcription of hypoxia-inducible VEGF. Our results demonstrate that radicicol specifically inhibits DNA binding of HIF-1 α /Arnt, thereby reducing hypoxia-induced VEGF expression, which results in the reduction of hypoxia-induced angiogenesis.

Materials and Methods

Cell Culture and Hypoxia Treatment. Hep 3B cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in MEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Gaithersburg, MD), 50 IU of penicillin, and 50 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO) under humidified air containing 5% CO₂ at 37°C. Cells were exposed to hypoxia (0.1% O₂) by incubating cells in an anaerobic incubator (model 1029; Forma Scientific, Marietta, OH) in 5% CO₂/10% H₂/85% N₂ at 37°C. Hypoxia was also induced chemically by treating cells with 100 μ M CoCl₂ (Sigma-Aldrich) (Mukhopadhyay et al., 1995).

Inhibitors, Antibodies, and Plasmids. Hep 3B cells were treated with 150 μ M of genistein (Sigma-Aldrich) in dimethyl sulfoxide (DMSO), 10 μ M PP2 (Calbiochem-Novabiochem Co., San Diego, CA) in DMSO, 1 μ g/ml geldanamycin (Sigma-Aldrich) in DMSO, and 0.5 μ g/ml radicicol in DMSO. Radicicol was purified as described previously (Kwon et al., 1992). Anti-HIF-1 α antibody and anti-Arnt antibody were obtained from Transduction Laboratories (Lexington,

KY). The immunogen region for the anti-HIF-1 α antibody is located between amino acids 610 and 727. The p(HRE)₄-luc reporter plasmid contains four copies of the hypoxia-responsive element (5'-GATCGC-CCTACGTGCTGTCTCA-3') of erythropoietin (EPO), the simian virus 40 promoter, and the firefly luciferase gene (Ema et al., 1997). The p(EPO)-luc reporter plasmid contains one copy of the 3' enhancer of EPO (nucleotides 3449–3470).

Preparation of Nuclear Extracts. Hep 3B cells were serum starved by incubation in MEM containing 0.5% FBS for 24 h and then incubated in 0.1% O₂ for 6 h. Nuclear extracts were prepared as described previously (Hur et al., 2001). Confluent (70%) Hep 3B cells in 100-mm tissue culture plates were washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in four packed cell volumes of buffer A (10 mM Tris-HCl, pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, 2 μ g/ml aprotinin, and 1 mM Na₃VO₄) and incubated on ice for 10 min. Subsequently, the cells were homogenized by 15 strokes with a Dounce type-B pestle. The nuclei were pelleted by centrifugation at 3,300g for 15 min at 4°C and resuspended in two packed nuclei volumes of buffer B (20 mM Tris-HCl, pH 7.8, 1.5 mM MgCl₂, 450 mM KCl, 20% glycerol, 0.5 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, 2 μ g/ml aprotinin, and 1 mM Na₃VO₄). The suspensions were incubated with gentle rocking at 4°C for 1 h and centrifuged at 25,000g for 30 min at 4°C. Supernatants were frozen at -80°C. Protein concentrations were measured by the Bradford (1976) assay.

Electrophoretic Mobility Shift Assay. Oligonucleotides for W18 (sense, 5'-agcttGCCCTACGTGCTGTCTCAg-3'; antisense, 5'-aatteTGAGACAGCACGTAGGGCa-3') were annealed and labeled (1.75 pmol) with [α -³²P]dATP and Klenow fragment. Unincorporated nucleotides were removed by gel filtration over a Sephadex G25 column. Nuclear extracts were preincubated with poly(dI/dC) (500 ng) in 20 μ l of buffer C (10 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM MgCl₂, 1 mM EDTA, 5 mM DTT, and 5% glycerol). The labeled W18 probe (5 \times 10⁵ cpm) was incubated with nuclear extract (10 μ g) for 15 min at room temperature. The reactions were separated on a 5% polyacrylamide gel at 250 V in 0.5 \times Tris/borate/EDTA at 4°C. Gels were vacuum-dried and autoradiographed. For supershift assays, either anti-HIF-1 α antibody or anti-Arnt antibody was added to the reaction mixture and incubated for 2 h at 4°C before loading (Semenza and Wang, 1992).

Western Analysis of HIF-1 α and Coimmunoprecipitation. Hep 3B cells were serum-starved by incubation in MEM containing 0.5% FBS for 40 to 48 h before treatment with inhibitors or hypoxia. Cells were washed once with ice-cold PBS and lysed in radioimmunoprecipitation assay buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 100 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 50 mM β -glycerophosphate, 25 mM NaF, 20 mM EGTA, 1 mM DTT, and 1 mM Na₃VO₄. The lysates were centrifuged at 10,000g for 10 min at 4°C. The protein concentrations of the supernatants were measured by the Bradford (1976) assay. An equal amount of each protein sample (30 μ g) was used and Western analysis was performed as described previously (Hur et al., 2001). Nuclear extracts (250 μ g) were prepared as described above and precleared by incubating with 1 μ g of anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and 20 μ l of 0.5% ImmunoPure immobilized protein A/G gel (Pierce, Rockford, IL) for 10 min at 4°C. The cleared extracts were mixed with 2 μ g of anti-Arnt antibody. After addition of 20 μ l of 0.5% ImmunoPure immobilized protein A/G gel, mixtures were rotated for 4 h at 4°C and centrifuged. Immunoprecipitated pellets were washed three times in buffer and then resuspended in SDS sample buffer. The entire samples were boiled for 3 to 5 min before electrophoresis through an 8% SDS-polyacrylamide gel. The proteins were transferred onto nitrocellulose membrane by semidry transfer (Trans-Blot SD; Bio-Rad, Hercules, CA). Coimmunoprecipitated proteins were immunoblotted with either anti-HIF-1 α antibody or anti-

Arnt antibody. HIF-1 α or Arnt proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ) with an anti-mouse Ig conjugated with horseradish peroxidase as a secondary antibody.

