Molecular Mechanisms of Activation of Vascular Endothelial Growth Factor Gene by Nitric Oxide

一酸化窒素による血管内皮増殖因子遺伝子転写活性化機構の解析

木村 英生

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TABLE OF CONTENTS

INTRODUCTION

Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is a multistep process that involves migration and proliferation of endothelial cells, remodeling of the extracellular matrix and functional maturation of the newly assembled vessels (1,2). Physiologically, angiogenesis is a tightly regulated process, resulting from the balance of angiogenic and angiostatic stimuli. These stimuli are regulated temporally and spatially, as for example during early embryonic development, organogenesis, and wound healing. At other times, angiogenesis is completely inhibited (3). Unregulated angiogenesis, in fact, is the cause of severe tissue dysfunction and has been directly implicated in the pathogenesis of various diseases including retinopathies, psoriasis, rheumatoid arthritis, and other chronic inflammatory diseases (4). Moreover, angiogenesis is essential for solid tumor outgrowth (5).

The endothelial cell-specific vascular endothelial growth factor (VEGF) exerts a pivotal role in normal and pathological angiogenesis (6). Its production by stromal or epithelial cells is sufficient to trigger angiogenesis, and inactivation of the corresponding gene results in abnormal blood vessel development and embryonic lethality in mice (7). Indeed, synthesis of VEGF, followed by its secretion into the extracellular environment, is one of the first steps in the angiogenic cascade and controls the onset, extent, and duration of this process. A number of angiogenic stimuli have been found to induce VEGF gene expression including several growth factors, cytokines, hormones, phorbol esters, oncogenes, nitric oxide (NO), and hypoxia (17). VEGF gene regulation in hypoxic cells occurs during the 'rescue' phase (18), and is characterized by its transcriptional activation (19-24), primarily through the hypoxia-response element (HRE) that includes cis-acting DNA elements recognized by multiple trans-activators (10,20,23,25,26).

The hypoxia-inducible factor 1 (HIF-1) is the best-characterized regulator of the VEGF gene transcription. In its active form, it is a dimer composed of two distinct subunits, both of which belong to the basic helix-loop-helix-per-arnt-sim (bHLH-PAS) protein family:

HIF-1α and HIF-1β, the aryl hydrocarbon receptor nuclear translocator (ARNT) (27). They bind to the target DNA sequence (HIF-1 binding site; HBS) within the HRE and enhance the transcription rate of hypoxia-inducible genes (23,27).

Nitric oxide is an intracellular and intercellular signaling molecule, generated in eukaryotic cells from L-arginine by a reaction catalyzed by NO synthases (28). A widespread range of biological effects are attributed to this molecule (29,30). Some effects are linked to its intracellular second messenger nature, while others result from its paracrine actions, mediated by activation of the guanylate cyclase/3',5'-cyclic guanosine monophosphate (GC/cGMP) pathway (30,31). Indeed, although NO is highly reactive and believed to be quite unstable *in vivo*, once produced in sufficient amounts it can travel significant distances in the tissue to reach multiple cellular targets (32).

There is a considerable body of evidences that NO downregulates the expression of VEGF gene (12,14,33-35). In spite of these observations, production of angiogenic activity by human monocytes has been found to depend on NO (16), and NO-generating compounds have been shown to stimulate the VEGF gene transcription in human glioblastoma and hepatoma cells in culture (15). Furthermore, a strong positive correlation between NO synthase (NOS) activity, cGMP levels and tumor angiogenesis have been recently described in head and neck (36) and gynecological cancers (37,38). We have investigated the mechanism of NO-mediated regulation of the human VEGF gene in human glioblastoma and hepatoma cells. We demonstrate that the VEGF gene transcription is activated by NO as well as by hypoxia via the HBS and an adjacent 'ancillary' sequence within the HRE of this gene. This response to NO is mediated, at least in part, by activation of the HIF-1 complex independent of the GC/cGMP pathway. In addition, we show that a common structure of the HRE, consisting of the HBS and its ancillary sequence, is widely seen among hypoxia-inducible genes including VEGF, erythropoietin (Epo), and some glycolytic enzymes.

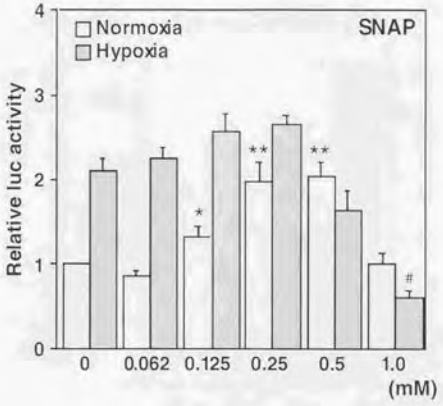
RESULTS

Analysis of the human VEGF gene promoter response to nitric oxide

Following exposure to NO donors, VEGF mRNA rapidly accumulates in human glioblastoma or hepatoma cells; this is prevented by pre-treatment of the cells with the RNA polymerase inhibitor actinomycin D (15). This finding suggests that VEGF mRNA accumulation by NO is mediated, at least in part, by transcriptional activation of the VEGF gene. To verify this possibility, a luciferase reporter phVEGF1 was used to test the effect of NO donors on the activity of the human VEGF promoter in A-172 cells. S-nitroso-N-acetyl-D, L-penicillamine (SNAP) enhanced the activity of the transfected promoter in a dose-dependent manner (Fig. 1A) within 6 to 12 h. The chemically distinct NO donor 3-(2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino)-1-propanamine (NOC5) was as effective as SNAP in inducing the reporter gene activation (Fig. 1B). NO concentration was represented by nitrite and determined by Griess reaction. Nitrite concentration of the culture medium was quite high in the presence of these NO donors (Fig. 1C). These results suggest that NO enhances the transcription of the VEGF gene. Similar dose-response correlations and induction kinetics were observed in the same cells for the endogenous VEGF gene activation by these NO donors (15).

For comparison, the effect of low oxygen tension on phVEGF1 expression was also determined in A-172 cells under the same experimental conditions. As shown in Fig. 1A and 1B, transcription of the transfected reporter gene was enhanced by hypoxia (1% O_2) ($p<0.01$ versus control). This is consistent with the fact that this reporter includes the HRE of the VEGF gene (19-21,23). When cells were stimulated with either SNAP or NOC5 under hypoxic conditions, maximal promoter activation was achieved by lower concentrations of NO donors, as compared with normoxic conditions (21% O_2). However, 1 mM of NOC5, the optimal concentration for reporter activation in normoxia, inhibited hypoxic induction of the reporter gene (Fig 1B). The response of the transfected VEGF promoter to SNAP and hypoxia in human hepatoma Hep3B cells was also tested.

