Mitogen-Activated Protein Kinase Kinase Inhibitor PD98059 Blocks the trans-Activation but Not the Stabilization or DNA Binding Ability of Hypoxia-Inducible Factor-1α

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ABSTRACT

Under low oxygen tension, cells increase the transcription of specific genes that are involved in angiogenesis, erythropoiesis, and glycolysis. Hypoxia-induced gene expression primarily depends on the stabilization of the α-subunit of hypoxia-inducible factor-1 (HIF-1α), which acts as a heterodimeric trans-activator. Our results indicate that stabilization of HIF-1α protein by treatment of proteasome inhibitors, is not sufficient for hypoxia-induced gene activation, and an additional hypoxia-dependent modification is necessary for gene expression by HIF-1α. Here, we demonstrate that mitogen-activated protein kinase kinase-1 (MEK-1) inhibitor PD98059 does not change either the stabilization or DNA binding ability of HIF-1α but it inhibits the trans-activation ability of HIF-1α, thereby it reduces the hypoxia-induced transcription of both an endogenous target gene and a hypoxia-responsive reporter gene. We found that hypoxia induced p42/p44 mitogen-activated protein kinases (MAPKs) that are target protein kinases of MEK-1, and that expression of dominant-negative p42 and p44 MAPK mutants reduced HIF-1-dependent transcription of the hypoxia-responsive reporter gene. Our results are the first to identify that hypoxia-induced trans-activation ability of HIF-1α is regulated by different mechanisms than its stabilization and DNA binding, and that these processes can be experimentally dissociated. MEK-1/p42/p44 MAPK regulates the trans-activation, but not the stabilization or DNA binding ability, of HIF-1α.

Cellular oxygen is an important regulatory stimulus for many physiological and pathological processes. Under low oxygen tension, cells adapt by up-regulating the transcription of specific genes that are 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 (EPO), and several glycolytic enzymes, such as lactate dehydrogenase (Shweit et al., 1992; Iyer et al., 1998). These diverse target genes are induced by a common transcription factor, hypoxia-inducible factor 1 (HIF-1) (Semenza and Wang, 1992).

HIF-1 was first identified as a heterodimeric trans-activator that recognizes a specific DNA sequence, termed the hypoxia-responsive element (HRE) in the 3′-untranslated region of the erythropoietin gene (Semenza and Wang, 1992). HIF-1 is composed of two subunits, HIF-1α and β, both of which belong to the growing family of basic helix-loop-helix (bHLH) period/aromatic hydrocarbon receptor nuclear translocator/simple-minded [Per/Arnt/Sim (PAS)] proteins. Cloning of the HIF subunits revealed that HIF-1β is identical to Arnt that previously was known as a partner protein of dioxin receptor (Wang and Semenza, 1995; Li et al., 1996). Embryonic stem cells that lack either the Arnt or the HIF-1α gene fail to respond to hypoxia and show defects in blood vessel formation, indicating that both are essential for adaptation to hypoxic conditions in normal cells (Maltepe et al., 1997; Iyer et al., 1998). Structural analyses of bHLH-PAS proteins reveal that interactions between HLH-PAS domains mediate the dimerization of the two subunits, and individual ABBREVIATIONS: VEGF, vascular endothelial growth factor; EPO, erythropoietin; HIF-1α, hypoxia-inducible factor-1α; HRE, hypoxia responsive element; bHLH, basic helix loop helix; Arnt, aromatic hydrocarbon receptor nuclear translocator; VHL, von Hippel-Lindau; CBP, cAMP response element-binding protein-binding protein; MEK-1, mitogen-activated protein kinase kinase-1; MAPK, mitogen-activated protein kinase; PAS, Per/Arnt/Sim (period/aromatic hydrocarbon receptor nuclear translocator/single-minded); EPAS1, endothelial Per/Arnt/Sim-domain protein 1; MEM, minimal essential medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; TPA, 12-O-tetradecanoylphorbol 13-acetate.
The C-terminal half of HIF-1α contains two hypoxia-inducible trans-activation domains that are regulated by different mechanisms in response to hypoxia. One, located on the N-terminal side, becomes stabilized and activated in response to hypoxia; the other, located on the C-terminal side, is constitutively stable, but becomes activated by hypoxia (Li et al., 1996; Pugh et al., 1997; Ema et al., 1999).