Northern Analysis. Hep 3B cells were grown to 80% confluence on 100-mm tissue culture plates. Total RNA was isolated using an RNeasy spin column according to the manufacturer's instructions (QIAGEN, Chatsworth, CA). Total RNA (12 μ g) was electrophoresed through a 1% agarose gel containing formaldehyde and transferred to nitrocellulose membrane. Blots were hybridized with α -³²P-labeled cDNA of VEGF, phosphoglycerate kinase-1 (PGK-1), or actin, washed, dried, and autoradiographed with Hyperfilm MP (Amersham Biosciences). The expression levels of VEGF, PGK-1 and actin were measured with a radioisotope imaging analyzer (Bas2000; Fuji, Tokyo, Japan).

Transient Transfection and Luciferase Assay. Hep 3B cells were plated at 1 \times 10⁵ cells per well in a 12-well plate. Eighteen hours later, transfection was carried out using Superfect reagent (QIAGEN) according to the manufacturer's instructions. Twelve hours before hypoxic treatment, transfected Hep 3B cells were serum starved with medium containing 0.5% FBS. Forty-eight hours after transfection, cell extracts were prepared and analyzed with a luminometer (Berthold Lumat LB9501) using the luciferase assay system (Promega, Madison, WI). Each measured luciferase activity was normalized for total protein concentration, as measured by the Bradford (1976) assay using bovine serum albumin as a standard. The transfection efficiency was monitored by measuring cotransfection of the β -galactosidase encoding plasmid (pCHO110) and β -galactosidase activity.

Fluorescence Microscopy. Hep 3B cells were cultured on a cover-slide and treated with the control vehicle (DMSO) or radicicol (0.5 μ g/ml) at 30 min before the 4 h exposure to CoCl₂ (75 μ M). Cells were washed twice with PBS and fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100. Cells were then incubated for 1 h at 20°C with anti-HIF- α antibody diluted to 1:100 in PBS containing 3% FBS and 1% bovine serum albumin. After washing twice with PBS, cells were incubated for 30 min at 20°C with fluorescein isothiocyanate-coupled secondary anti-mouse antibody staining (1:100 dilution). Cells were washed twice with PBS and incubated with 500 mM propidium iodide for 5 min at 20°C. Nuclei were visualized by staining propidium iodide. After washing three times with PBS, stained cells and nuclei were observed with fluorescence microscope.

Capillary Tube Formation Assay. The bovine fibroblast growth factor (bFGF) was obtained from Upstate Biotechnology (Lake Placid, NY), the Matrigel from Collaborative Biomedical Products (Bedford, MA), and Microcon YM-10 from Millipore (Bedford, MA). The early passages (5–7 passages) of bovine aortic endothelial cells (BAECs) were kindly provided by Dr. I. Jo at the National Institutes of Health of Korea. BAECs were grown in MEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO₂. Hep 3B cells were treated with geldanamycin (1 μ g/ml in DMSO) or radicicol (0.1, 0.5 or 2.5 μ g/ml in DMSO) 1 h before a 16-h exposure to 0.1% O₂. The cell medium was prepared from treated Hep 3B cells in low serum medium (0.5% FBS). Each cell medium was filtered through Microcon YM-10 to filter out low molecular mass materials of less than 10 kDa. Agent removal was confirmed by thin-layer chromatography of the ethyl acetate extract from the Microcon YM-10 concentrated cell medium. To conduct the capillary tube formation assay, 250 μ l of matrigel (10 mg/ml) was placed in a 24-well culture plate and polymerized for 30 min at 37°C. The BAECs (1 \times 10⁵ cells) were seeded on the surface of the Matrigel and treated with bFGF (30 ng/ml). Then 50 μ l of concentrated cell medium was added to each sample and incubated for 18 h. The morphological changes in the cells and tubes were observed under a microscope and photographed at \times 100 magnification using a JVC digital camera (TK-C1380U; JVC, Yokohama, Japan) (Kim et al., 2001).

Results

The Hsp90 Inhibitor Radicicol Represses Hypoxia-Induced Gene Expression. To investigate the possibility that Hsp90 modulates hypoxia-induced gene expression, we first measured the changes in hypoxia-induced expression of both endogenous genes and HRE-driven reporter genes in Hep 3B cells that had been pretreated with several inhibitors. For the following analyses, we serum-starved Hep 3B cells before hypoxic stimulation because serum itself slightly activates the function of HIF-1 α under normoxic conditions and thereby reduces the hypoxia-inducibility of HIF-1 α activity (D'Angelo et al., 2000; Richard et al., 2000). Then, several inhibitors were added to the cell medium of Hep 3B cells 1 h before hypoxic exposure. We exposed cells to hypoxic conditions by incubating cells in a hypoxic chamber (0.1% O₂) or treating cells with the hypoxia-mimicking agent, CoCl₂. Treatment with radicicol (0.5 μ g/ml, 1.4 μ M) partially reduces hypoxia-induced expression of the endogenous *VEGF* and *PGK*-1 genes, as shown in Fig. 1A. We also found that geldanamycin (1 μ g/ml, 1.8 μ M), another Hsp90 inhibitor, reduces hypoxia-induced *VEGF* and *PGK*-1 expression. Treatment with the tyrosine kinase inhibitor genistein (150 μ M) and the Src specific inhibitor PP2 (10 μ M) also reduces hypoxia-induced expression of endogenous *VEGF* and *PGK*-1 as shown in Fig. 1A. We used two hypoxia-inducible reporter plasmids containing either four copies of HRE or 50 base pairs of the EPO 3' enhancer region upstream of the simian virus 40 promoter and luciferase gene (Huang et al., 1996). Treatment with radicicol decreased the hypoxia-induced expression of both reporter genes dose dependently (Fig. 1B).