A


Normoxia
Hypoxia
SNAP
Relative luc activity
0
1
2
3
4
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0.062
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(mM)
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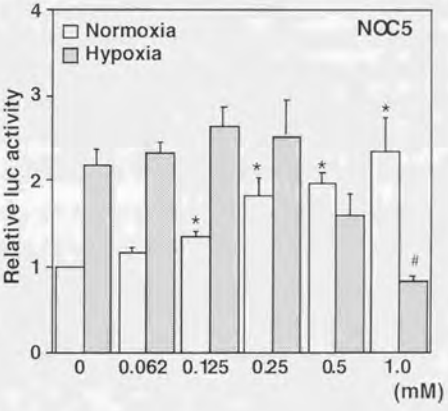
B


Normoxia
Hypoxia
NCC5
Relative luc activity
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1
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C

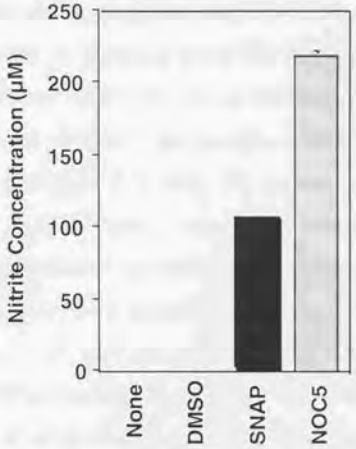


Figure 1. Effect of NO and hypoxia on the expression of the VEGF reporter gene in A-172 cells. A-172 cells were stimulated with 0.5 mM SNAP (A) or 0.5 mM NOC5 (B) for 12 h under normoxic (21% O_2) or hypoxic (1% O_2) conditions. The final concentration of DMSO was 0.1% in (A). (NOC5 was dissolved in PBS.) (A) n=8; *p<0.05, **p<0.01 versus control in normoxia, #p<0.01 versus control in hypoxia. (B) n=8; *p<0.01 versus control in normoxia, #p<0.01 versus control in hypoxia. Relative luciferase activity indicates ratios of mean luciferase/β-galactosidase activity. Error bars represent SEM in a given measurement. (C) Nitrite concentration of the culture medium after 12 h exposure to NO donors under normoxia (0.1% DMSO or 0.5 mM SNAP or 0.5mM NOC5). (n=3)

Maximum induction was obtained in 36 h after exposure to hypoxia and SNAP (data not shown).

Studies with GC inhibitors have suggested that VEGF mRNA accumulation in response to NO donors is mediated by an increase in intracellular cGMP (15). The response of the reporter to NO in the presence of GC inhibitors was therefore analyzed. When cells were incubated with SNAP in the presence of either methylene blue (MB, 25 μM) or 6-anilino-5,8-quinolinequinone (LY83583, 1.25 μM), NO-induced promoter activation was completely inhibited ($p<0.01$ versus control in SNAP). In contrast, 1H-[1,2,4]oxidiazolo[4,3-a]quinoxalin-1-one (ODQ, 50 μM), another specific GC inhibitor, did not inhibit the NO-induced transcriptional activation even at the higher concentration. However, LY83583 (1.25 μM) could attenuate the activation to the levels of the untreated cells in the presence of ODQ (25 μM) ($p<0.05$ versus ODQ+SNAP) (Fig. 2). In addition, to test if an increase of cGMP levels could enhance VEGF promoter activity, 8-Br-cGMP (a protein kinase G activator) was added to the culture medium at concentration of 800 μM in the presence or absence of LY83583 (1.25 μM). In both cases, 8-Br-cGMP did not show any effect on NO-induced promoter activity. Hypoxic induction of the same reporter gene was unaffected by either GC inhibitor or by 8-Br-cGMP when tested under the same experimental conditions (Fig. 2). Despite the suppression of NO-induced VEGF promoter activation by MB and LY83583, these results suggest that the NO-induced activation is not mediated by the GC/cGMP pathway.

It has been recently reported that NO suppresses hypoxic induction of the VEGF gene by using sodium nitroprusside (SNP) as an NO donor in human hepatoma cell lines (33,35). Our studies with SNP demonstrated that it attenuated the VEGF promoter activation by hypoxia in a dose-dependent manner. Moreover, it had no significant effect under normoxia at concentrations up to 100 μM in A-172 cells (Fig. 3) and Hep3B cells (data not shown), in contrast to our results with SNAP and NOC5.

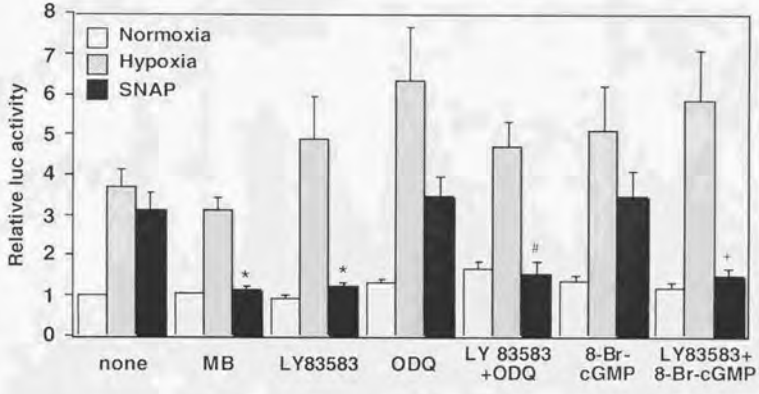


Figure 2. Effect of the guanylate cyclase inhibitor and/or protein kinase G activator on human VEGF promoter activity in A-172 cells. The cells were exposed to 25 μM MB, 1.25 μM LY83583, or 25 μM ODQ and/or 800 μM 8-Br-cGMP under the same conditions as in Figure 1 for 12 h. n=6; *p<0.01 versus control in SNAP, #p<0.05 and +p<0.01 versus ODQ in SNAP, and 8-Br-cGMP in SNAP, respectively.

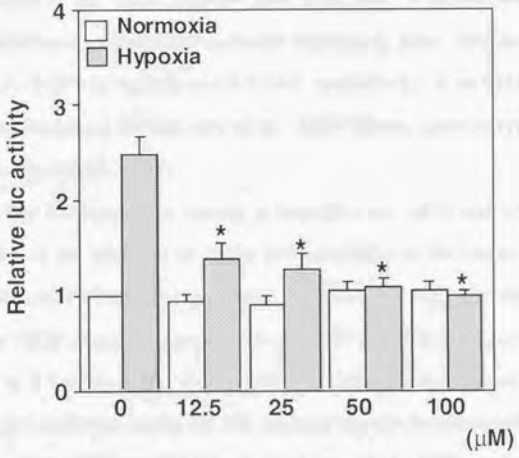


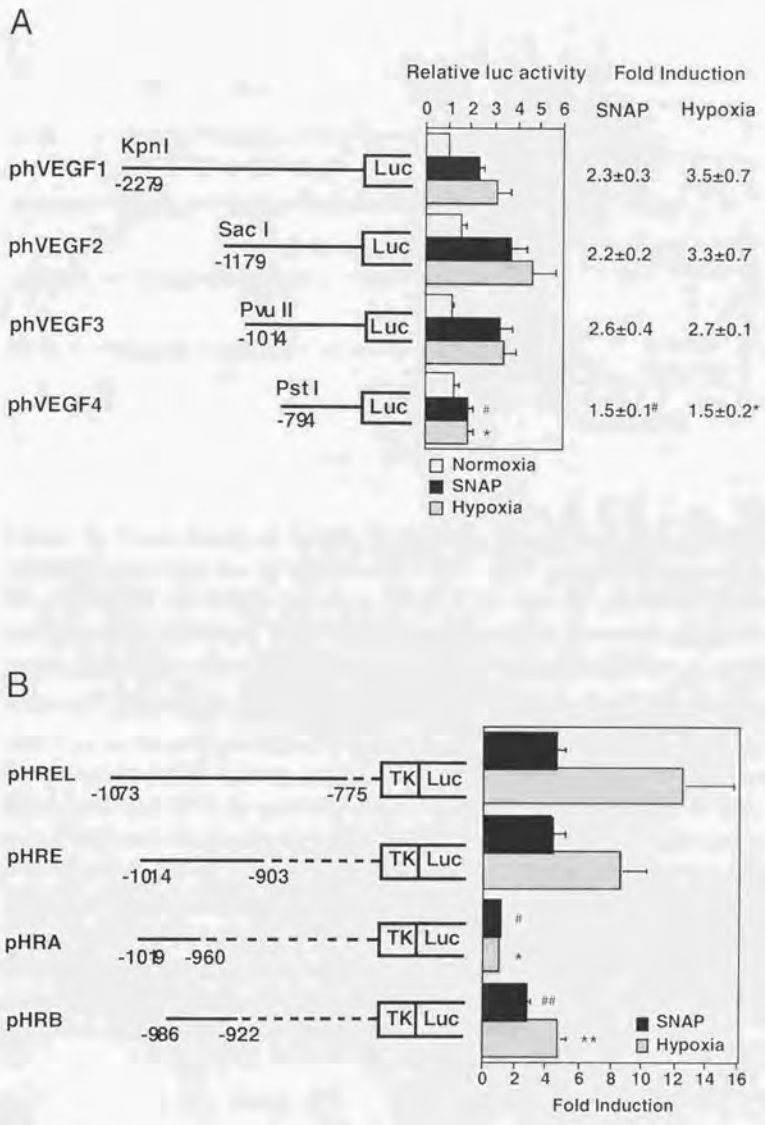
Figure 3. Effect of SNP on human VEGF promoter in A-172 cells. The cells were exposed to various concentrations of SNP under normoxic and hypoxic conditions for 12 h. n=6; *p<0.01 versus control in hypoxic conditions.