Recent studies show that the functional activity of HIF-1 is primarily regulated by hypoxia-induced accumulation of HIF-1α protein, which is otherwise rapidly degraded by the ubiquitin-proteasome pathway in normoxic cells (Huang et al., 1998). In contrast, the protein level of Arnt is constant (Salceda and Caro, 1997; Kallio et al., 1999). Several lines of evidence imply that tumor suppressors such as p53 and von Hippel-Lindau (VHL) promote ubiquitination and proteasomal degradation of HIF-1α, whereas oncogenic growth factors increase the stability of HIF-1α (Feldser et al., 1997; Maxwell et al., 1999; Zhong et al., 1999; Ravi et al., 2000). However, we still do not understand the nature of the signal and mechanism that renders the degradation process inactive during hypoxia. Several lines of evidence indicate that, in addition to stabilization, hypoxia causes HIF-1α to recruit its coactivator, the cAMP response element-binding protein-binding protein (CBP/p300) (Arany et al., 1996; Carrero et al., 1998). In contrast, the protein level of Arnt is constant (Salceda and Caro, 1997; Kallio et al., 1999). Several lines of evidence imply that tumor suppressors such as p53 and von Hippel-Lindau (VHL) promote ubiquitination and proteasomal degradation of HIF-1α, whereas oncogenic growth factors increase the stability of HIF-1α (Feldser et al., 1997; Maxwell et al., 1999; Zhong et al., 1999; Ravi et al., 2000). However, we still do not understand the nature of the signal and mechanism that renders the degradation process inactive during hypoxia. Several lines of evidence indicate that, in addition to stabilization, hypoxia causes HIF-1α to recruit its coactivator, the cAMP response element-binding protein-binding protein (CBP/p300) (Arany et al., 1996; Carrero et al., 1998). As one possible mechanism, hypoxic stimulation may cause conformational changes in HIF-1α (Kallio et al., 1997). Another possibility is that redox signals or post-translational modification by protein kinases plays a role in regulating HIF-1α (Kallio et al., 1997; Ema et al., 1999; Richard et al., 1999). However, the post-translational modifications of HIF-1 and the possible role of phosphorylation are not fully understood.

Recent findings indicated that MEK-1/p42/p44 MAPK pathway is involved in hypoxia action (Conrad et al., 1999; Richard et al., 1999; Mint et al., 2000). It was demonstrated that HIF-1α is phosphorylated by p42/p44 MAPK in vitro, not by p38 MAPK or c-Jun NH2-terminal kinase, and that phosphorylation and mobility shift of HIF-1α protein is inhibited by PD98059. Conrad et al. (1999) demonstrated that another hypoxia-regulated transcription factor, endothelial PAS-domain protein 1 (EPAS1/HIF-2α/HLF/HRF), is phosphorylated in hypoxic PC12 cells and p42/p44 MAPK is a critical mediator of HIF-2α activation. However, PD98059 has no effect on EPAS1 phosphorylation. They suggested that MAPK pathway does not directly phosphorylate EPAS1, but instead targets other associated protein for EPAS1 function. Extending these previous findings, here we investigate how MEK-1/p42/p44 MAPK pathway modulates the function of HIF-1α.

We dissect the HIF-1α activation process into several steps, including stabilization, DNA binding, and emergence of trans-activation ability. Our findings indicate that MEK-1/p42/p44 MAPK pathway is specifically involved in activation of trans-activation ability of HIF-1α, but not in its stabilization and DNA binding activity. We show that the stabilization of HIF-1α is not sufficient for hypoxia-induced gene activation, and MEK-1/p42/p44 MAPK brought about by hypoxia is necessary for full activation of HIF-1α as a trans-activator.

Materials and Methods

Cell Culture and Hypoxic Treatment. Hep3B cells were purchased from American Type Culture Collection (HB-8064) and cultured in MEM supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) and penicillin/streptomycin (50 IU and 50 μg/ml, respectively; Sigma Chemical, St. Louis, MO) under humidified air containing 5% CO2 at 37°C. Cells were exposed to hypoxia (0.01% O2) by incubating cells in an anaerobic incubator (model 1029; Forma Scientific, Marietta, OH) in 5% CO2, 10% H2, and 85% N2 at 37°C. Hypoxia was also induced chemically by treating cells with 100 μM CoCl2 (Sigma Chemical) (Pugh et al., 1997).

Inhibitors, Antibodies, and Plasmids. Hep3B cells were pretreated with 100 μM PD98059 (New England BioLabs, Beverly, MA) in dimethyl sulfoxide (DMSO), 150 μM genistein (Sigma Chemical Co.) in DMSO, 260 μM ALLN (N-Ac-Leu-Leu-norleucinal; Calbiochem-Novabiochem Co., San Diego, CA) in 50% (v/v) ethanol/DMSO, or 100 μM MG-132 (carbobenzoxy-I-leucyl-l-leucinal; Calbiochem-Novabiochem) in DMSO. Anti-HIF-1α antibody was obtained from Transduction Laboratories (Lexington, KY). Anti-phospho-p42/p44 antibody and anti-p42/p44 antibody were obtained from New England Biolabs (Beverly, MA). The immunogen region for anti-HIF-1α antibody is located in amino acids 610 to 727. The pGAL4/HIF-1α construct contains the DNA binding domain (1–147 amino acids) of yeast GAL4 linked to the full-length coding region of mouse HIF-1α, as described previously (Li et al., 1996). GAL4-driven reporter plasmid pG-tk-luc contains GAL4 binding sites upstream of the thymidine kinase promoter and the luciferase gene. The p(HRE)-luc reporter plasmid contains four copies of the erythropoietin hypoxiareponsive element (5′-GATGCGCCCTACGCTGTCTCA-3′; nucleotides 3449–3470), the simian virus 40 promoter, and the firefly luciferase gene (Ema et al., 1997). pERK1KK and pERK2KK encode dominant-negative mutants of p44 (K71R) and p42 (K52R) MAP kinase, respectively (Frost et al., 1994).