Because both geldanamycin and radicicol inhibit tyrosine kinases, and treatment with the tyrosine kinase inhibitors genistein and PP2 block hypoxia-induced gene expression, Hsp90 may modulate hypoxia-induced activation of HIF-1 α directly or indirectly through tyrosine kinase action (Bijl-maker and Marsh, 2000).

Effects of Radicicol on Hypoxia-Induced Stabilization of HIF-1 α . The activation process of HIF-1 α is initiated by the hypoxia-induced stabilization of the HIF-1 α protein. Hsp90 acts as a chaperone to protect cells from nonnative proteins, which increase in response to heat shock and other stresses. Thus, we sought to investigate whether Hsp90 is involved in the accumulation of HIF-1 α upon hypoxic stress. We measured the changes in HIF-1 α protein levels in Hep 3B cells that were treated with inhibitors before hypoxic exposure. Western analyses displayed multiple bands that were recognized by anti-HIF-1 α antibody, indicating that stabilized HIF-1 α undergoes strong post-translational modifications (Fig. 2A). In hypoxic cells, slowly migrating HIF-1 α is more stable than fast-migrating HIF-1 α . Although treatment with radicicol slightly reduces the stability of slowly migrating HIF-1 α by 23%, it increases the stability of the fast migrating one by 35%. Overall, treatment with radicicol (0.5 μ g/ml) decreases the total amount of hypoxia-induced HIF-1 α protein by 5% (Fig. 2A). However, an equivalent dose of radicicol reduces hypoxia-induced gene expression of both reporter and endogenous genes (Fig. 1). In contrast, treatment with the tyrosine kinase inhibitors genistein or PP2 significantly reduced the total amount of stabilized HIF-1 α protein. When hypoxic conditions were mimicked by CoCl₂ treatment, similar results were observed (Fig. 2B).

We found that radicicol and tyrosine kinase inhibitors have different effects on hypoxia-induced stabilization of HIF-1 α , suggesting that radicicol-induced reduction of hypoxia-in-