Identification of the nitric oxide response elements of the human VEGF gene

To determine the NO-response element of the human VEGF promoter, we constructed a series of deletion mutants of phVEGF1 and tested their response to SNAP (0.5 mM for 12 h) following transient transfection in A-172 cells. Removal of DNA sequences between positions -2,279 and -1,014, did not cause any significant change in the hypoxia- or NO-induced activation of the VEGF reporter gene (Fig. 4A). A further deletion to -794 (phVEGF4) significantly reduced the promoter response to either stimulus ($p<0.01$ and <0.05 versus phVEGF3 in hypoxia and in SNAP, respectively). It should be noted that a similar stepwise decrease in the response of the VEGF reporter genes to hypoxia has been reported previously (20,22,23,44).

To confirm that NO-responsive element is located in the -1014 and -794 region, we tested the ability of this sequence to confer NO-inducibility to the herpes simplex virus thymidine kinase (HSV-TK) gene promoter. As shown in Fig. 4B, this is the case. Deletion of the VEGF promoter sequence between -960 and -903 (pHRA) completely lost the response to SNAP ($p<0.01$ versus pHRE in SNAP). Analysis of an additional recombinant (pHRB) further locates the NO-response element between positions -986 and -922 ($p<0.01$ versus pHRA in SNAP). It overlaps with the HRE of the VEGF gene. Sequence comparisons between the human, mouse, and rat VEGF genes in this DNA region reveal a high degree of evolutionary conservation (data not shown). In particular, there is conformity in 4 sequences. These correspond to the HBS 5'-TACGTGGG (-975 to -968); the AP-1 site 5'-TGACTAA (-937 to -931); the NF-κB 'like' sequence 5'-GGGTTTTGCC (-1,000 to -991); and the sequence 5'-ACAGGTC (-962 to -956), which we call the HIF-1 ancillary sequence (HAS). The last has been previously suggested to be essential for hypoxic induction of the VEGF promoter (20).

To determine the role of each of these sequence elements in the NO-mediated responses, we tested responses of pHRE and its related mutants to SNAP. The response of pHRE was quantitatively and qualitatively comparable to that of phVEGF3 (Fig. 4A). A mutation in


A
Relative luc activity
Fold Induction
0 1 2 3 4 5 6
SNAP
Hypoxia
phVEGF1
KpnI
-2279
Luc
2.3±0.3
3.5±0.7
phVEGF2
Sac I
-1179
Luc
2.2±0.2
3.3±0.7
phVEGF3
Pvu II
-1014
Luc
2.6±0.4
2.7±0.1
phVEGF4
Pst I
-794
Luc
#
*
1.5±0.1#
1.5±0.2*
Normoxia
SNAP
Hypoxia
B
pHREL
-1073
-775
TK Luc
pHRE
-1014
-903
TK Luc
pHRA
-1019
-960
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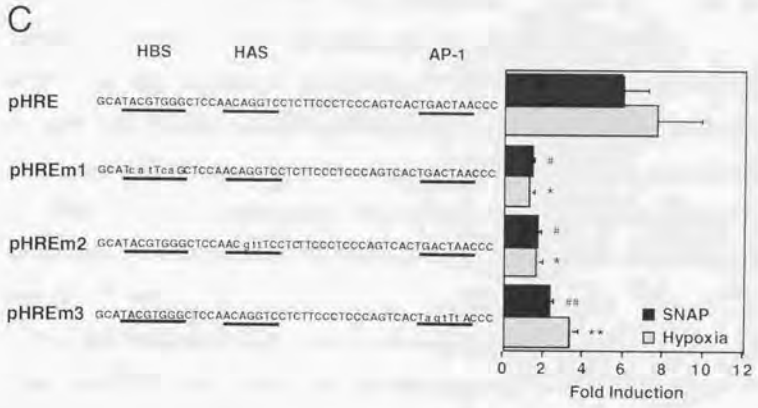


Figure 4. Localization of VEGF 5'-flanking sequences which mediate transcriptional response to hypoxia or SNAP. VEGF sequences were cloned to the promoterless pGL2 basic vector (A), or 5' to an HSV-TK promoter-luciferase transcription unit of pT81luc0 (B, C). The locations of restriction sites are shown relative to the transcription start site. Relative luciferase activity indicates ratios of mean luciferase/β-galactosidase activity. Fold induction, by hypoxia or by SNAP, represents the ratio of relative luc activity in cells at 1% O_2 or 0.5 mM SNAP in 0.1% DMSO, to those at 21% O_2 or 0.1% DMSO, respectively. (C) Nucleotides of transcription factor binding sites (HBS, HAS, and AP-1) are underlined (-975 to -968, -962 to -956, and -937 to -931, respectively), and substituted bases are shown in lower case letters. (A) n=8 (hypoxia) or n=11 (SNAP); *p<0.05 versus phVEGF1 and <0.01 versus phVEGF3 in hypoxia, #p<0.01 versus phVEGF1 and <0.05 versus phVEGF3 in SNAP. (B) n=8; *p<0.01 versus pHRB in hypoxia, **p<0.05 versus pHRE in hypoxia, #p<0.01 versus pHRB in SNAP, ##p<0.05 versus pHREL in SNAP. (C) n=6; *p<0.01 versus pHRE and <0.05 versus pHREm3 in hypoxia, **p<0.05 versus pHRE in hypoxia, #p<0.01 versus pHRE and <0.05 versus pHREm3 in SNAP, ##p<0.05 versus pHRE in SNAP.

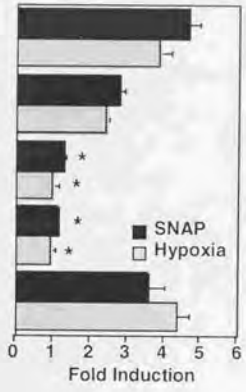
the HBS (pHREm1), or in the HAS (pHREm2) completely abolished the NO-induced activation of the promoter ($p<0.01$ versus pHRE), and a mutation in the AP-1 site (pHREm3) inhibited, partially but significantly, the response to NO ($p<0.05$ versus pHRE) (Fig. 4C). As controls, the response of these reporters to hypoxia was also measured under the same conditions and was found to be superimposable to that of NO. The effects of NO and hypoxia on these reporters were also the same when tested in Hep3B cells (data not shown). Taken together, these results indicate that the NO- and hypoxia-response sequences of the human VEGF gene co-localize with the HBS, the HAS, and, in part, the AP-1 site.