Western Analysis of HIF-1α and p42/p44 MAPK. Hep3B cells were serum starved by incubation in MEM containing 0.5% fetal bovine serum for 40 to 48 h before treatment with inhibitors or hypoxia. Cells were washed once with ice-cold phosphate-buffered saline and lysed in radiocimmunoprecipitation assay buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml antipain, 10 μg/ml aprotinin, 50 mM β-glycerocephosphate, 25 mM NaF, 20 mM EGTA, 1 mM DTT, and 1 mM Na3VO4. The lysates were centrifuged at 10,000 g for 10 min at 4°C. The protein concentrations of the supernatants were measured by a Bradford assay. An equal amount of each protein sample (30 μg) was resolved using 10% SDS-PAGE and transferred in transfer buffer (39 mM glycine, 48 mM Tris-HCl, pH 7.5, 0.057% SDS, 20% methanol) onto nitrocellulose membrane by semidy transfer (Trans-Blot SD; Bio-Rad, Hercules, CA). The primary antibody was applied for 1 h, blots were washed, horseradish peroxidase-conjugated secondary antibody was applied for 1 h, and blots were rewashed. Bound antibody was visualized using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ).

Northern Analysis. Hep3B cells were grown to 80% confluency 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 (10 μg) was electrophoresed through a 1% agarose gel containing formaldehyde and transferred to Nytran filter. Blots were hybridized with α-32P-labeled cDNA of VEGF EPO or actin, washed, dried, and autoradiographed with Hyperfilm MP (Amersham Pharmacia Biotech) as described previously (Li et al., 1996) The expression levels of VEGF, EPO, and actin were detected and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The results were analyzed using the Student’s t-test to determine the significance of differences between the experimental and control groups.

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were measured with a Fujix bioimaging analyzer (model BAS2000; Fuji, Japan).

**Transient Transfection and Luciferase Assay.** Hep3B cells were plated at 1 x 10^5 cells/well of 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 Hep3B cells were serum starved with medium containing 0.5% fetal bovine serum. 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 Bradford assay using bovine serum albumin as a standard.

**Preparation of Nuclear Extracts.** Hep3B cells were serum starved by incubation in MEM containing 0.5% fetal bovine serum for 24 h, and then incubated in 0.01% O2 for 6 h. Nuclear extracts were prepared as described previously (Semenza and Wang, 1992). Seventy percent confluent Hep3B cells in 100-mm tissue culture plates were washed twice with cold phosphate-buffered saline, resuspended in four packed cell volumes of buffer A [10 mM Tris-HCl (pH 7.8), 150 mM MgCl2, 10 mM KCl, 0.5 mM EDTA], and 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 1 mM Na3VO4, and incubated on ice 10 min. Subsequently, the cells were homogenized by 15 strokes with a Dounce type-B pestle. The nuclei were pelleted by centrifugation at 3300g for 15 min at 4°C and resuspended in two packed nuclei volumes of buffer B [20 mM Tris-HCl (pH 8.0), 150 mM MgCl2, 450 mM KCl, 20% glycerol, 0.5 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/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 a Bradford assay.

**Electrophoretic Mobility Shift Assay (EMSA).** Oligonucleotides for W18 (sense: 5’-aattcTGAGACAGCACGTAGGGCa-3’; antisense: 5’-agcttGCCCTACGTGCTGTCTCAg-3’), or M18 (70 pmol; sense: 5’-aattcTGAGACAGCACGTAGGGCa-3’; antisense: 5’-agcttGCCCTACGTGCTGTCTCAg-3’), or M18 (70 pmol; sense: 5’-aattcTGAGACAGCACGTAGGGCa-3’; antisense: 5’-agcttGCCCTACGTGCTGTCTCAg-3’) was also added to the reaction mixture. For supershift assays, anti-HIF-1α antibody was added to the reaction mixture and incubated for 2 h at 4°C before loading (Semenza and Wang, 1992).

