The MEK Inhibitor, PD98059 Blocks the Transactivation, but not the Stabilization or DNA Binding Ability, of Hypoxia-Inducible Factor-1α

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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 subunit of Hypoxia-Inducible Factor-1 (HIF-1), which acts as a heterodimeric transactivator. Our results indicate that stabilization of HIF-1a 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-1 but it inhibits the transactivation ability of HIF-1, 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 (MAPK) 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 transactivation ability of HIF-1a 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 transactivation, but not the stabilization or DNA binding ability, of HIF-1α.

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Abbreviations used: Arnt, aromatic hydrocarbon receptor nuclear translocator; bHLH, basic helix loop helix; CBP, CREB-binding protein; EMSA, electrophoretic mobility shift assay; EPAS1, endothelial PAS–domain protein 1; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; HIF-1• , hypoxia-inducible factor-1 • ; HRE, hypoxia responsive element; MAPK, mitogen-activated protein kinase; MEK-1, mitogen-activated protein kinase kinase-1; PAS, Per/Arnt/Sim; TPA, 12-O-Tetradecanoylphorbol 13-acetate; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau.

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 transactivator. Our results indicate that stabilization of HIF-1• 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-1• . 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 transactivation 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 (MAPK) 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 transactivation ability of HIF-1• 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 transactivation, but not the stabilization or DNA binding ability, of HIF-1• .

Introduction

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 upregulated during hypoxia include vascular endothelial growth factor (VEGF), erythropoietin (EPO), and several glycolytic enzymes, such as lactate dehydrogenase (Goldberg *et al.* 1988; Iyer *et al.* 1998; Shweit *et al.*, 1992). 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 transactivator 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-1• and • , both of which belong to the growing family of basic-helix-loop-helix Per, Arnt, Sim (bHLH-PAS) proteins. Cloning of the HIF subunits revealed that HIF-1• is identical to aromatic hydrocarbon receptor nuclear translocator (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-1• 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 (Iyer *et al.* 1998; Maltepe *et al.* 1997). Structural analyses of bHLH-PAS proteins reveal that interactions between HLH-PAS domains mediate the dimerization of the 2 subunits, and individual basic regions of the 2 subunits make contact with their corresponding DNA sequences. Thus, dimerization of bHLH-PAS proteins is a prerequisite for their DNA binding (Jiang *et al.*, 1996; Reisz-Porszasz *et al.*, 1994). The C-terminal half of HIF-1• contains two hypoxia-inducible transactivation 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 in response to hypoxia. (Ema *et al.*, 1999; Li *et al.*, 1996; Pugh *et al.*, 1997)

Recent studies show that the functional activity of HIF-1 is primarily regulated by hypoxia-induced accumulation of HIF-1• 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 (Kallio *et al.*, 1999; Salceda and Caro, 1997). Several lines of evidence imply that tumor suppressors such as p53 and von Hippel-Lindau (VHL) promote ubiquitination and proteasomal degradation of HIF-1• , whereas oncogenic growth factors increase the stability of HIF-1• (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-1• to recruit its coactivator, the CREB-binding protein (CBP/p300) (Arany *et al.*, 1996; Carrero *et al.*, 2000). As one possible mechanism, hypoxic stimulation may cause conformational changes in HIF-1• and β, which then recruit coactivators (Kallio *et al.*, 1997). Another possibility is that redox signals or post-translational modification by protein kinases play a role in regulating HIF-1• (Ema *et al.*, 1999; Kallio *et al.*, 1997; 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/p44MAPK pathway is involved in hypoxia action. (Conrad *et al.*, 1999; Minet *et al.*, 2000; Richard *et al.*, 1999). It was demonstrated that HIF-1• is phosphorylated by p42/p44 MAPK *in vitro*, not by p38 MAPK or c-Jun N-terminal kinase, and that phophorylation and mobility shift of HIF-1• is inhibited by PD98059 (Richard *et al.*, 1999; Minet *et al.*, 2000). Conrad *et al.* demonstrated that other hypoxia-regulated transcription factor, endothelial PAS-domain protein 1 (EPAS1/HIF-2• /HLF/HRF) is phosphorylated in hypoxic PC12 cells and p42/p44 MAPK is a critical mediator of HIF-2 • 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-1• .