The HBSs are located in the promoter or enhancer regions of several hypoxia-inducible genes, and the consensus sequence was described as either 5'-(G/C/T)ACGTGC(G/C)-3' (20) or 5'-RCGTG-3' (45). To determine the exact extent of the HBS in the VEGF gene, a series of pHRE mutants with 1-3 nucleotides (nt) exchanges (pHREm1a to 1k) were synthesized, and their reporter expression was compared with that of the wild-type (pHRE) after NO and hypoxic treatments. The pHRE mutants, pHREm1b, 1c, 1e, 1f, and 1g, that encompass the substitution of a 6-nt sequence, lost their response to both 0.5 mM SNAP and hypoxia (Fig 5A and 5B). Therefore, the 6-nt sequence, TACGTG, contains the core of the HBS. In addition, a mutation at the last G (pHREm1i) of this sequence eliminated its response to both stimuli, while a mutation at T, the first nucleotide of TACGTG (pHREm1h) partially attenuated the activity of the luciferase reporter (Fig. 5B). Thus, at least the sequence, ACGTG, is required for the HBS to function.

To clarify the importance of the initial T within the sequence, TACGTG, we substituted the T with an A, C, or G, and tested the reporter activity of the resultant constructs. Figure 5C shows that the promoter responded better to NO and hypoxia if the first nucleotide was a T or G, rather than an A or C ($p<0.05$). A compilation of HBSs (Table 1) showed that the HRE contains a sequence T/GACGTG as a functional HBS in many hypoxia-inducible genes, whereas A or C in the first nucleotide of this sequence is found in only a few genes.

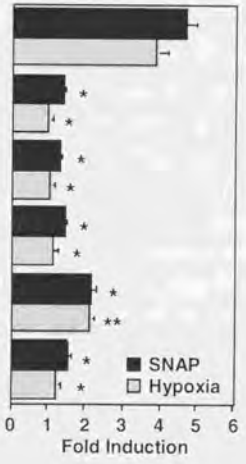
A

pHRE	5'-GCATACGTGGGCTCCAACAGGTCCTCT-3'
pHREm1a	5'-tagTACGTGGGCTCCAACAGGTCCTCT-3'
pHREm1b	5'-GCAcgaGTGGGCTCCAACAGGTCCTCT-3'
pHREm1c	5'-GCATActctGGCTCCAACAGGTCCTCT-3'
pHREm1d	5'-GCATACGTGttaTCCAACAGGTCCTCT-3'



B

pHRE	5'-GCATACGTGGGCTCCAACAGGTCCTCT-3'
pHREm1e	5'-GCAcgCGTGGGCTCCAACAGGTCCTCT-3'
pHREm1f	5'-GCATAatTGGGCTCCAACAGGTCCTCT-3'
pHREm1g	5'-GCATACGctGGCTCCAACAGGTCCTCT-3'
pHREm1h	5'-GCAaACGTGGGCTCCAACAGGTCCTCT-3'
pHREm1i	5'-GCATACGTaGGCTCCAACAGGTCCTCT-3'



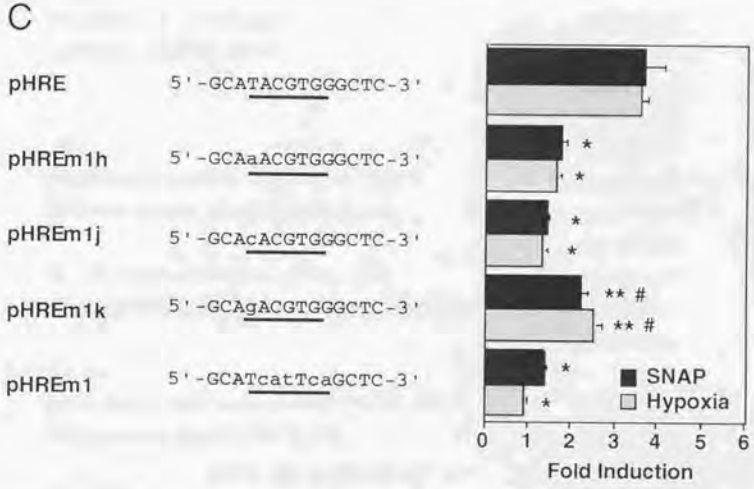


Figure 5. The HIF-1 binding site of VEGF gene is determined as TACGTG. A-172 cells were collected after 12 h exposure to either normoxia (21% O_2), hypoxia (1% O_2), DMSO (0.1%), or SNAP (0.5 mM in 0.1% DMSO). Nucleotides of a putative HBS are underlined, and substituted bases are shown in lowercase letters. (A) A series of pHRE-related mutants with 3-nt substitutions within the HRE were prepared, and their responses to SNAP and hypoxia were tested. n=4; *p<0.01 versus pHREm1a and pHREm1d. (B) A series of pHRE-related mutants with 1- or 2-nt substitutions within the HRE were prepared, and their responses to both stimuli were tested. n=6; *p<0.01, **p<0.05 versus pHRE. (C) A series of pHRE-related mutants with substitution of the first nucleotide of TACGTG were prepared, and their responses to both stimuli were tested. n=6; *p<0.01, **p<0.05 versus pHRE. #p<0.05 versus pHREm1h and pHREm1j.

Hypoxia-inducible genes	HIF-1 binding sites
Glycolysis	
(h) aldolase A (ALDA) (46)	GACGTGAC
(h) enolase 1 (ENO-1) (46)	GACGTGGG
	TGCGTGCG
	TACGTGAC
	CACGTGCG
(m) glucose transporter 1 (GLUT-1) (51)	GGCGTGCC
(h) lactate dehydrogenase A (LDHA) (46)	CACGTGGG
	GACGTGCG
(m) phosphofructokinase L (PFKL) (96)	TACGTGCT
(h) phosphoglycerate kinase 1 (PGK-1) (46,50)	GACGTGAC
	GACGTGCG
Vasodilation	
(m) inducible nitric oxide synthase (iNOS) (97)	TACGTGCT
(m) heme oxygenase 1 (HO-1) (98)	GACGTGCT
	GACGTGCC
Vasoconstriction	
(h) endothelin 1 (ET-1) (99)	CACGTTGC
Angiogenesis	
(h) vascular endothelial growth factor (VEGF) (20)	TACGTGGG
Erythropoiesis	
(h) erythropoietin (Epo) (77)	TACGTGCT
Iron transport	
(h) transferrin (Tf) (100)	TACGTGCA
	TACGTGCG
Synthesis of catecholamines	
(h) tyrosine hydroxylase (TH) (101)	TACGTGTA

Table 1. HIF-1 binding sites of hypoxia-inducible genes. Note that most of the HIF-1 sites have a common sequence 'T/GACGTG'. The core sequences are underlined. The species are: (h), human; (m), mouse.

This finding is consistent with our results that the sequence A/CACGTG was less functional (Fig. 5C). Together, our findings suggest that a 6-nt sequence, TACGTG, is the core motif of the HBS in the VEGF promoter.

We have demonstrated that not only the HBS, but also its downstream HAS, is essential for NO and hypoxic induction of the VEGF reporter gene. To identify the extent of the HAS, we tested the response of pHRE and its related mutants (pHREm2a to 2k) to NO and hypoxia. Figures 6A and 6B illustrate the results of the mutation analysis of the HAS. Since pHREm2b, 2e, and 2d lost NO- and hypoxia-induced luciferase activity (Fig. 6A), a 9-nt sequence, AACAGGTCC, was found to contain the core of the HAS. A further analysis of the sequence requirement (Fig. 6B) revealed that any 2-bp mutation within ACAGGT (pHREm2g, 2h and 2i) resulted in the loss of response, but a mutation at the first A (pHREm2k) did not abrogate luciferase activity. A mutation at TCC (pHREm2d), but not at CC (pHREm2j), within the sequence ACAGGTCC, attenuated the reporter activity. Thus, any substitution within CAGGT eliminated the promoter response to either stimulus. This result suggests that these 5 nt constitute the HAS and the strict sequence requirement might be indicative of the binding of some factor to the HAS. These experiments indicate that NO and hypoxia similarly enhance the VEGF promoter activity, and both the HBS and the HAS are required as cis-elements for the activation of VEGF by these stimuli.