**Results**

**Effects of Kinase Inhibitors and Proteasome Inhibitors on Hypoxia-Induced Stabilization of HIF-1α.** To investigate the possibility that specific kinases or proteases modulate hypoxia-induced gene expression, we first measured the changes in protein level of HIF-1α in Hep3B cells that were treated with several inhibitors by using a HIF-1α-specific antibody. The immunogen region for anti-HIF-1α antibody is located amino acids 610 to 727 that share relatively low sequence similarity with HIF-2α and other HIF-like factors (O’Rourke et al., 1999). Immunoblotting with anti-HIF-1α antibody detects the hypoxia or cobalt chloride induced HIF-1α protein that migrates at 110 to 140 kDa with diffused pattern. We cannot exclude the possibility that this diffused band may represent covalently modified HIF-1α or other HIF-like factors. For the following analyses, including immunoblotting, we used serum starved Hep3B cells before hypoxic stimulation because serum itself slightly activates function of HIF-1α in normoxic condition, thereby reducing hypoxia-inducibility of HIF-1α activity (D’Angelo et al., 2000; Richard et al., 2000). Pretreatment with genistein, a tyrosine kinase inhibitor, abolishes the hypoxia-induced accumulation of HIF-1α (Fig. 1). In contrast, pretreatment with MEK-1 inhibitor PD98059 does not decrease the level of hypoxia-induced HIF-1α protein. We found that pretreatment with PD98059 (100 μM) efficiently inhibits MEK-1 activity, thereby blocking the phosphorylation of p42/p44 MAPK at 6- and 16-h exposure to hypoxia under condition used (Fig. 5B). Our results imply that a tyrosine kinase pathway is involved in hypoxia-induced stabilization of HIF-1α, but an MEK-1 pathway is not. Several lines of evidence indicate that hypoxia stabilizes the HIF-1α protein by reducing the ubiquitin-dependent proteasomal degradation of HIF-1α protein. We found that pretreatment with PD98059 (100 μM) efficiently inhibits MEK-1 activity, thereby blocking the phosphorylation of p42/p44 MAPK at 6- and 16-h exposure to hypoxia under condition used (Fig. 5B). Our results imply that a tyrosine kinase pathway is involved in hypoxia-induced stabilization of HIF-1α, but an MEK-1 pathway is not. Several lines of evidence indicate that hypoxia stabilizes the HIF-1α protein by reducing the ubiquitin-dependent proteasomal degradation of HIF-1α protein.
HIF-1α. To investigate the possibility that blocking protease activity may be sufficient for the stabilization of HIF-1α protein in normoxic conditions, we treated HepB3 cells with proteasome inhibitors ALLN and MG132, and then measured the protein level of HIF-1α. ALLN is a broad-spectrum protease inhibitor, whereas MG132 specifically inhibits 26S proteasome, thereby reducing the degradation of ubiquitin-conjugated proteins. These protease inhibitors partially stabilized HIF-1α, even in normoxic conditions (Fig. 1). This finding suggests that inhibition of proteasomes can rescue HIF-1α from degradation, even without hypoxic stimulation.

Effects of Kinase Inhibitors and Proteasome Inhibitors on Hypoxia-Induced DNA Binding Ability of HIF-1 Complex. To investigate the effect of kinases and proteasomes on the ability of HIF-1α/Arnt complex to bind HRE in response to hypoxia, we prepared nuclear extracts from HepB3 cells that were treated with inhibitors. The nuclear extracts were mixed with a radiolabeled oligonucleotide, W18, which contains the HRE sequence from the 3’ enhancer region of the erythropoietin gene, and then were subjected to EMSA (Semenza and Wang, 1992). As observed in other previous EMSA with HRE, our results revealed hypoxia-induced, constitutive, and nonspecific complexes. Hypoxia-induced complex was detected specifically when nuclear extracts from hypoxic HepB3 cells were assayed. Constitutive complexes were present when either induced or uninduced extracts were assayed and therefore are due to the constitutively expressed factors. The hypoxia-inducible complex and the constitutive complexes were completely abolished by the presence of 100-fold molar excess of unlabeled oligonucleotide W18, but not by oligonucleotide M18, which has three single-nucleotide substitutions that abolish its ability to interact with HIF-1α/Arnt, indicating that these DNA-protein complexes are specific for the HRE sequences. In contrast, nonspecific complexes were abolished by the presence of unlabeled M18, indicating that this complex is not specific for HRE sequences (Fig. 2A). To examine the composition of the hypoxia-induced complexes, nuclear extracts were mixed with anti-HIF-1α antibody and then subjected to EMSA. Supershifts confirm the presence of HIF-1α in the complex (Fig. 2B). In accord with the results in Fig. 1, genistein abolishes the hypoxia-induced DNA binding of the HIF-1 complex. In contrast, PD98059 does not affect HIF-1α/Arnt binding to HRE in response to hypoxia (Fig. 2C). These findings suggest that the MEK-1 pathway does not affect the preceding steps, including stabilization, nuclear localization, and heterodimerization between HIF-1α and Arnt that are prerequisite processes for HRE binding.