We dissect the HIF-1• activation process into several steps, including stabilization, DNA binding, and emergence of transactivational ability. Our findings

indicate that MEK-1/p42/p44 MAPK pathway is specifically involved in activation of transactivational ability of HIF-1• , but not in its stabilization and DNA binding activity. We show that the stabilization of HIF-1• 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-1• as a transactivator.

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 Gibco BRL) and Pen/Strep (50 IU and 50 μg/ml respectively, Sigma Chemical Co.) under humidified air containing 5% CO_2 at 37°C. Cells were exposed to hypoxia (0.01% O_2) by incubating cells in an anaerobic incubator (Model 1029, Forma Scientific, Inc.) in 5% CO2, 10% H_2 and 85% N_2 at 37°C. Hypoxia was also induced chemically by treating cells with 100 μM $CoCl_2$ (Sigma Chemical Co.)(Pugh *et al.*, 1997)

Inhibitors, antibodies and plasmids

Hep3B cells were pretreated with 100μM of PD98059 (New England BioLabs, Inc.) in dimethyl sulfoxide (DMSO), 150 μM of Genistein (Sigma Chemical Co.) in DMSO, 260 μM of ALLN (N-Ac-Leu-Leu-norleucinal; Calbiochem-Novabiochem Co.) in 50%v/v ethanol/DMSO, or 100 μM of MG-132 (Carbobenzoxy-L-leucyl-L-leucinal; Calbiochem-Novabiochem Co.) in DMSO. Anti-HIF-1α antibody was obtained from Transduction Laboratories. Anti-phospho-p42/p44 antibody and Anti-p42/p44 antibody were obtained from New England BioLab. The immunogen region for anti-HIF-1α antibody is located in amino acids 610-727. The pGAL4/HIF-1α construct contains the DNA binding domain (1-147aa) of yeast GAL4 linked to the full length coding region of mouse HIF-1α, as described (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)$_4$-luc reporter plasmid contains 4 copies of the erythropoietin hypoxia-responsive element (5'-GATCGCCCTACGTGCTGTCTCA-3'; nucleotides 3449-3470), the SV40 promoter, and the firefly luciferase gene (Ema *et al.* 1997). pERK1KR and pERK2KR 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 40-48 hours before treatment with inhibitors or hypoxia. Cells were washed once with ice-cold PBS and lysed in RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris pH7.4, 100 μg/ml PMSF, 10 μg/ml Leupeptin, 1 μg/ml Antipain, 10 μg/ml Aprotinin, 50 mM β -Glycerophosphate, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4. The lysates were centrifuged at 10,000x g for 10 min at 4℃. 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], 0.037% SDS, 20% Methanol) onto nitrocellulose membrane by semi-dry transfer (Trans-Blot SD, BioRad Laboratories). The primary antibody was applied for 1 hour, blots were washed, horseradish peroxidase (HRP) conjugated secondary antibody was applied for 1 hour, and blots were rewashed. Bound antibody was visualized using

enhanced chemiluminescence according to the manufacturer's instructions (ECL, Amersham Pharamcia Biotech).

Northern analysis

Hep3B 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 Inc.). Total RNA (10 μg) was electrophoresed through a 1% agarose gel containing formaldehyde and transferred to Nytran filter. Blots were hybridized with [α-^{32}P]-labeled cDNA of VEGF or actin, washed, dried, and autoradiographed with Hyperfilm MP (Amersham Pharmacia Biotech) as described. (Li *et al.*, 1996) The expression levels of VEGF and actin were measured with a phosphoimager (Model Bas2000, Fuji, Japan).