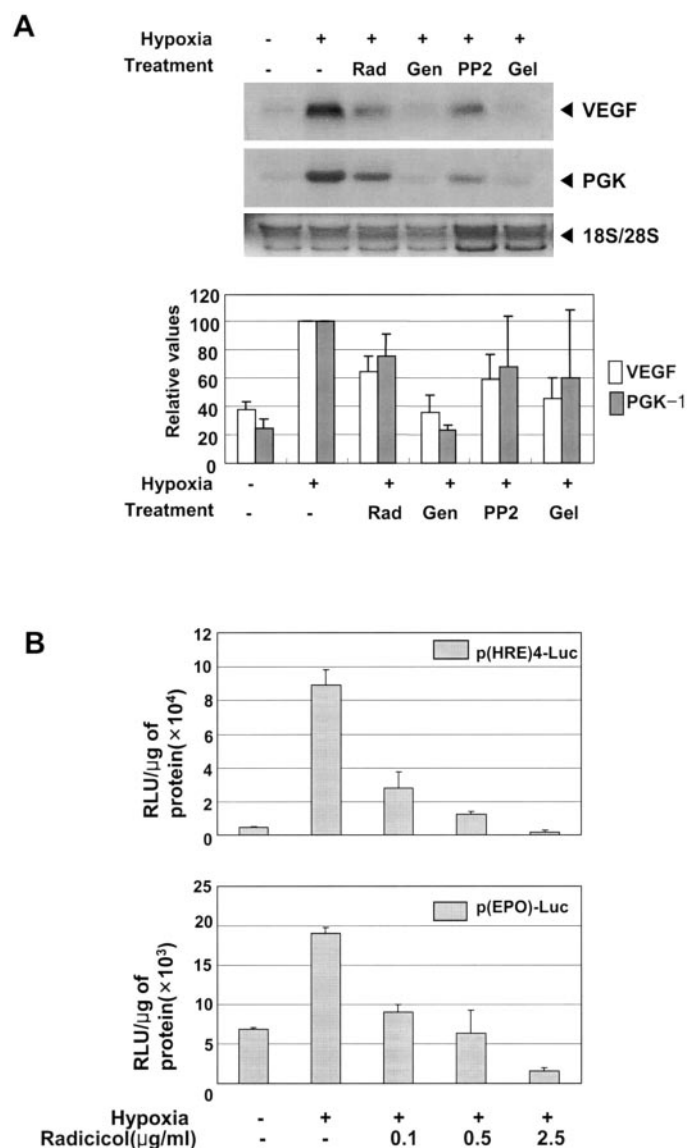


Fig. 1. Effects of radicicol on hypoxia-induced expression of endogenous genes and reporter genes. A, Hep 3B cells were treated with radicicol (0.5 μ g/ml in DMSO), genistein (150 μ M in DMSO), PP2 (10 μ M in DMSO), or geldanamycin (1 μ g/ml in DMSO) 1 h before a 16 h exposure to 0.1% O₂. Total RNA was isolated and 12 μ g of total RNA was separated on the 1% formaldehyde agarose gel. RNA was transferred onto a nitrocellulose membrane and hybridized with ³²P-labeled human VEGF and PGK-1. mRNA of VEGF and PGK-1 was visualized by exposure to X-ray film. The mRNA level of VEGF and PGK-1 was quantified by filmless radioisotope imaging analysis of the bands labeled "VEGF" and "PGK-1". The level of VEGF or PGK-1 mRNA in hypoxic cells was defined as 100%. The values represent the means and S.D. of three experiments. B, Hep 3B cells (1.0 × 10⁵ cells/well in a 12-well plate) were transfected with either reporter plasmids p(HRE)₄-luc (50 ng) (top) or p(EPO)-luc (50 ng) (bottom). Transfected cells were serum-starved for 20–24 h with medium containing 0.5% FBS and then treated with the indicated dose of the Hsp90 inhibitor radicicol 1 h before a 16-h exposure to 0.1% O₂. Forty-eight hours after transfection, cell extracts were prepared and analyzed by the luciferase assay system. Luciferase activity was normalized for total protein concentration and the value represents the mean and S.D. of three experiments. The transfection efficiency was monitored by measuring cotransfection of the β -galactosidase encoding plasmid (pCHO110) and β -galactosidase activity.

duced gene expression is not caused by decreased tyrosine kinase activity. The question, then, is how does radicicol reduce hypoxia-induced activation of HIF-1 α ?

Effects of Radicicol on Nuclear Localization of HIF-1 α . Release of Hsp90 is critical for unmasking functional activities such as nuclear localization, dimerization, and DNA binding of the AhR and glucocorticoid receptor. Because the partner protein Arnt constitutively locates in the nucleus, dimerization between Arnt and cytosolic HIF-1 α requires nuclear translocation of HIF-1 α (Shaknovich et al., 1992; Pratt, 1997). Kallio et al. (1998) demonstrated that hypoxia-inducible nuclear accumulation of HIF-1 α depends on a nuclear localization signal within the C-terminal region of HIF-1 α . Nuclear translocation of many *trans*-activators is conditionally stimulated by signal-induced conformational changes and the unmasking of nuclear localization signals. To test whether hypoxia-induced nuclear localization of HIF-1 α is modulated by radicicol, we visualized HIF-1 α proteins in radicicol treated cells by immunofluorescence. Our results indicate that upon hypoxic exposure, the HIF-1 α protein is exclusively detected in the nucleus, whereas HIF-1 α is not visible in normoxic cells (shown in Fig. 3). Treatment with radicicol did not change either the amount of HIF-1 α

protein or the hypoxia-induced nuclear localization of HIF-1 α . This observation using fluorescence microscopy is consistent with the finding that radicicol does not change the hypoxia-induced stability of HIF-1 α (Fig. 2). Our finding suggests that the Hsp90 inhibitor radicicol does not block nuclear localization of HIF-1 α to reduce hypoxia-induced gene expression.

Effects of Radicicol on the Hypoxia-Induced DNA Binding Ability of the HIF-1 Complex. To test whether radicicol affects the protein-protein interaction between HIF-1 α and Arnt, we treated Hep 3B cells with radicicol before exposure to hypoxia and then prepared nuclear protein extracts. Immuno-precipitation was performed using anti-Arnt antibody and coprecipitated HIF-1 α was detected by Western analysis using anti-HIF-1 α antibody. Our results indicate that treatment with radicicol does not reduce the interaction between Arnt and HIF-1 α in hypoxic cells. To investigate the effect of radicicol on the ability of the HIF-1 α /Arnt complex to bind HRE in response to hypoxia, the nuclear extracts were mixed with a radiolabeled oligonucleotide (W18) that contains the HRE sequence and the mixture was subjected to the electrophoretic mobility shift assay (EMSA) (Semenza and Wang, 1992). As observed in previous EMSAs with HRE, our results revealed the presence of hypoxia-induced, constitutive and nonspecific complexes (Hur et al., 2001). The hypoxia-induced complex was detected only when nuclear extracts from hypoxic Hep 3B cells were assayed. To examine the composition of the hypoxia-induced complexes, nuclear extracts were mixed with either anti-HIF-1 α antibody or anti-Arnt antibody and were then subjected to EMSA. Supershifts confirmed the presence of HIF-1 α and Arnt in the complex (Fig. 4B). Treatment with radicicol significantly reduced the hypoxia-induced DNA binding of the HIF-1 complex, indicating that Hsp90 does affect the DNA binding of the HIF-1 α /Arnt heterodimer (Fig. 4B). As shown in Figs. 2 and 3, radicicol does not change