To investigate whether endogenous HIF-1α that has been stabilized by treatment with a proteasomal inhibitor is able to interact with the HRE, we prepared nuclear extracts from HepB3 cells that had been incubated under normoxic conditions for 6 h in the presence of protease inhibitors MG132 or ALLN and then the nuclear extracts were subjected to EMSA. To a lesser extent, ALLN and MG132 stimulate the formation of a complex with HRE under normoxic conditions (Fig. 2D). The lesser amount of HRE/HIF-1 complex might reflect the fact that less HIF-1α is stabilized by the inhibition of proteasome (Fig. 1). This observation implies that, even in the absence of a hypoxic signal, the accumulation of HIF-1α is sufficient to cause HIF-1α to dimerize with nuclear protein Arnt and to bind the HRE.

Effects of Kinase Inhibitors and Proteasome Inhibitors on Hypoxia-Induced trans-Activation Ability of HIF-1α. In general trans-activators have two separable functions, DNA binding ability and trans-activation ability for recruiting a target coactivator near by the promoter. To measure trans-activation ability of HIF-1α, we used a GAL4-driven reporter system. The GAL4 reporter plasmid encodes the firefly luciferase gene under the control of the GAL4 binding site and tk promoter. We generated a pGAL4/HIF-1α plasmid, which encodes full-length mouse HIF-1α linked to the DNA binding domain of yeast protein GAL4 (1–147 amino acids). Because only the GAL4 fusion protein is able to bind GAL4 binding sites, the reporter gene is transcribed only when HIF-1α has trans-activational ability. In this system, we can only measure the trans-activational ability of HIF-1α, not its DNA binding ability, because the GAL4/HIF-1α chimera interacts with DNA through the GAL4 do-

![Fig. 2. Effects of kinase inhibitors and proteasome inhibitors on hypoxia-induced HRE binding of HIF-1 complex. Oligonucleotide W18 contains the HRE from the erythropoietin enhancer. M18 has three single-nucleotide substitutions that abolish its ability to interact with HIF-1α/Arnt.](Image 312x179 to 557x516)
main, not through HIF-1α (Li et al., 1996). To evaluate the effect of inhibitors on the trans-activation ability of HIF-1α, we transfected Hep3B cells with pGALA/HIF-1α together with reporter plasmid and treated the transfected Hep3B cells with inhibitors. As shown in Fig. 3, the DNA binding domain of GAL44 fails to induce hypoxia-induced transcription of reporter gene, whereas the GAL4/VP16 chimera constitutively activates transcription of reporter gene. In contrast, the GAL4/HIF-1α chimera mediates hypoxia-dependent activation of the reporter gene, indicating that trans-activation ability of HIF-1α is also induced by hypoxia. Treatment with genistein reduces the hypoxia-induced trans-activation ability of HIF-1α, possibly by blocking the hypoxia-induced stabilization of the GAL4/HIF-1α chimeric protein, as it does the endogenous HIF-1α. Interestingly, pretreatment of MEK-1 inhibitor PD98059 abolishes the hypoxia-induced trans-activation ability of HIF-1α. This finding that MEK-1 pathway specifically blocks trans-activation ability but not DNA binding ability of HIF-1α suggests that stabilization/DNA binding of HIF-1α is regulated in MEK-1-independent pathway but trans-activation ability of HIF-1α is regulated in MEK-1-dependent pathway.

In contrast, treatment of proteasomal inhibitors ALLN and MG132 induces stabilization and DNA-binding ability of HIF-1α, whereas it fails to induce the trans-activation ability of HIF-1α under normoxic conditions. This finding emphasizes that mere up-regulation of HIF-1α protein levels by proteasome inhibitors is not sufficient to elicit transcription by HIF-1α and that MEK-1 pathway is additionally required for hypoxia-induced trans-activation ability of HIF-1α. Our results first demonstrate that the stabilization and DNA binding of HIF-1α occur through different mechanisms than the process of its trans-activation.

**Effects of Kinase Inhibitors and Proteasome Inhibitors on Hypoxia-Induced Gene Expression.** Our findings indicate that hypoxia-induced gene activation is mediated primarily by accumulation of HIF-1α, and this activation process is mediated by protein kinases. Among protein kinases, tyrosine kinase is involved, at a minimum, in rescuing HIF-1α from ubiquitin-proteasomal degradation. On the other hand, the MEK-1/p42/p44 MAPK pathway seems to be involved in hypoxia-induced trans-activational ability but not in the hypoxia-induced stabilization and DNA binding activity of HIF-1α. The ultimate goal of HIF-1 activation is the transcription of target genes such as VEGF and erythropoietin. To investigate the effect of tyrosine kinase, the MEK-1 pathway, and proteasomes on HIF-1-dependent gene expression, we used a luciferase reporter plasmid driven by four copies of HRE core sequences (Ema et al., 1999). By using HRE-driven reporter plasmid, we can measure both DNA binding ability and trans-activation ability of HIF-1α. We transfected Hep3B cells with an HRE-driven reporter and treated the cells with inhibitors. Pretreatment of Hep3B cells with genistein or PD98059 causes a pronounced reduction in hypoxia-induced gene expression of HRE-driven reporter plasmid in response to hypoxia (Fig. 4A). Both proteasomal inhibitors, MG132 and ALLN, fail to induce the expression of HRE reporter gene under normoxic conditions.