Transient transfection and luciferase assay

Hep3B cells were plated at 1×10^5 cells per well of a 12-well plate. 18 hours later, transfection was carried out using Superfect reagent (Qiagen) according to the manufacturer's instructions. 12 hours prior to hypoxic treatment, transfected Hep3B cells were serum starved with medium containing 0.5% fetal bovine serum. 48 hours after transfection, cell extracts were prepared and analyzed with a luminometer (Berthold Lumat LB9501) using the Luciferase Assay System (Promega). 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 hours, then incubated in 0.01% O_2 for 6 hours. Nuclear extracts were prepared as described (Semenza and Wang 1992). 70% confluent Hep3B cells in 100-mm tissue culture plates were washed twice with cold phosphate-buffered saline, resuspended in 4 packed cell volumes of buffer A (10 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.4 mM Phenylmethylsulfonyl fluoride, 2 μg/ml Leupeptin, 2 μg/ml Pepstatin, 2 μg/ml Aprotinin, and 1 mM Na_3VO_4) 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 3,300g for 15 min at 4°C and resuspended in 2 packed nuclei volumes of buffer B (20 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, 450 mM KCl, 20% glycerol, 0.5 mM DTT, 0.4 mM Phenylmethylsulfonyl fluoride, 2 μg/ml Leupeptin, 2 μg/ml Pepstatin, 2 μg/ml Aprotinin, 1 mM Na_3VO_4). The suspensions were incubated with gentle rocking at 4°C for 1 hour 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 (BioRad Laboratories).

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides for W18 (sense: 5'- agcttGCCCTACGTGCTGTCTCAg-3', antisense:

5'-aattcTGAGACAGCACGTAGGGCa-3') were annealed and labeled (1.75 pmoles) with [• -^{32}P]dATP and Klenow. Unincorporated nucleotides were removed by gel filtration over a Sephadex G25 column. Nuclear extracts were preincubated with poly dIdC (500ng) in 20 • l of buffer (10 mM Tris–HCl [pH 7.5], 100 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5% glycerol). The labeled W18 probe ($5x10^5$ cpm) was incubated with nuclear extract (10 • g) for 15 min at room temperature. The reactions were separated on 5% PAGE at 250 V in 0.5x TBE at 4°C. Gels were vacuum-dried and autoradiographed. For competition assays, a 100-fold molar excess of unlabelled double stranded oligonucleotide W18 (70 pmoles; sense: 5'-agcttGCCCTACGTGCTGTCTCAg-3', antisense: 5'-aattcTGAGACAGCACGTAGGGCa-3'), or M18 (70 pmoles; sense: 5'-agcttGCCCTAAAAGCTGTCTCAG-3', antisense: 5'-aattcTGAGACAGCTTTTAGGGCA-3') was also added to the reaction mixture. For supershift assays, anti-HIF-1• antibody was added to the reaction mixture and incubated for 2 hours at 4°C prior to loading (Semenza and Wang 1992).

Results

Effects of kinase inhibitors and proteasome inhibitors on hypoxia-induced stabilization of HIF-1•

To investigate the possibility that specific kinases or proteases modulate hypoxia-induced gene expression, we first measured the changes in protein level of HIF-1• in Hep3B cells that were treated with several inhibitors by using a HIF-1α specific antibody. The immunogen region of HIF-1α is located amino acids 610-727 region that shares relatively low sequence similarity with HIF-2α and other HIF like factors (O'Rourke *et al.* 1999). Immunoblotting with anti-HIF-1• antibody detects the hypoxia or cobalt chloride induced HIF-1α protein which migrates at 110-140 kDa with diffused pattern. We cannot exclude the possibility that this diffused band may represent covalently modified HIF-1• or other HIF like factors. For the following analyses, including immunoblotting, we serum-starved Hep3B cells prior to hypoxic stimulation because serum itself slightly activates function of HIF-1α in normoxic condition, thereby reduces 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-1• (Fig. 1). In contrast, pretreatment with MEK-1 inhibitor, PD98059 does not decrease the level of hypoxia-induced HIF-1• protein. We found that pretreatment with PD98059 (100μM) efficiently inhibits MEK-1 activity, thereby blocks the phosphorylation of p42/p44 MAPK at 6 hour and 16 hour exposure to hypoxia under

condition used (see Fig. 5B). Our results imply that a tyrosine kinase pathway is involved in hypoxia-induced stabilization of HIF-1• , but a MEK-1 pathway is not. Several lines of evidence indicate that hypoxia stabilizes the HIF-1• protein by reducing the ubiquitin-dependent proteasomal degradation of HIF-1• . To investigate the possibility that blocking protease activity may be sufficient for the stabilization of HIF-1• protein in normoxic conditions, we treated HepB3 cells with proteasome inhibitors ALLN and MG132, and then measured the protein level of HIF-1• . 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-1• , even in normoxic conditions (Fig. 1). This finding suggests that inhibition of proteasomes can rescue HIF-1• 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-1• /Arnt complex to bind HRE in response to hypoxia, we prepared nuclear extracts from Hep3B 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 electrophoretic mobility shift assay (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 Hep3B 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 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-1• /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-1• antibody and then subjected to EMSA. Supershifts confirm the presence of HIF-1• 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-1• /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-1• and Arnt, which are prerequisites for HRE binding.