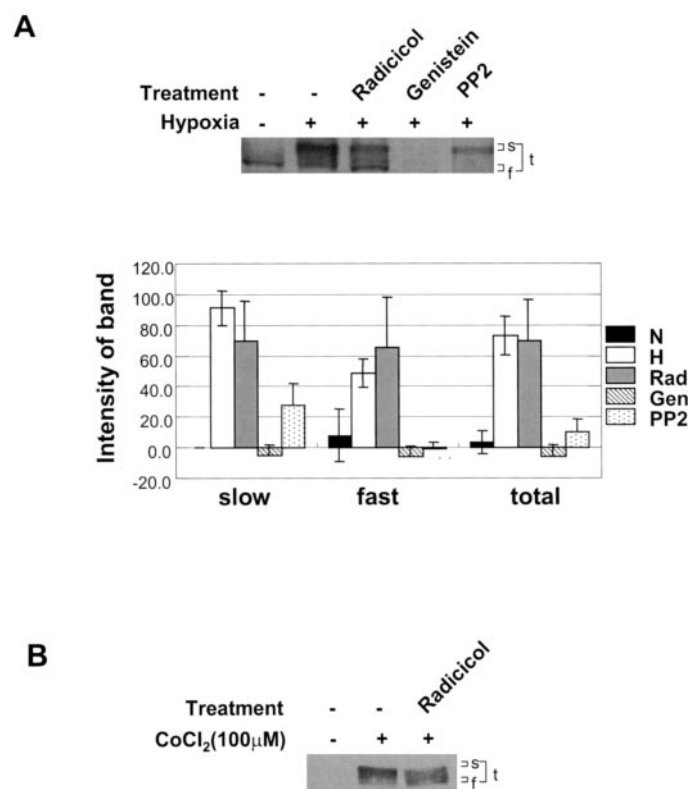


Fig. 2. Effects of radicicol on the hypoxia-induced stabilization of HIF-1 α . Before stimulation, Hep 3B cells were serum-starved with medium containing 0.5% FBS for 36 h and then treated with radicicol (0.5 μ g/ml), genistein (150 μ M), or PP2 (10 μ M) 1 h before a 6-h exposure to 0.1% O₂. A, CoCl₂ (100 μ M). B, the cells were lysed and 30 μ g of whole-cell lysates were used for Western analysis. Immunoblot analyses were performed using anti-HIF-1 α antibody and visualized by a chemiluminescence system. The slowly migrating, high molecular mass HIF-1 α is marked by "s", whereas the fast migrating, low molecular mass HIF-1 α is marked by "f". The total HIF-1 α band is marked "t". The density of each band, "s", "f", and "t" was quantified by densitometry (Bio-Rad GS700). The density of the slow band in the normoxic sample was defined as the background. The values represent the means and S.D. of three experiments.

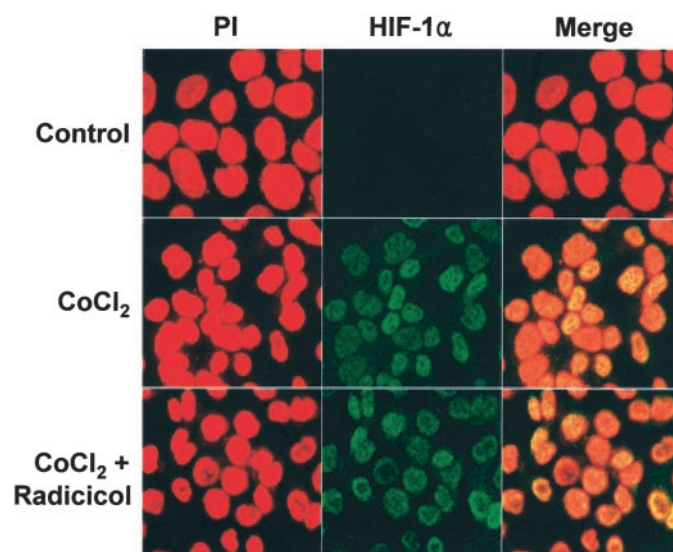


Fig. 3. Effects of radicicol on the nuclear localization of HIF-1 α . Hep 3B cells were treated with the control vehicle (DMSO) or radicicol (0.5 μ g/ml in DMSO) 30 min before a 4-h exposure to CoCl₂ (75 μ M). Cells were fixed and incubated with anti-HIF antibody (1:100 dilution) followed by fluorescein isothiocyanate-coupled secondary anti-mouse antibody staining (1:100 dilution). Cells were then incubated with 500 nM propidium iodide (PI) for 5 min and subjected to fluorescence microscopy.

hypoxia-induced stability, nuclear localization of HIF-1 α , or the protein interaction between Arnt and HIF-1 α , although it specifically reduces the DNA binding ability of the HIF-1 complex. We speculated that the Hsp90 inhibitor radicicol restrains the proper conformation of the bHLH domains of HIF-1, resulting in the loss of DNA binding activity of the HIF-1 heterodimer, thereby reducing hypoxia-induced gene expression.

The Effect of Radicicol-Treated Cell Medium on Capillary Tube Formation of Endothelial Cells. To verify that radicicol actually reduces the secretion of VEGF from hypoxic cells, which may affect angiogenesis, we tested the effect of radicicol treated cell medium from Hep 3B cells on capillary tube formation in bovine aortic endothelial cells (BAECs). Capillary tube formation is a key phenotype of angiogenesis induced by VEGF (Kim et al., 2001). We removed radicicol or geldanamycin from the cell medium by filtering out low molecular mass materials of less than 10 kDa and confirmed the absence of radicicol or geldanamycin in the cell medium by thin-layer chromatography of an ethyl acetate extract from the cell medium. As shown in Fig. 5,

cultured BAECs on the Matrigel formed an extensive network of thick tubes in the presence of bovine fibroblast growth factor (bFGF). Hypoxia-treated cell medium increased tube formation as much as the positive control treated with bFGF, suggesting the expression of VEGF in hypoxic Hep 3B cells (Fig. 5, A to C). However, geldanamycin (1 μ g/ml) or radicicol treated hypoxic cell medium displayed reduced tube formation in BAECs in a dose-dependent manner compared with the untreated hypoxic cell medium (Fig. 5, D to G). The staining of cell medium-treated BAECs with trypan blue confirmed that the reduction in tube formation was not caused by cytotoxic effects (data not shown). Accordingly, these results demonstrate that radicicol reduces the secretion of the angiogenic factor VEGF from hypoxic cells.