The HBS and the HAS are located adjacent to each other. To investigate whether these two elements function interdependently, we constructed pHRE mutants, with either a 2-nt deletion within (pHREm3a), or a 5-nt insertion (pHREm3b) into the spacer, and tested the reporter activity after NO and hypoxic treatments. As shown in Fig. 7, either mutation resulted in a loss of reporter activation, suggesting that the spacing between these motifs is crucial for the promoter activity. This result raises the possibility that some putative factor, what we call it an HIF-1 ancillary factor (HAF), may bind to the HAS and interact with HIF-1 for VEGF gene induction.

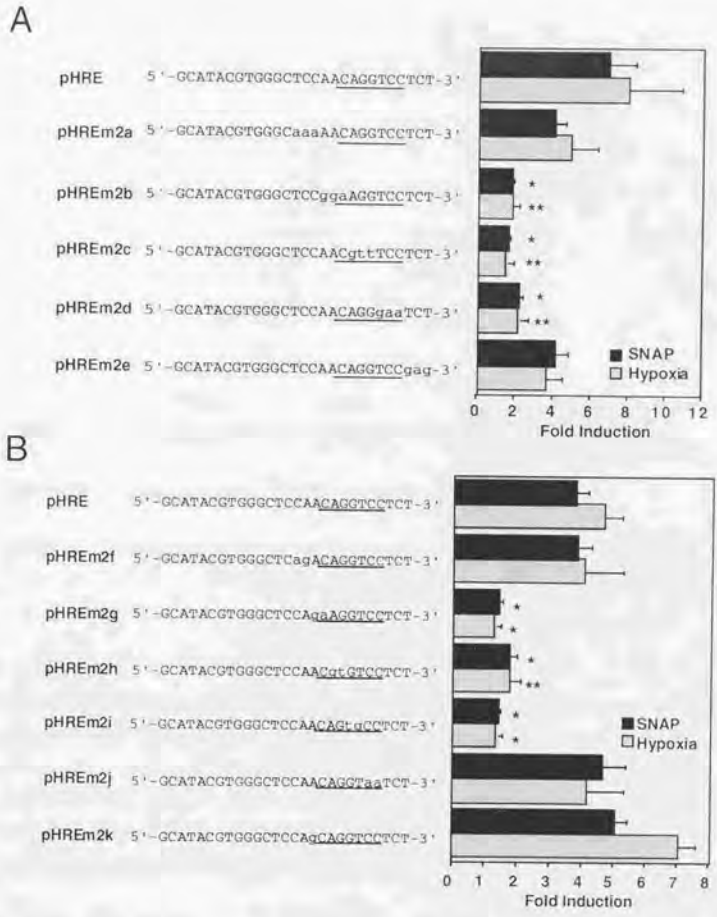


Figure 6. The HIF-1 ancillary sequence of VEGF gene is determined as CAGGT. A-172 cells were exposed to the same conditions as in Figure 1 for 12 h. n=6; *p<0.01, **p<0.05 versus pHRE. Nucleotides of a putative HAS are underlined, and substituted bases are shown in lowercase letters. (A) A series of pHRE-related mutants with 3-nt substitutions within the HRE were prepared, and their responses to SNAP and hypoxia were tested. (B) A series of pHRE-related mutants with 1- or 2-nt substitutions within the HRE were prepared, and their responses to both stimuli were tested.

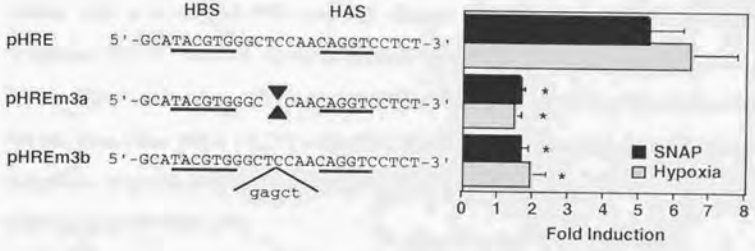


Figure 7. The spatial alignment of the HBS and the HAS is crucial for NO and hypoxic induction of VEGF gene. A-172 cells were exposed to the same conditions as in Figure 1 for 12 h. pHRE-related mutants with either a 2-nt deletion or a 5-nt insertion within the spacer between the HBS and the HAS were prepared. $n=6$; $^*p<0.01$ versus pHRE. Nucleotides of putative HBS and HAS are underlined, and substituted bases are shown in lowercase letters.

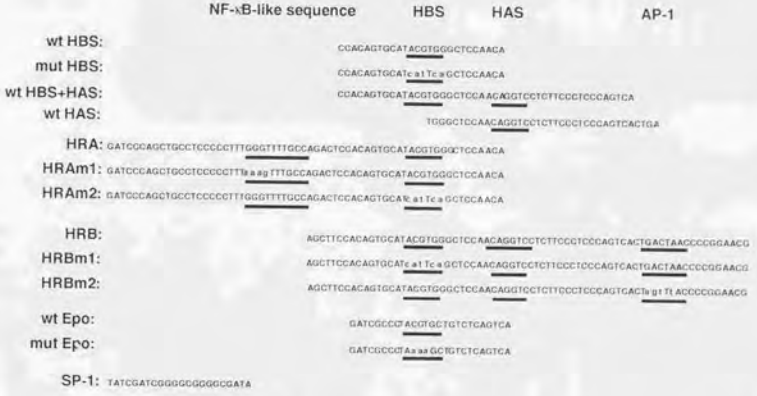
Characterization of nitric oxide-responsive nuclear proteins that bind to the HBS of the human VEGF gene

To identify the transcription factor(s) present in A-172 cells that may interact with the NO-response element, and in particular with the HBS, we analyzed the *in vitro* binding of nuclear proteins to a labeled WT HBS (-985 to -960) double-stranded oligonucleotide (Fig. 8A). Several DNA-protein complexes were detected, as shown in Fig. 8B. Four bands are present when using nuclear extracts from both control and SNAP-stimulated cells (NS, C1, C2 and C3). The lowest band (NS) does not represent a specific complex, since it can interact with a variety of wild-type and mutated oligonucleotides. The other three complexes (C1, C2 and C3) represent proteins interacting specifically with the HBS or with its flanking sequences. These three complexes are detected using probes containing this site from either VEGF (20,23) or Epo (23,47,48) genes. It has been suggested that the complexes represent constitutive binding of ATF-1 and CREB-1 transcription factors within or near the HBS (49).

The remaining two upper bands (H1 and H2) were faint in extracts from untreated cells, but quite intense following cell exposure to SNAP (Fig. 8B). These labeled complexes were inhibited by an excess of unlabeled oligonucleotides containing the wild-type HBS from either the human VEGF or Epo genes (wt HBS, HRA, HRAm1, HRB, HRBm2, and wt Epo). However, they were not inhibited by an excess of oligonucleotides containing mutation in the HBS (mut HBS, HRAm2, HRBm1, and mut Epo) or by an SP-1-binding oligonucleotide of unrelated sequence (SP-1) (Fig. 8B). Nuclear extracts from hypoxic A-172 cells showed a similar pattern of binding (Fig. 8C). However, the intensity of H2 is generally stronger than that of H1 in the NO-treated cells, while both are similarly enhanced in the hypoxic cells.

To verify the presence of HIF-1 protein in H1 and H2 complexes, we then used antibodies against both HIF-1α and HIF-1β/ARNT in supershift assays. As shown in Fig.