To investigate whether endogenous HIF-1• that has been stabilized by treatment with a proteasomal inhibitor is able to interact with the HRE, we prepared nuclear

extracts from Hep3B cells that had been incubated under normoxic conditions for 6 hours 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-1• is stabilized by the inhibition of proteasome (see Fig. 1). This observation implies that, even in the absence of a hypoxic signal, the accumulation of HIF-1• is sufficient to cause HIF-1• to dimerize with nuclear protein, Arnt and to bind the HRE.

Effects of kinase inhibitors and proteasome inhibitors on hypoxia-induced transactivation ability of HIF-1•

In general transactivators have two separable functions, DNA binding ability and transactivation ability for recruiting a target coactivator near by the promoter. To measure transactivation ability of HIF-1• , 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-1• plasmid, which encodes full-length mouse HIF-1• linked to the DNA binding domain of yeast protein GAL4 (1-147aa). Since only the GAL4 fusion protein is able to bind GAL4 binding sites, the reporter gene is transcribed only when HIF-1• has transactivational ability. In this system, we can only measure the transactivational ability of HIF-1• , not its DNA binding ability, because the GAL4/HIF-1• chimera interacts with DNA through the

GAL4 domain, not through HIF-1• (Li *et al.* 1996). To evaluate the effect of inhibitors on the transactivation ability of HIF-1• , we transfected Hep3B cells with pGAL4/HIF-1• together with reporter plasmid and treated the transfected Hep3B cells with inhibitors. As shown in Fig. 3., the DNA binding domain of GAL4 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-1• chimera mediates hypoxia-dependent activation of the reporter gene, indicating that transactivational ability of HIF-1• is also induced by hypoxia. Treatment with Genistein reduces the hypoxia-induced transactivational ability of HIF-1• , possibly by blocking the hypoxia-induced stabilization of the GAL4/HIF-1• chimeric protein, as it does the endogenous HIF-1• . Interestingly, pretreatment of MEK-1 inhibitor PD98059 abolishes the hypoxia-induced transactivational ability of HIF-1• . This result demonstrates that in response to hypoxia, MEK-1 pathway specifically blocks transactivation ability but not DNA binding ability of HIF-1α, suggesting that stabilization/DNA binding of HIF-1α is regulated in MEK-1 independent pathway but transactivation 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-1• , whereas it fails to induce the transactivational ability of HIF-1• under normoxic conditions. This finding emphasizes that mere up-regulation of HIF-1• protein levels by proteasome inhibitors is not sufficient to elicit transcription by HIF-1• and that MEK-1 pathway is important for

hypoxia-induced transactivation ability of HIF-1• . Our results first demonstrate that the stabilization and DNA binding of HIF-1α occur through different mechanisms than the process of its transactivational 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-1• , and this activation process is mediated by protein kinases. Among protein kinases, tyrosine kinase is involved, at least, in rescuing HIF-1• from ubiquitin-proteasomal degradation. On the other hand, the MEK-1/p42/p44MAPK pathway appears to be involved in hypoxia-induced transactivational ability but not in the hypoxia-induced stabilization and DNA binding activity of HIF-1• . The ultimate goal of HIF-1 activation is the transcriptional activation 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 transactivation ability of HIF-1• . 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.

In order 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 (100μM) decreases the hypoxia-induced expression of the endogenous VEGF about 52% compared to that in hypoxic conditions (See Fig. 4B). Higher dose of PD98059 (200μM) is required for about 43% reduction of hypoxia-induced EPO expression. 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 transactivation ability of HIF-1α by MEK-1 inhibitor decreased the hypoxia-induced transcription of both reporter and endogenous genes.