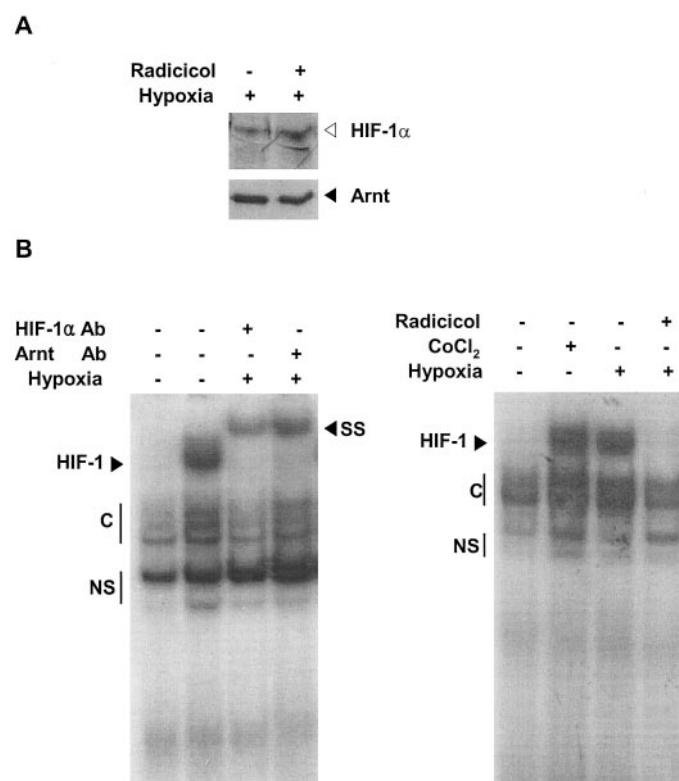


Fig. 4. Effects of radicicol on the hypoxia-induced DNA binding ability of the HIF-1 complex. Hep 3B cells were serum-starved for 36 to 48 h with medium containing 0.5% FBS. Cells were pretreated with radicicol (0.5 μ g/ml) 1 h before a 6-h exposure to 0.1% O₂ or CoCl₂ (100 μ M). Nuclear extract was prepared as described under *Materials and Methods*. A, the nuclear extracts (250 μ g) were precipitated with anti-Arnt antibody and immobilized by protein A/G gel. The precipitates were separated by SDS-polyacrylamide gel electrophoresis and then coimmunoprecipitated proteins were analyzed by immunoblotting with either anti-HIF1 α antibody (\triangleright) or anti-Arnt antibody (\blacktriangleleft). B, the nuclear extracts were analyzed by EMSA. The nuclear extracts (10 μ g) were mixed with radiolabeled W18, followed by incubation with either anti-HIF1 α antibody or anti-Arnt antibody (left). Hep 3B cells were pretreated with radicicol (0.5 μ g/ml) for 1 h and then stimulated for 6 h with 0.1% O₂ or CoCl₂ (100 μ M). The nuclear extracts (10 μ g) were mixed with radiolabeled W18 and EMSA was performed (right).

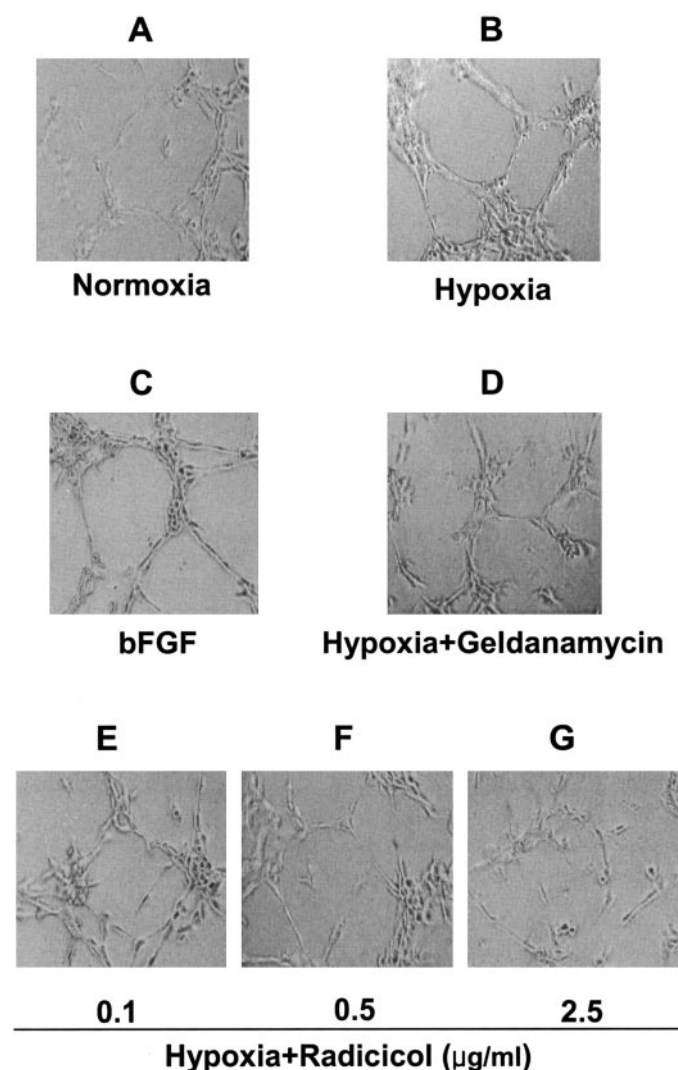


Fig. 5. The effects of radicicol-treated cell medium on capillary tube formation in endothelial cells (BAECs). Cell media were prepared from Hep 3B cells that had been treated with inhibitors and hypoxia as described under *Materials and Methods*. BAECs were seeded on Matrigel-coated wells at a density of 1×10^5 cells/well. A, the BAECs were stimulated with normoxic cell medium. B, the BAECs were stimulated with hypoxic cell medium treated with the control vehicle. C, the BAECs were stimulated with bFGF (30 ng/ml). D, the BAECs were stimulated with cell medium from hypoxic cells treated with geldanamycin (1 μ g/ml). E to G, the BAECs were stimulated with cell medium from hypoxic cells treated with 0.1, 0.5, and 2.5 μ g/ml of radicicol, respectively. Figures were selected as representative photographs from two independent experiments.