A


NF-κB-like sequence
HBS
HAS
AP-1
wt HBS:
mut HBS:
wt HBS+HAS:
wt HAS:
HRA:
HRAm1:
HRAm2:
HRB:
HRBm1:
HRBm2:
wt Epo:
mut Epo:
SP-1:
TATCGATCGGGGCGGGGCGATA

B

competitor
molar ratio
SNAP
- + 0 + 10 + 50 + 250 + 10 + 50 + 250 + 250 + 250 + 250 + 250 + 250 + 250 + 250 + 250
wt HBS
mut HBS
HRA
HRAm1
HRAm2
HRB
HRBm1
HRBm2
wt Epo
mut Epo
SP-1
H1
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C1
C2
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competitor
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wt HBS
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H1
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C1
C2
C3
NS

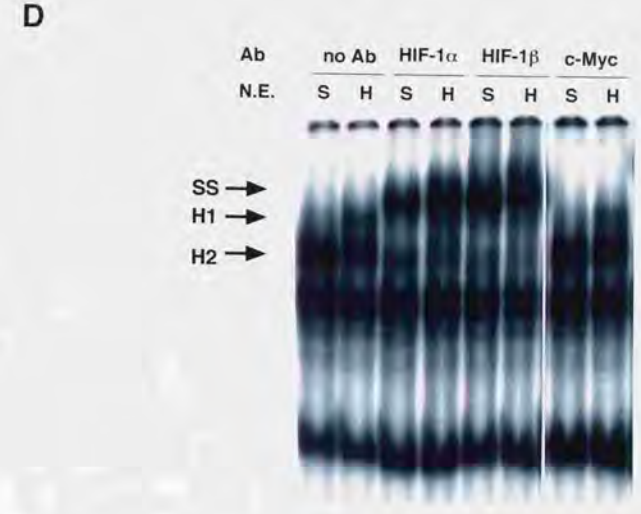

Figure 8. NO and hypoxia enhance HIF-1 binding activity.

(A) Oligonucleotide sequences for EMSA. Nucleotides of functional transcription factor binding sites are underlined, and substituted bases are shown in lowercase letters. WT HBS was used as a labeled probe.

(B, C) EMSAs showing the binding specificity of nuclear factors from SNAP- (B) or hypoxia- (C) treated cells. Nuclear extracts (5 μg) from A-172 cells, treated by 0.1 % DMSO or 0.5 mM SNAP (B), or under normoxic (21% O_2) or hypoxic (1% O_2) conditions (C), were incubated with wt HBS probe for 30 min in the presence of no competitor (0), or 10-, 50-, or 250-fold molar excess of unlabeled competitor oligonucleotides. SNAP- or hypoxia-induced (H1 and H2), constitutive (C1, C2 and C3) and nonspecific (NS) complexes are indicated.

(D) Supershift of HRE binding complexes. Nuclear extracts (5 μg) from A-172 cells treated by 0.5 mM SNAP or by hypoxia were incubated with labeled wt HBS probe, in the presence or absence of monoclonal antibodies against HIF-1α, HIF-1β or c-Myc as potential supershifting reagents. The shifted complexes (SS) are indicated. Ab: antibody, NE: nuclear extract, S: SNAP; H: hypoxia.

8D, both NO- and hypoxia-induced H complexes were indeed completely supershifted (SS) by either anti-HIF-1α or anti-HIF-1β antibodies, but not by unrelated anti-c-Myc antiserum.

Gel shift assays of Hep3B nuclear extracts were also performed for HIF-1 binding to the wt HBS probe. Inducible bands H1 and H2 were quite visible in NO- and hypoxia-treated cell extracts, and the patterns of relative amounts of these bands were quite similar to those seen in A-172 nuclear extracts. The supershift assay demonstrated that these inducible bands also contained HIF-1 α and β protein (data not shown). These results indicate that NO and hypoxia induce HIF-1 binding activity in A-172 and Hep3B cells.

It has been reported that the amount of HIF-1α protein is significantly increased under hypoxic conditions. This response depends upon the stabilization of HIF-1α rather than increased HIF-1α mRNA levels, and the abundance of HIF-1α protein primarily determines the enhancement of HIF-1 binding activity (52,53). To examine whether NO affects the HIF-1α accumulation, we performed Western blot analysis with an anti-HIF-1α monoclonal antibody. The nuclear and the whole-cell extracts were prepared from A-172 cells. Figures 9A and 9B show that NO as well as hypoxia significantly induced HIF-1α accumulation in both the nucleus and the whole cell. This indicates that the abundance of HIF-1α also accounts for NO-induced HIF-1 activation.

Characterization of Nuclear Proteins That Bind to the HAS of the Human VEGF Gene

We have demonstrated that hypoxia- and NO-induced HIF-1 activity mediates transcriptional activation of the VEGF gene. HIF-1 forms DNA-binding complexes containing the p300/CREB-binding protein when bound to its target HBS under hypoxic conditions (54). However, no previous study has described (a) binding factor(s) to the

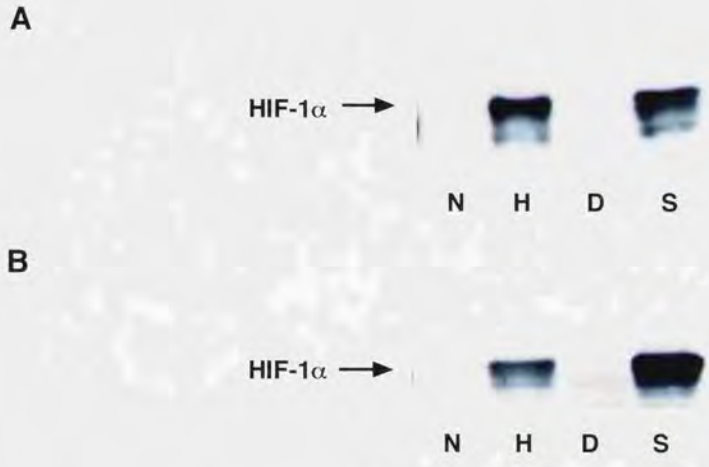

Figure 9. Expression of HIF-1α proteins in A-172 cells. Nuclear extracts (A) and whole-cell extracts (B) were prepared from untreated or hypoxia- (8 h), DMSO- (3 h), or SNAP- (3 h) treated A-172 cells, and subjected to Western blot analysis using anti-HIF-1α monoclonal antibody.

adjacent HAS. To identify proteins that bind to the HAS of the VEGF gene, we analyzed, *in vitro*, the binding of nuclear proteins to three kinds of ^{32}P-labeled oligonucleotides corresponding to the HBS (wt HBS), the HAS (wt HAS), and both elements (wt HBS+HAS) in NO- or hypoxia-treated cells. Nuclear proteins were extracted from A-172 cells cultured under 1 % O_2 or 0.5 mM SNAP, or each respective control cells. The extracts were incubated with labeled probes for 30 min at room temperature in the same binding buffer as in Figure 8 (100 mM KCl), and the mixtures were electrophoresed on non-denaturing TAE-acrylamide gels. As shown in Fig. 10A, electrophoretic mobility shift assays (EMSA) revealed that protein-DNA complexes (C) were always present when either wt HBS or wt HBS+HAS was used as a probe. The bands of less mobility (H1 and H2) appeared only when nuclear extracts from the NO- or hypoxia-treated cells were used. Similar patterns of H1 and H2 bands were observed when nuclear extracts from the NO- or hypoxia-treated cells were used with wt HBS and wt HBS+HAS probes. However, no additional bands were observed in any extract with wt HBS+HAS and wt HAS probes. Various binding conditions (KCl concentration between 40 to 160 mM) or a different running buffer (TBE instead of TAE) failed to reveal any additional band when wt HBS+HAS was used as a probe or any inducible bands when wt HAS probes were used (data not shown).