Hypoxia-induced activation of p42/p44 MAPK is necessary for HRE dependent gene expression

Since PD98059 is a specific inhibitor of MEK-1, we wanted to investigate if

hypoxia could ultimately activate p42/p44 MAPK, which are downstream protein kinases of MEK-1. Hep3B cells were serum starved for 48 hours, 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. Since 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, 10ng/ml) plus 10% serum for 10 minutes induced an activation of p42/p44 MAPK and exposure to hypoxia increased phosphorylation of p42/p44 MAPK, while 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 hour hypoxic treatments, whereas Genistein very slightly inhibits p42/p44 MAPK activation by 6 and 16 hour hypoxic treatments (Fig. 5B, C). This finding suggests that PD98059 inhibits activity of HIF-1α in a mechanism that blocks the hypoxia-induced p42/p44 MAPK activation.

In order 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). 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 multi-step activation process of HIF-1 in response to hypoxia. We dissected the activation process of HIF-1 into several steps including stabilization of HIF-1• , DNA binding of the HIF-1• /Arnt complex, and transactivation. Consistent with other reports, we found that treatment with Genistein, a tyrosine kinase inhibitor, abolishes hypoxia-induced accumulation of HIF-1• protein (Wang et al, 1995). Extending this finding, we demonstrated that Genistein thereby blocks subsequent steps, including DNA binding and transcriptional activation by HIF-1• . 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 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 other tyrosine kinase inhibitor, Herbimycin A (Kansra *et al.* 1999; Takahashi and Berk, 1996). 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 other mechanisms than tyrosine kinase pathway. The possibility that tyrosine kinase Src conveys hypoxic signal, has been investigated. Mukhopadhay *et al.* (1995) found that hypoxia increases the catalytic

activity of endogenous Src kinase and also that the expression of a dominant-negative 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 signalling. 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 endogeneous genes and HIF-1-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 (Carmeliet *et al.,* 1998; Graeber *et al.,* 1996; Maxwell *et al.,* 1999; Ravi *et al.,* 2000). The von Hippel-Lindau tumor suppressor protein (pVHL) 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 pVHL 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 reproduce the phosphorylation of HIF-1α, which was inhibited by treatment of PD98059 (Minet *et al.*, 2000; Richard *et al.* 1999). These findings <u>suggest that cooperation between hypoxia and growth factor leads the activation of HIF-1α via MEK-1/p42/p44 MAPK pathway.</u>

Extending these previous findings, we propose that MEK-1/p44/p42 MAPK pathway is specifically involved in activation of transactivational 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-1• nor HIF-1's HRE binding ability, whereas MEK-1 inhibitor specifically blocks the hypoxia-induced transactivational 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 transactivational ability. Escaping from proteasomal

degradation induces both stabilization and DNA binding ability of HIF-1α, whereas transactivation ability of HIF-1α is induced by MEK-1 dependent pathway. Now, the 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*, whether point mutations of p42/p44 MAPK concensus site (PXSP) of HIF-1α change function of HIF-1α.

PD98059, a MEK-1 inhibitor, more dramatically reduced the hypoxia-induced transcription of HRE-driven reporter gene than that of endogenous VEGF and EPO (data not shown). This result may occur because transactivation 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 CREB/ATF 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 multi-protein complex that includes HIF-1 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, EGF, PDGF, 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 contains many other regulatory regions not only for HIF-1 but also for other transactivators such as Sp1, NF-1 and AP-1 and 2 (Klagsburn and D'amore *et al.* 1996). In the context of the endogenous regulatory region, the other transactivator in this multiprotein complex may compensate for impairment of transactivational ability of HIF-1• to recruit coactivator and/or general transcription factors near the promoter of the target gene. In contrast, the impairment of transactivational ability of HIF-1• may greatly impact the transcription of the reporter gene, which is driven only by the core HRE sequence. HRE-reporter plasmid contains only 4 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 transactivation capability of HIF-1a by MEK-1 inhibition abolishes the HRE reporter gene expression completely.

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References

Arany Z, Huang LE, Eckner R, Bhattacharya S, Jiang C, Goldberg MA, Bunn HF and Livingston DM (1996) An essential role for p300/CBP in the cellular response to hypoxia. *Proc Natl Acad Sci U S A* **93**:12969-12973.