Discussion

We investigated the modulatory effects of the Hsp90 inhibitor radicicol on the HIF-1 activation process in response to hypoxia. We dissected the HIF-1 activation process into several steps, including stabilization of the HIF-1 α protein, nuclear translocation, heterodimerization with Arnt, DNA binding of the HIF-1 α /Arnt complex, and the ultimate expression of both hypoxia-inducible endogenous and reporter genes. We found that hypoxia-induced DNA binding of HIF-1 α is more sensitive to inhibition by radicicol than is the stability of the HIF-1 α protein. Reduced gene expression of VEGF and PGK-1 results from the hypoxia-induced inhibition of HIF-1 α DNA binding. Our results suggest that Hsp90 regulates the DNA binding step of the HIF-1 α activation process. The addition of recombinant or purified Hsp90 to EMSAs has been shown to increase the DNA binding ability of the bHLH protein MyoD. This activation process involves a transient interaction between Hsp90 and MyoD that does not involve the formation of a stable tertiary complex such as DNA-MyoD-Hsp90 (Shaknovich et al., 1992). The results suggest that transient interaction of DNA-binding proteins with Hsp90 can affect their DNA binding activity.

Richard et al. (1999) demonstrated that phosphatase treatment of hypoxic cell extracts removed slowly migrating HIF-1 α , but not fast-migrating proteins, indicating that hypoxia-induced phosphorylational modification of HIF-1 α slows electrophoretic migration. In hypoxic cells, the slowly migrating HIF-1 α is more stable than the fast-migrating HIF-1 α . Our results show that radicicol decreases the proportion of slowly migrating HIF-1 α to fast-migrating HIF-1 α (Fig. 2). Further investigation is needed to determine whether these proportional changes affect the function of HIF-1 α or Hsp90.

Recently, HIF-1 α was also recognized as a novel, tumor-specific target for anticancer therapy because 1) it is an essential transcription activator of VEGF and many glycolytic enzymes, which are required for angiogenesis and continued tumor growth; 2) it is present only in hypoxic cells, which are common inside solid tumors (Brown, 2000). Hsp90 inhibitors result in the destabilization and degradation of Hsp90 client proteins, including mutated p53, Raf-1, and ErbB2. Furthermore, Hsp90 inhibitors have already exhibited promising antitumor activity such as antiproliferative and antiangiogenic effects (Oikawa et al., 1993; Neckers et al., 1999). Another Hsp90 inhibitor, the oxime derivative of radicicol known as KF58333 (1 μ M), decreased the HIF-1 α protein level under both normoxia and hypoxia in human breast cancer cells in vitro and inhibited growth and angiogenesis in human breast cancer xenografts (Kurebayashi et al., 2001). Therefore, Hsp90 inhibitors could become useful as hypoxia-sensitive and therapeutic antiangiogenic and antitumor agents.

The subcellular distribution of Hsp90 in hypoxic cells is not well understood. Minet et al. (1999) demonstrated that Hsp90 does not cotranslocate with HIF-1 α into the nucleus upon hypoxia in COS-7 cells. In contrast, Katschinski et al. (2002) showed that hypoxia does not increase the total amount of Hsp90 and induces nuclear translocation of Hsp90 in HepG2 cells. Our immunofluorescence studies demonstrated that the concentration and subcellular distribution of Hsp90 in Hep 3B cells are not significantly changed by CoCl₂ treatment (data not shown). Although the subcellular local-

ization of Hsp90 in hypoxic cells is not clear, coimmunoprecipitation analyses showed that Hsp90 interacts with the bHLH-PAS domain of HIF-1 α in normoxia but not in hypoxia (Gradin et al., 1996; Minet et al., 1999). However, the effects of the interaction between HIF-1 α and Hsp90 on the functions of HIF-1 α , such as DNA binding and transcriptional activation of target genes in hypoxic cells, must be investigated in the future.