As 'CAGGT', a core motif of the HAS, and a sequence 'ACGTG' in the HBS form an imperfect inverted repeat, competition assays were performed using labeled wt HBS as a probe to see if the HAS could be a binding site for the HIF-1 protein. HIF-1 bands induced by SNAP (H1 and H2) were displaced by excessive unlabeled wt HBS oligonucleotides, but not by wt HAS (Fig. 10B). This result suggests that these bands specifically bind to the HBS, but the HAS is not a candidate for the HBS. The same result was obtained with hypoxia-treated nuclear extracts (data not shown).

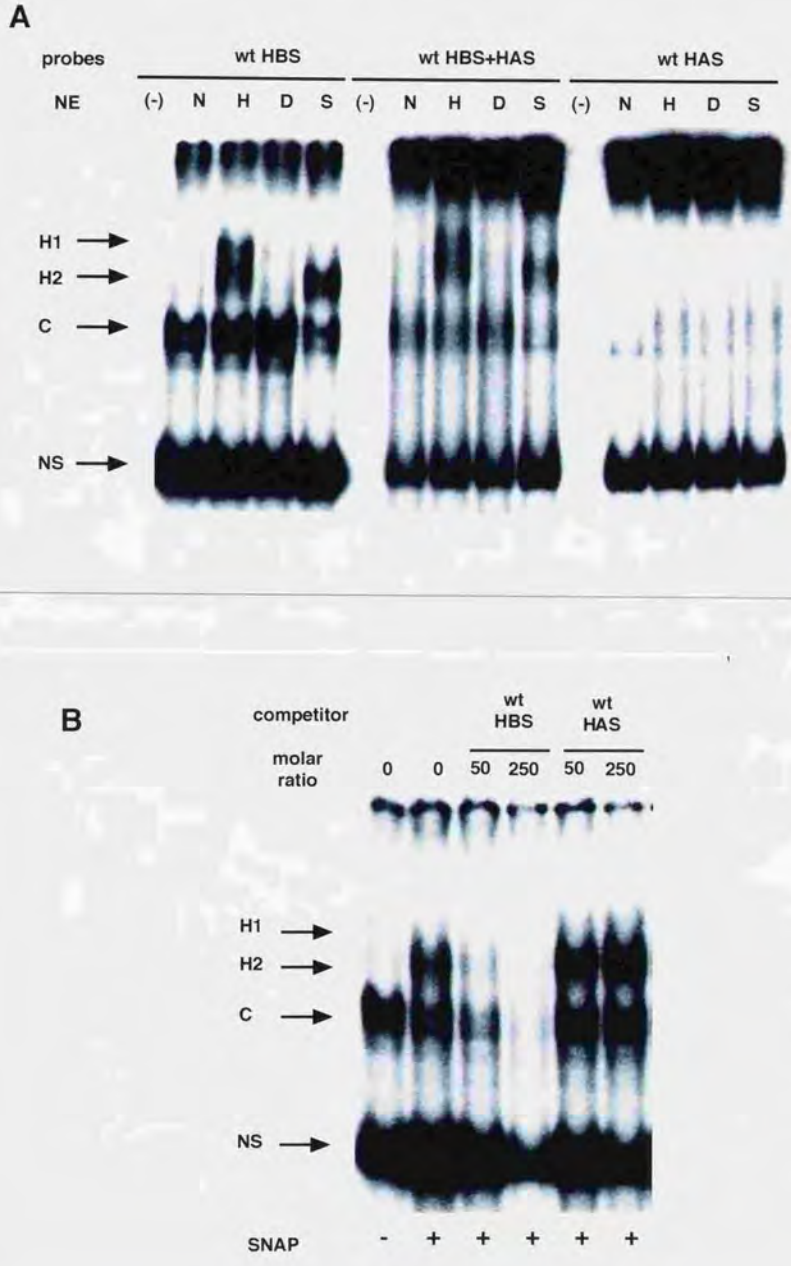
A
probes
wt HBS
wt HBS+HAS
wt HAS
NE
(-) N H D S (-) N H D S (-) N H D S
H1
H2
C
NS
B
competitor
wt HBS
wt HAS
molar ratio
0 0 50 250 50 250
H1
H2
C
NS
SNAP
- + + + + +

Figure 10. A putative HIF-1 ancillary factor is not identical to HIF-1. Oligonucleotides for EMSA were wt HBS, which contains the wild-type HBS, but not the HAS; wt HBS+HAS, which contains both the wild-type HBS and the wild-type HAS; and wt HAS, which contains the wild-type HAS, but not the HBS. A-172 cells were exposed to either 0.5 mM SNAP or 0.1% DMSO for 3 h, or to either hypoxia or normoxia for 8 h. Nuclear extracts (5 μg per lane) from these cells were incubated with ^{32}P-labeled probes (3.0×10^4 cpm) at room temperature for 30 min, with or without competitors, prior to electrophoresis. DNA-protein complexes are indicated by arrows. (A) Comparison of DNA-binding activities recognized by wt HBS, wt HBS+HAS, and wt HAS. Hypoxia- or NO-induced complexes with slower (H1) and faster (H2) mobility, constitutively binding complexes to the HRE (C), and non-specific bands (NS) are indicated. (-): no serum, N: normoxia, H: hypoxia, D: DMSO, S: SNAP. (B) Competition assays with either normoxia or hypoxia-treated nuclear extracts using wt HBS as a labeled probe in the presence of 0-, 50-, 250-fold molar excess of competitors.

DISCUSSION

Nitric oxide and transcription factors activity

Since the discovery of its role in intercellular signaling, NO has been shown to be involved in an almost endless list of biological processes, including cell proliferation, differentiation, death, defense against pathogens, intercellular communications, cell motility and contraction. The variety of different responses evoked by this molecule in widely different cell types is to be ascribed to its intrinsic ability to interact with, and thereby regulate, multiple, independent pathways controlling cellular functions. How this relatively simple molecule controls these pathways, however, have been clarified only in part. This is particularly true for its effects on gene transcription, a process which not only drives complex tasks such as those required for cell proliferation, differentiation and death, but is also a prerequisite for stabilization and enforcement of the cellular and tissue responses to NO.

The best-characterized genetic control exerted by NO involves activation of the genes involved in the cellular resistance to oxidative stress in prokaryotes, governed by the SoxR and OxyR transcription factors (55,56). In these cases, covalent redox reactions including oxidation of the [2Fe-2S] clusters of SoxR (55) or nitrosylation of cysteine residues of OxyR (56) result in activation of these factors from a latent, reduced state to a transcriptionally active conformation capable of regulating the expression of sets of genes responsive to each of these transcriptional regulators. In lower eukaryotes, NO appears to be capable of inhibiting zinc finger-type yeast transcription activators, such as LAC9, by interacting with and thereby destroying their zinc-sulfur clusters (57), while in mammalian cells NO has been shown to regulate the activity of the NF-κB (58) and AP-1 (59) transcription factor complexes. In these cases, NO can exert a dual regulatory action on these factors, activating or inhibitory, depending upon the cellular conditions and via different mechanisms. For example, NF-κB is activated by NO in lymphoid cells (56,60), but it is instead inhibited in other cell types (61-64), and the same applies for AP-1 in other

cellular contexts (59,65,66). This is one of the characteristic features of many cellular responses to NO, and it is not unexpected, given the chemical nature of this molecule that, because of its instability, can easily react with different chemical groups in biological molecules, such as thiol groups, metals and reactive oxygen intermediates.