Carmeliet P, Dor Y, Herbert J-M and Keshet E (1998) Role of HIF-1• in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**: 485-490.

Carrero P, Okamoto K, Coumailleau P, O'Brien S, Tanaka H and Poellinger L (2000) Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. *Mol Cell Biol* **20**:402-411.

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ and Maxwell PH. (2000) Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J Biol Chem* **275**:25733-25741

Conrad PW, Freeman TL, Beitner-Johnson D and Millhorn DE (1999) EPAS1 trans-activation during hypoxia requires p42/p44 MAPK. *J Biol Chem* **274**:33709-33713

D'Angelo G, Ladoux A, and Frelin C (2000) Hypoxia-induced transcriptional activation of vascular endothelial growth factor is inhibited by serum. *Biochem Biophys Res Commun* **267**:334-338

Ebert BL and Bunn HF (1998) Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor and p300/CREB binding protein. *Mol Cell Biol* **18**:4089-4096.

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 1alpha regulates the VEGF expression and is potentially involved in lung and vascular development. *Proc Natl Acad Sci U S A* **94**:4273-4278.

Ema M, Hirota K, Mimura J, Abe H, Yodoi J, Sogawa K, Poellinger L and Fujii-Kuriyama Y (1999) Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J* **18**:1905-1914.

Feldser D, Agani F, Iyer NV, Pak B, Ferreira G and Semenza GL (1997) Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. *Cancer Res* **59**:3915-3918.

Frost J, Geppert T, Cobb M and Feramisco J (1994) A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP-1 by Ha-Ras, phorbol 12-myristate 13-acetate, and serum. *Proc Natl Acad Sci U S A* **91**: 3844-3848.

Gleadle JM and Ratcliffe PJ (1997) Induction of hypoxia-inducible factor-1, erythropoietin, vascular endothelial growth factor, and glucose transporter-1 by hypoxia: Evidence against a regulatory role for Src Kinase. *Blood* **89**: 503-509.

Goldberg MA, Dunning SP and Bunn F (1988) Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science*. **242**: 1412-1414.

Graeber TG, Osmanian C, Jack T, Housman DE, Koch CJ, Lowe SW and Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* **379**:88-91.

Huang LE, Gu J, Schau M and Bunn HF (1998) Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* **95**:7987-7992.

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M,

Gearhart JD, Lawler AM, Yu AY and Semenza GL (1998) Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. *Genes Dev* **12**:149-162.

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.

Jiang BH, Agani F, Passaniti A and Semenza GL (1997) v-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res* **57**:5328-5335.

Kallio PJ, Pongratz I, Gradin K, Mcguire J and Poellinger L. (1997) Activation of hypoxia-inducible factor 1• : Posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc Natl Acad Sci USA* **94**: 5667-5672.

Kallio PJ, Wilson WJ, O'Brien S, Makino Y and Poellinger L (1999) Regulation of the hypoxia-inducible transcription factor1• by the ubiquitin-proteasome pathway. *J Biol Chem* **274**: 6519-6525.

Kansra SV, Reddy MA, Weng YI and Shukla SD (1999) Activation of mitogen activated

protein kinase in human platelets by Genistein. *Pharmacol Res* **39**:21-31

Kvietikova I, Wenger RH, Marti HH and Gassmann M (1995) The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. *Nucleic Acid Res* **23**: 4542-4550.

Li H, Ko HP and Whitlock, JP Jr. (1996) Induction of phosphoglycerate kinase 1 gene expression by hypoxia : Roles of Arnt and HIF1α . *J Biol Chem* **271**:21262-21267.

Maltepe E, Schmidt JV, Baunoch D, Bradfield CA and Simon MC (1997) Abnormal angiogenesis and responses to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature* **386**:403-407.

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* **399**:271-275.

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* **375**:577-581.

Pugh CW, O'Rourke JF, Nagao M, Gleadle JM and Ratcliffe PJ (1997) Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. *J Biol Chem* **272**:11205-11214.

Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL and Bedi A (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha.*Genes Dev* **14**:34-44.