References

- Antonsson C, Whitelaw ML, McGuire J, Gustafsson JA, and Poellinger L (1995) Distinct roles of the molecular chaperone hsp90 in modulating dioxin receptor function via the basic helix-loop-helix and PAS domains. *Mol Cell Biol* **15**:756–765.
- Bijlmakers M-JE and Marsh M (2000) Hsp90 is essential for the synthesis and subsequent membrane association, but not the maintenance, of the Src-Kinase p56^{lck}. *Mol Biol Cell* **11**:1585–1595.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254.
- Brown JM (2000) Exploiting the hypoxic cancer cell: mechanisms and therapeutic strategies. *Mol Med Today* **6**:157–162.
- Bruick RK and McKnight SL (2001a) A conserved family of prolyl-4-hydroxylases that modify HIF. *Science (Wash DC)* **294**:1337–1340.
- Bruick RK and McKnight SL (2001b) Building better vasculature. *Genes Dev* **15**:2497–2502.
- Coumault L, Poellinger L, Gustafsson JA, and Whitelaw ML (1995) Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild type ligand binding affinity and specificity. *J Biol Chem* **270**:25291–25300.
- Crews ST (1998) Control of cell lineage-specific development and transcription by bHLH-PAS proteins. *Genes Dev* **12**:607–620.
- D'Angelo G, Ladoux A, and Frelon C (2000) Hypoxia-induced transcriptional activation of vascular endothelial growth factor is inhibited by serum. *Biochem Biophys Res Commun* **267**:334–338.
- Ema M, Taya S, Yokotani N, Sogawa K, Matsuda Y, and Fujii-Kuriyama Y (1997) A novel bHLH-PAS factor with close sequence similarity to hypoxia-inducible factor 1 α regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci USA* **94**:4273–4278.
- Epstein AC, Gleadow JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, et al. (2001) *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**:43–54.
- Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgard R, Tora L, Gassmann M, and Poellinger L (1996) Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol Cell Biol* **16**:5221–5231.
- Huang LE, Arany Z, Livingston DM, and Bunn HF (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its α -subunit. *J Biol Chem* **271**:32253–32259.
- Hur E, Chang KY, Lee E, Lee S-K, and Park H (2001) Mitogen-activated protein kinase kinase inhibitor PD98059 blocks the trans-activation but not the stabilization or DNA binding ability of hypoxia-inducible factor-1. *Mol Pharmacol* **59**:1216–1224.
- Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG Jr (2001) HIF α Targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science (Wash DC)* **292**:464–468.
- Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, et al. (1998) Cellular and developmental control of O₂ homeostasis by hypoxia-inducible factor 1 α . *Genes Dev* **12**:149–162.
- Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim AV, Hebestreit HF, Mukherji M, Schofield CJ, et al. (2001) Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science (Wash DC)* **292**:468–472.
- Jiang BH, Rue E, Wang GL, Roe R, and Semenza GL (1996) Dimerization, DNA binding and transactivation properties of hypoxia-inducible factor 1. *J Biol Chem* **271**:17771–17778.
- Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, Tanaka H, and Poellinger L (1998) Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1 α . *EMBO (Eur Mol Biol Organ) J* **17**:6573–6586.
- Kallio PJ, Wilson WJ, O'Brien S, Makino Y, and Poellinger L (1999) Regulation of the hypoxia-inducible transcription factor 1 α by the ubiquitin-proteasome pathway. *J Biol Chem* **274**:6519–6525.
- Katschinski DM, Le L, Heinrich D, Wagner KF, Hofer T, Schindler SG, and Wenger RH (2002) Heat induction of the unphosphorylated form of hypoxia-inducible factor-1 α is dependent on heat shock protein-90 activity. *J Biol Chem* **277**:9262–9267.
- Kim MS, Kwon HJ, Lee YM, Baek JH, Jang J-E, Lee S-W, Moon E-J, Kim H-S, Lee S-K, Chung HY, et al. (2001) Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med* **7**:437–443.
- Kurebayashi J, Otsuki T, Kurosaki M, Soga S, Akinaga S, and Sonoo H (2001) A radicicol derivative, KF58333, inhibits expression of hypoxia-inducible factor-1 α and vascular endothelial growth factor, angiogenesis and growth of human breast cancer xenografts. *Jpn J Cancer Res* **92**:1342–1351.
- Kwon HJ, Yoshida M, Fukui Y, Horinouchi S, and Beppu T (1992) Potent and specific inhibition of p60v-src protein kinase both in vivo and in vitro by radicicol. *Cancer Res* **52**:6926–6930.

- Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature (Lond)* **399**:271–275.
- McGuire J, Coumailleau P, Whitelaw ML, Gustafsson JA, and Poellinger L (1995) The basic helix-loop-helix/PAS factor Sim is associated with hsp90. Implications for regulation by interaction with partner factors. *J Biol Chem* **270**:31353–31357.
- Minet E, Mottet D, Michel G, Roland I, Raes M, Remacle J, and Michiels C (1999) Hypoxia-induced activation of HIF-1: role of HIF-1 α -Hsp90 interaction. *FEBS Lett* **460**:251–256.
- Mukhopadhyay D, Tsiokas L, Zhou XM, Foster D, Brugge JS, and Sukhatme VP (1995) Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature (Lond)* **375**:577–581.
- Neckers L, Schulte TW, and Mimnaugh E (1999) Geldanamycin as a potential anti-cancer agent: its molecular target and biochemical activity. *Investig New Drugs* **17**:361–373.
- Oikawa T, Ito H, Ashino H, Toi M, Tominaga T, Morita I, and Murota S (1993) Radicol, a microbial cell differentiation modulator, inhibits in vivo angiogenesis. *Eur J Pharmacol* **241**:221–227.
- Pratt WB (1997) The role of the hsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via MAP kinase. *Annu Rev Pharmacol Toxicol* **37**:297–326.
- Richard DE, Berra E, Gothi   E, Roux D, and Pouyssegur J (1999) p42/p44 Mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1 α (HIF-1 α) and enhance the transcriptional activity of HIF-1*. *J Biol Chem* **274**:32631–32637.
- Richard DE, Berra E, and Pouyssegur J (2000) Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1 α in vascular smooth muscle cells. *J Biol Chem* **275**:26765–26771.
- Schulte TW, Akinaga S, Soga S, Sullivan W, Stensgard B, Toft D, and Neckers LM (1998) Antibiotic radicol binds to the N-terminal domains of Hsp90 and shares important biologic activities with geldanamycin. *Cell Stress Chaperones* **3**:106–108.
- Semenza GL (2002) Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med* **7**:345–350.
- Semenza GL and Wang GL (1992) A nuclear factor induced by hypoxia via *de novo* protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* **12**:5447–5454.
- Shaknovich R, Shue G, and Kohtz S (1992) Conformational activation of a basic helix-loop-helix protein (MyoD) by the C-terminal region of murine HSP90 (HSP84). *Mol Cell Biol* **12**:5059–5068.
- Sharma SV, Agatsuma T, and Nakano H (1998) Targeting of the protein chaperone, HSP90, by the transformation suppressing agent, radicol. *Oncogenes* **16**:2639–2645.
- Swanson HI, Chan WK, and Bradfield CA (1995) DNA binding specificities and pairing rules of the Ah receptor, ARNT and SIM proteins. *J Biol Chem* **270**:26292–26302.
- Taylor BL and Zhulin IB (1999) PAS domains: internal sensors of oxygen, redox potential and light. *Microbiol Mol Biol Rev* **63**:479–506.

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