To test the effect of these inhibitors on the hypoxia-induced expression of the endogenous target gene, we treated Hep3B cells with inhibitors and measured the mRNA levels of VEGF, EPO, and actin by Northern analysis. Pretreatment with genistein almost completely blocks hypoxia-induced VEGF expression, whereas pretreatment with PD98059 (100 μM) decreases the hypoxia-induced expression of the endogenous VEGF about 52% compared with that in hypoxic conditions (Fig. 4B). We also found that PD98059 partially blocks the hypoxia-induced EPO expression and twice more amount of PD98059 (200 μM) is required to decrease the hypoxia-induced EPO expression about 43% compared with that in hypoxia. ALLN did not induce the expression of VEGF under normoxic conditions. The transient transfection and Northern analysis demonstrate that inhibition of both tyrosine kinase and MEK-1 ultimately reduced the hypoxia-induced gene expression, but inhibition of proteasomes is not sufficient to induce hypoxia-responsive transcription. These results indicate that mere up-regulation of stabilization/HRE binding of HIF-1 by treatments of proteasome inhibitor is not enough to induce transcription of both the reporter gene and the endogenous genes, and that inhibition in trans-activation
Hypoxia-Induced Activation of p42/p44 MAPK Is Necessary for HRE-Dependent Gene Expression. Because PD98059 is a specific inhibitor of MEK-1, we wanted to investigate whether hypoxia could ultimately activate p42/p44 MAPK, which are downstream protein kinases of MEK-1. Hep3B cells were serum starved for 48 h, and then exposed to hypoxia for various times. Whole cell lysates were immunoblotted with either an antibody specific for tyrosine-phosphorylated p42/p44 MAPK or an antibody that equally recognizes phospho- and dephospho-p42/p44 MAPK. Because phosphorylation of p42/p44 MAPK is a clear indication of activation, we used an anti-phospho-p42/p44 MAPK antibody to evaluate the activity of p42/p44 MAPK. Stimulation with 12-O-tetradecanoylphorbol 13-acetate (TPA, 10 ng/ml) plus 10% serum for 10 min induced an activation of p42/p44 MAPK and exposure to hypoxia increased phosphorylation of p42/p44 MAPK, whereas hypoxia and TPA did not change the total amount of p42/p44 MAPK (Fig. 5A). Pretreatment of PD98059 blocks the phosphorylation of p42/p44 MAPK induced by both 6- and 16-h hypoxic treatments, whereas genistein does not strongly inhibit p42/p44 MAPK activation by 6- and 16-h hypoxic-treatments (Fig. 5, B and C). This finding suggests that PD98059 inhibits activity of HIF-1α in a mechanism that blocks the hypoxia-induced p42/p44 MAPK activation.

To test whether increased p42/p44 MAPK activity is involved in the hypoxia-induced transcription of genes that are under the control of HREs, we cotransfected Hep3B cells with the HRE-driven reporter plasmid together with increasing amounts of plasmids that encode dominant-negative p44 and p42 MAPK mutants, pERK1-KR, and pERK2-KR, respectively (Frost et al., 1994) (Fig. 6). Cotransfection of 50 ng of pERK1-KR dramatically reduced HIF-1-dependent transcription of the HRE-reporter gene, whereas cotransfection of pERK2-KR influences the hypoxia-induced gene expression of HRE-reporter plasmid to a much lesser extent. The inhibition of p44 MAPK more dramatically reduces the hypoxia-induced gene activation. Thus, the activity of p42/p44 MAPK is required for HIF-1-dependent transcription.

Discussion

Here, we investigated how phosphorylation pathways are involved in the multistep activation process of HIF-1 in response to hypoxia. We dissected the activation process of HIF-1 into several steps, including stabilization of HIF-1α, DNA binding of the HIF-1α/Arnt complex, and trans-activation. Consistent with other reports, we found that treatment with genistein, a tyrosine kinase inhibitor, abolishes hypoxia-induced accumulation of HIF-1α protein (Wang et al., 1995). Extending this finding, we demonstrated that genistein thereby blocks subsequent steps, including DNA binding and transcriptional activation by HIF-1α. However, we cannot exclude the possibility that tyrosine kinase itself, or its downstream protein kinases, may regulate the other activation processes of HIF-1 as well. Our finding that genistein does not strongly inhibit hypoxia-induced phosphorylation of p42/p44 MAPK suggests that hypoxia activates p42/p44 MAPK via genistein-insensitive pathway. Other reports have demonstrated that genistein activates the ability of HIF-1α by MEK-1 inhibitor decreases the hypoxia-induced transcription of both reporter and endogenous genes.
MAPK activity in platelets, and that p42/p44 MAPK activation by adhesion and shear stress in endothelial cells, is inhibited not by genistein, but by another tyrosine kinase inhibitor, herbimycin A (Takahashi and Berk, 1996; Kansra et al., 1999). Our results of pharmacological inhibitors raise the necessity to investigate which specific tyrosine kinase is involved in hypoxia-induced activation of HIF-1α, and whether genistein can inhibit function of HIF-1α via different mechanisms than tyrosine kinase pathway. Several reports investigated the possibility that tyrosine kinase Src conveys hypoxic signal. Mukhopadhyay et al. (1995) found that hypoxia increases the catalytic activity of endogenous Src kinase and also that the expression of a dominant-nega-