Reisz-Porszasz S, Probst MR, Fukunaga BN and Hankinson O (1994) Identification of functional domains of the aryl hydrocarbon receptor nuclear translocator protein (ARNT). *Mol Cell Biol* 14:6075-6086.

Richard D.E, Berra E., Gothié E., Roux D. and Pouysségur J. (1999) p42/p44 Mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1• (HIF-1•) and enhance the transcriptional activity of HIF-1*. *J. Biol. Chem.* **274**: 32631-32637.

Richard D.E, Berra E, and Pouyssegur J (2000) Nonhypoxic pathway mediates the induction of hypoxia-inducible factor 1alpha in vascular smooth muscle cells. *J Biol Chem* **275**:26765-26771

Salceda S and Caro J (1997) Hypoxia-inducible factor1• (HIF-1•) protein is rapidly

degraded by the ubiquitin-proteasome system under normoxic conditions. *J Biol Chem* **272**: 22642-22647.

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.

Shweit D, Itin A, Soffer D and Kreshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**:843-848.

Shima, D. T., Deutsch, U., and D' Amore, P. A. (1995) Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells mediated by increase in mRNA stability. *FEBS Lett.* **370**: 203-208

Wang GL, Jiang B-H, and Semenza GL (1995) Effect of protein kinase and phosphatase inhibitors on expression of hypoxia-inducible factor 1. *Biochem Biophys Res Commun* **216**:669-675

Wang GL and Semenza GL (1995) Purification and charaterization of hypoxia-inducible factor 1. *J Biol Chem* **270**:1230-1237.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL and Simons JW (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* **59**:5830-5835.

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Figure Legends

Fig. 1. Effect of kinase inhibitors and proteasome inhibitors on hypoxia-induced stabilization of HIF-1α

Before stimulation, Hep3B cells were serum-starved with medium containing 0.5% fetal bovine serum for 48 hours and then treated with PD98059 (100 μM) or Genistein (150 μM) for 1 hour, followed by 6 hours exposure to $CoCl_2$ (100 μM) or 0.01% oxygen. Serum-starved Hep3B cells were treated with MG132 (100 μM) or ALLN (260 • M) for 6 hours. (A) Immunoblot analysis of protein level of HIF-1α in Hep3B cells treated with several inhibitors in the presence or absence of $CoCl_2$ (100μM). (B) Immunoblot analysis of protein level of HIF-1α in Hep3B cells treated with several inhibitors under hypoxic (0.01% O_2) or normoxic conditions (20% O_2). The HIF-1α protein was visualized by chemiluminescence and quantitated by densitometry. The numbers represent the relative induction levels of HIF-1α. The value of HIF-1α in uninhibited hypoxic cells is arbitrarily defined as 100%.

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-1. Hep3B cells were serum-starved with medium containing 0.5% fetal bovine serum, then incubated in normoxia or 0.01% O_2 for 6 hours. (A) Nuclear extracts (10 μg) were incubated with radiolabeled W18 in the presence or absence of a 100-fold molar excess of unlabelled W18 and M18. The mixtures were analyzed by EMSA. (B) Nuclear extracts were mixed with radiolabeled W18, followed by incubation with anti-HIF1α antibody. (C) Hep3B cells were pretreated with MEK-1 inhibitor PD98059 (100μM) or tyrosine kinase inhibitor Genistein (150μM) for one hour, then stimulated for 6 hrs with 0.01% O_2 or $CoCl_2$ (100μM). Nuclear extracts were mixed with radiolabeled W18. The mixtures were analyzed by EMSA. (D) Hep3B cells also were incubated with 26S proteasome inhibitor MG132 (100 μM) or protease inhibitor ALLN (260 • M) for 6 hrs under normoxic conditions.

Fig. 3. Effects of kinase inhibitors and proteasome inhibitors on hypoxia-inducible transactivation by GAL4/HIF-1α.