![Fig. 5](image)

**Fig. 5.** Effects of hypoxia on the activity of p42/p44 MAP kinase. A, phosphorylation of p44 MAPK and p42 MAPK by hypoxia. Hep3B cells at 70% confluency were serum starved with 0.5% serum-containing media for 48 h and then exposed to hypoxia for the indicated times or 10 ng/ml TPA plus 10% serum for 10 min. Whole cell lysates (30 μg) were separated on 10% SDS-PAGE and transferred to a Nytran filter. Immunoblot analysis was performed using anti-phospho-p42/p44 MAPK antibody (top). The lower arrow and the upper arrow at right, respectively, indicate the position of phosphorylated p42 and p44 MAPK, which are both detected by the antibody. The same blot used to detect phospho-p42/p44 MAPK above was stripped and reprobed with an anti-p42/p44 MAPK that detects both phospho- and dephospho-p42/p44 (bottom). The lower arrow and the upper arrow on the right, respectively, indicate the position of unphosphorylated p42 and p44 MAPK, which are both detected by the antibody. B, Hep3B cells at 70% confluency were serum starved with 0.5% serum-containing media for 48 h and then pretreated with PD98059 (100 μM) for 1 h before hypoxic exposure for the indicated times or 10 ng/ml TPA plus 10% serum for 10 min. Immunoblot analysis was performed using anti-phospho-p42/p44 MAPK antibody (top) or anti-p42/p44 MAPK antibody (bottom). C, Hep3B cells at 70% confluency were serum starved with 0.5% serum-containing media for 48 h and then pretreated with genistein (150 μM) for 1 h before hypoxic exposure for the indicated times or 10 ng/ml TPA plus 10% serum for 10 min. Immunoblot analysis was performed using anti-phospho-p42/p44 MAPK antibody (top) or anti-p42/p44 MAPK antibody (bottom). All experiments were done at least three times.

![Fig. 6](image)

**Fig. 6.** Effect of dominant-negative mutants of p44 and p42 MAP kinase on the hypoxia-induced expression of an HRE-driven reporter gene. A, Hep3B cells (1.0 × 10^5 cells/well in 12-well plate) were cotransfected with reporter plasmids p(HRE)_4-luc (200 ng) and pERK1KR (p44 MAPK mutant) (0, 50, 100, 500 ng) plus an amount of empty vector to produce a total transfection of 700 ng of DNA. B, Hep3B cells (1.0 × 10^5 cells/well in 12-well plate) were cotransfected with reporter plasmid p(HRE)_4-luc (200 ng) with pERK2KR (p42 MAPK mutant) (0, 50, 100, 500 ng) plus an amount of empty vector to produce a total transfection of 700 ng of DNA. Twelve hours before hypoxic treatment, transfected Hep3B cells were serum starved with medium containing 0.5% fetal bovine serum, and then treated for 16 h with 0.01% O_2 exposure. Forty-eight hours after transfection, cell extracts were prepared and analyzed by luciferase assay. Luciferase activity was normalized for total protein concentration. Values represent the mean and S.D. of three experiments.
tive mutant of c-Src reduces hypoxia-induced VEGF expression. They also showed that a dominant-negative Raf-1 blocked c-Src-activated VEGF expression under hypoxic conditions, indicating that Raf-1 is acting downstream of c-Src in hypoxic signaling. In contrast, Gleadle and Ratcliffe (1997) demonstrated that Src kinase activity is not activated by exposure to 1% oxygen that markedly induces activity of HIF-1, and that stably transfected Hep3B cells with plasmids encoding dominant-negative or inactive Src, are able to express both the endogenous genes and HRE-responsive reporter gene in response to hypoxia. They suggested that Src is not critical for the hypoxic activation of HIF-1 and VEGF.

Roles of tyrosine kinases in activation of HIF-1α remain to be investigated. Other reports have indicated that several growth factors that induce tyrosine kinase activity also stabilize the protein of HIF-1α even in normoxic conditions (Feldser et al., 1997; Jiang et al., 1997; Richard et al., 2000). Several observations suggest that both gain of function in oncogenes and loss of function in tumor suppressor genes increase the accumulation of HIF-1α, which consequently induces angiogenesis and glycolysis during cancer development (Graeber et al., 1996; Carmeliet et al., 1998; Maxwell et al., 1999; Ravi et al., 2000). The von Hippel-Lindau tumor suppressor protein interacts with HIF-1α and recruits an ubiquitin-ligase complex, thereby leading the oxygen-dependent proteolysis of HIF-1α in normoxic condition. The angiogenic phenotype of VHL-associated tumors is due to the loss of interaction between mutated von Hippel-Lindau tumor suppressor protein and HIF-1α (Cockman et al., 2000). These findings greatly emphasize that HIF-1α has a pivotal role in the process of cancer development. However, it is not clear how signals of hypoxia fit together with signals from growth factors and tumor suppressors to activate HIF-1α. Recent reports demonstrated that endogenous HIF-1α is highly phosphorylated in vivo and p42/p44 MAPK can reprove the phosphorylation of HIF-1α, which was inhibited by treatment of PD98059 (Richard et al., 1999; Minet et al., 2000). These findings suggest that cooperation between hypoxia and growth factor leads the activation of HIF-1α via MEK-1/p42/p44 MAPK pathway.

Extending these previous findings, we propose that MEK-1/p44/p42 MAPK pathway is specifically involved in activation of trans-activational ability of HIF-1, but not in its stabilization and DNA binding activity, based on the following findings. First, up-regulation of HIF-1α by proteasome inhibitors MG132 and ALLN results in accumulation of HIF-1α in nuclei. This accumulated HIF-1α is able to bind to an HRE site but not to activate transcription of the target gene. Second, treatment with MEK-1 inhibitor PD98059 does not affect either hypoxia-induced stabilization of HIF-1α nor HIF-1’s HRE binding ability, whereas MEK-1 inhibitor specifically blocks the hypoxia-induced trans-activational ability of HIF-1α. Third, hypoxia activates endogenous p42/p44 MAPK. Fourth, expression of dominant-negative mutants of p42/p44 MAPK and treatment with MEK-1 inhibitor reduced HIF-1-dependent transcription. Taken together, our findings indicate that the activation process of HIF-1α by hypoxia consists of at least two separable steps: stabilization/DNA binding of HIF-1α, and oxygen-dependent activation of HIF-1 trans-activational ability. Escaping from proteasomal degradation induces both stabilization and DNA binding ability of HIF-1α, whereas p42/p44 MAPK pathway induces trans-activational ability of HIF-1α. Now, questions are how hypoxia triggers both signals that are blocking ubiquitin-proteasome pathway and activating MEK-1 pathway, and how both signals are interconnected. It remains to be investigated whether stabilized HIF-1α by treatment of proteasome inhibitors actually binds to HRE in the endogenous genes in vivo, and whether point mutations of p42/p44 MAPK consensus site (PXSP) of HIF-1α change trans-activation ability of HIF-1α in vivo.

PD98059, an MEK-1 inhibitor, more dramatically reduced the hypoxia-induced transcription of HRE-driven reporter gene than that of endogenous VEGF and EPO. This result may occur because trans-activation by HIF-1α contributes less to the transcription of the endogenous gene than to the transcription of the HRE-driven reporter gene. Studies of hypoxia-inducible transcription of lactate dehydrogenase and erythropoietin suggest that their enhancers contain, adjacent to the HIF-1 site, additional binding sites for cAMP response element-binding protein/activating transcription factor and steroid hormone receptors (Kvietikova et al., 1995). These proteins also interact with CBP/p300. Thus, a common mechanism in hypoxia-induced gene expression may be the formation of a hypoxia-inducible multiprotein complex that includes HIF-1α and other transcription factors that bind adjacent to HIF-1, both of which interact with same coactivator, CBP/p300 (Ebert and Bunn, 1998). Expression of the endogenous VEGF is regulated by many different stimuli, including hypoxia, epidermal growth factor, platelet-derived growth factor, serum and phorbol ester, and among them hypoxia is the most intensive stimulator for VEGF induction. Hypoxia-induced increase of VEGF mRNA includes not only transcriptional activation but also post-transcriptional regulation of VEGF gene. The half-life of VEGF mRNA in cells grown under hypoxic condition is significantly increased (Shima et al., 1995). The regulatory region of the endogenous VEGF gene contains many other cis-acting elements not only for HIF-1 but also for other trans-activators such as Sp1, nuclear factor-1, and activator proteins 1 and 2 (Klagsburn and D’Amore, 1996). In the context of the endogenous regulatory region, the other trans-activators in this multiprotein complex may compensate for impairment of trans-activational ability of HIF-1α to recruit coactivator and/or general transcription factors near the promoter of the target gene. In contrast, the impairment of trans-activational ability of HIF-1α may greatly impact the transcription of the reporter gene, which is driven only by the core HRE sequence. HRE-reporter plasmid contains only four copies of HRE as regulatory elements. Therefore, the expression of reporter plasmid exclusively represents the effect through HRE and its target trans-acting protein, HIF-1 complex. For this reason, the block of trans-activation capability of HIF-1α by MEK-1 inhibition abolishes the HRE reporter gene expression completely.

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