(A) Schematic diagrams of plasmids used. pGALO encodes the DNA binding domain (1-147 amino acids) of yeast GAL4 protein. pGAL/VP16 and pGAL4/HIF-1α encode transactivation domain (412-490 amino acids) of VP16 and full-length mouse HIF-1α (1-

822 amino acids) linked to the DNA binding domain of yeast GAL4 (1-147 amino acids), respectively. pGAL-tk-luc contains a luciferase gene driven by GAL4 DNA binding sites (UAS) and tk promoter. (B) Hep3B cells (2.5 x 10^5 cells per well in a 6-well plate) were cotransfected with pGAL4/HIF-1α (2 • g) and reporter plasmid pGAL-tk-luc (1 • g). 12 hours prior to hypoxic treatment, transfected Hep3B cells were serum starved with medium containing 0.5% fetal bovine serum, then treated with MEK-1 inhibitor, PD98059 (100 μM), and tyrosine kinase inhibitor, Genistein (150 μM) for 1 hour prior to 16-hour exposure to hypoxia (0.01% O_2). Transfected cells were treated with MG132 (100 μM), and with ALLN (260 • M) for 16 hours under normoxic conditions. Forty-eight hours after transfection, cell extracts were prepared and luciferase activities were analyzed. Luciferase activity was normalized by total protein in the extract. Value represents the mean and standard deviation of three experiments

Fig. 4. Effect of protein kinase inhibitors and proteasome inhibitors on hypoxia-induced expression of a reporter gene and an endogenous gene.

(A) Hep3B cells (1.0 x 10^5 cells per well in 12-well plate) were cotransfected with reporter plasmid p(HRE)$_4$-luc (500 ng), containing 4 copies of HRE, the SV40 promoter, and a luciferase gene. 12 hours prior to hypoxic treatment, transfected Hep3B cells were serum starved with medium containing 0.5% fetal bovine serum, then treated with MEK-

1 inhibitor PD98059 (100 • M), and tyrosine kinase inhibitor Genistein (150 μM) for 1 hour prior to 16 hours exposure to hypoxia (0.01% O_2). Or, transfected cells were treated with proteasome inhibitors MG132 (100 μM) or ALLN (260 • M) 16 hours prior to harvest. Forty-eight hours after transfection, cell extracts were prepared and analyzed by luciferase assay. The luciferase activity was normalized for total protein concentration. Value represents the mean and standard deviation of five experiments. (B) Hep3B cells were pretreated with PD98059 (100 μM [x1] and 200 μM [x2]), or Genistein (150 μM) for 1 hour prior to 16 hour exposure to 0.01% oxygen. Hep3B cells were treated with ALLN (260 μM) for 16 hours prior to harvest. Total RNA was isolated and 10 μg were separated on a 1% formaldehyde agarose gel. RNA was transferred onto a Nytran filter and hybridized with ^{32}P-labeled human VEGF cDNA. The same blot was stripped and rehybridized with ^{32}P-labeled actin cDNA. mRNA levels of VEGF and actin were visualized by exposing to X-ray film. Gene expression of VEGF was quantitated by Phosphoimage analysis of the VEGF band normalized to the expression of actin. The numbers represent to relative induction levels of VEGF. The value of VEGF expression in hypoxic cells is arbitrarily defined as 100%.

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 hours and then

exposed to hypoxia for the indicated times or 20 ng/ml TPA 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 (upper panel). 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 (lower panel). 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 hours and then pretreated with PD98059 (100• M) for 1 hour prior to hypoxic exposure for the indicated times or 20 ng/ml TPA for 10 min. Immunoblot analysis was performed using anti-phospho-p42/p44 MAPK antibody (upper panel) or anti-p42/p44 MAPK antibody (lower panel). (C) Hep3B cells at 70% confluency were serum starved with 0.5% serum-containing media for 48 hours and then pretreated with Genistein (150• M) for 1 hour prior to hypoxic exposure for the indicated times or 20 ng/ml TPA for 10 min. Immunoblot analysis was performed using anti-phospho-p42/p44 MAPK antibody (upper panel) or anti-p42/p44 MAPK antibody (lower panel). All experiments were done at least three times.

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 x 10^5 cells per 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 x 10^5 cells per 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. 12 hours prior to hypoxic treatment, transfected Hep3B cells were serum starved with medium containing 0.5% fetal bovine serum, then treated for 16 hours 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 standard deviation of three experiments.

Index Terms; Transcriptional regulation, Hypoxia, Hypoxia-inducible factor-1, MAP kinase, bHLH-PAS