As a consequence, NO effects on a given biological system are dependent not only upon its absolute concentration but also, perhaps more important, on the redox status of the target cell and on the presence or availability of other reactive groups in biomolecules (67,68). Optimal concentrations of NO donors for VEGF reporter gene expression in normoxia cause an inhibitory effect on the gene activation by hypoxia (Fig. 1B). This may be attributed to higher concentrations of NO released from NO donors under hypoxia than under normoxia, as exposure to excessive amounts of NO could be toxic (69). This indicates that the final effect of NO on VEGF expression (activation or suppression) could also depend on the redox status of the cellular environment. A better understanding of these aspects of the biological activities of NO can be achieved only with a better knowledge of the cellular factors that are targets for regulation by this molecule.

Nitric oxide regulation of VEGF gene transcription and HIF-1 binding activity

In the present work, NO and hypoxia have been found to significantly induce the expression of a human VEGF reporter gene in glioblastoma and hepatoma cells. It has been previously shown that VEGF mRNA rapidly accumulates following exposure to NO donors in these cells, and that this is prevented by pre-treatment of the cells with the RNA polymerase inhibitor actinomycin D (15). These results suggest that NO activates the transcription of the endogenous VEGF gene as well as the transfected VEGF reporter gene.

The transcription factor HIF-1 plays a central role in hypoxic induction of the VEGF gene by binding to its target DNA sequence. NO-induced VEGF expression is also, at least in part, mediated by activation and subsequent binding of HIF-1. Therefore, NO and

hypoxia may share common features in the pathways of VEGF induction.

The promoter analysis of this gene reveals that deletion of the HRE completely abolishes VEGF induction by NO and hypoxia. A further analysis of the HRE shows that not only the HBS, but also its downstream HAS is essential for induction by these stimuli, and that the AP-1 site is required for its optimal response (Fig. 4). Similar cooperativity among several domains within the HRE was also reported in Epo and lactate dehydrogenase A (LDHA) genes. In the case of human Epo gene, the HBS, its adjacent sequence CACAG, and the binding site for hepatic nuclear factor 4 are crucial for the enhancer activity, and a mutation of either site abolished its hypoxic response (70,71). The promoter analysis of the human LDHA gene showed that a mutation in the HBS entirely abrogated the response to hypoxia, and a mutation in either its upstream ACGT or its downstream cyclic AMP response element, significantly, but not completely, reduced the promoter activity (72). These data indicate that multiple factors mediate transcriptional regulation of these genes through a complex interaction among these factors.

HIF-1α protein levels were massively upregulated in various cell lines under hypoxia, while HIF-1α mRNA levels were unchanged under the same conditions. Similarly, in the present work a dramatic increase in HIF-1α protein levels was seen in NO-treated A-172 cells (Fig. 9A and 9B), although we were unable to detect a significant change in HIF-1α mRNA. Thus, an increase in HIF-1α mRNA may not be the main mechanism for HIF-1α protein accumulation, but rather post-transcriptional or post-translational mechanisms (52,53) may be involved. HIF-1α is rapidly degraded under normoxia by the ubiquitin-proteasome pathway, while, under hypoxia, it is stabilized and immediately translocated into the nucleus where it dimerizes with ARNT (73-75). It has been recently shown that the chaperone heat shock protein 90 (Hsp90) interacts with HIF-1α under normoxia and is

essential for hypoxic activation of HIF-1 (76). Our results demonstrate that HIF-1α protein levels were elevated not only in the nucleus, but also in the whole cell (Fig. 9A and 9B). This finding suggests that accumulation of HIF-1α, as well as its translocation to the nucleus, may play a central role in NO- and hypoxia-induced activation of this transcription factor.

Although HIF-1 binding activity and HIF-1α accumulation are similarly induced by NO and hypoxic stimulation, EMSA showed that relative amounts of doublet inducible bands are different in extracts from NO- and hypoxia-treated cells. There are some possible mechanisms that could account for this difference. It may be attributable to the different status of phosphorylation. DNA binding of HIF-1 is regulated by protein phosphorylation (77), and the status of phosphorylation can affect the mobility of the target protein in polyacrylamide gels. It is also possible that different co-activators may be involved in HIF-1 activation by hypoxia when compared with that activated by NO.

A number of NO effects appear to be mediated by soluble GC, heme-containing proteins that react directly with NO and thereby induce an increase in intracellular cGMP levels (30,31). An involvement of cGMP in NO-induced activation of the endogenous VEGF promoter was suggested by results obtained with the GC inhibitors MB and LY83583 (15). However, as described here, another GC inhibitor (ODQ) did not attenuate NO-induced activation of the transfected VEGF promoter (Fig. 2). Moreover, 8-Br-cGMP did not mimic VEGF reporter gene induction by NO, even when used in conjunction with an NO donor and LY83583. Hypoxic induction of this reporter gene and HIF-1 binding activity were unaffected by either MB or LY83583 (Fig. 2). These results indicate that NO-induced activation of the gene promoter and of the HIF-1 factor, does not occur via cGMP-mediated signal transduction. They also show that NO and hypoxia act through distinct pathways, or via different molecular components of a single pathway, because of the different responses to MB and LY83583.

Common structures of HRE in hypoxia-inducible genes

Hypoxia induces a number of genes whose promoter or enhancer region contains one or more HBSs. They include aldolase A (ALDA), enolase 1 (ENO-1), glucose transporter 1 (GLUT-1), LDHA, phosphofructokinase L (PFKL), inducible nitric oxide synthase (iNOS), phosphoglycerate kinase 1 (PGK-1), heme oxygenase 1 (HO-1), Epo, transferrin (Tf) and VEGF. Most of them have T/GACGTG as a consensus sequence for the HBS, although HBSs in GLUT-1 (GGCGTG), ENO-1 (TGCGTG) and ET-1 (CACGTT) do not meet this consensus sequence perfectly (Table 1).

In this study, we have determined the exact extents of the HBS and the HAS in VEGF, and found that both elements form an imperfect inverted repeat. Moreover, the spacing of 8 nt, in VEGF, is crucial. Surprisingly, analysis of the HREs of the above genes revealed that the HREs in 7 out of 11 hypoxia-inducible genes form an imperfect inverted repeat, and that the spacing is 8 nt in 6 genes and 9 nt in the rest (Fig. 11). These similar structures of HRE indicate that a single HBS within the HRE is not enough to confer full induction of gene expression in most of hypoxia-inducible genes, and that some trans-activating factor(s) other than HIF-1 may bind to the HAS and collaborate with HIF-1 protein for hypoxic and NO-induction.

All of these HASs share a common sequence, CAC, except for the VEGF gene. CAC in the sense or GTG in the antisense strand is known as a recognition site of ARNT, as seen in the HBS (T/GACGTG). ARNT is the central dimerization partner for bHLH-PAS family transcription factors including HIF-1α, EPAS1 (endothelial PAS domain protein 1), AhR, and Sim (78). It is possible that ARNT dimerizes with a member of PAS family and binds to its target HAS. Taken together, these results suggest that a common mechanism, other than an HIF-1-mediated pathway, may exist for NO- and hypoxia-induced expression of the hypoxia-inducible genes.

Genes	HRE Sequences	Spacing (nt)	HAS Sequences
VEGF	5'-CATACGTGGGCTCCAACAGGTCCT-3'	8	CAGGT
EPO	5'-CCTACGTGCTGTCTCACACAGCCT-3'	8	CACAG
ALDA	5'-GGGATGTGGTCCGAGTCACGTCCG-3'	8	CACAT
ENO-1	5'-CGCACGTGGCCCCGGACACGCAGC-3'	8	CACGC
LDHA	5'-CACACGTGGGTTCCCGCACGTCCG-3'	8	CACGT
GLUT-1	5'-CAGGCGTGCCGTCTGACACGCATC-3'	8	CACGC
HO-1	5'-CGGACGTGCTGGCGTGGCACGTCCT-3'	9	CACGT

Figure 11. The HREs of several hypoxia-inducible genes contain a common structure consisting of the HBS and the HAS. The sequences that contain the HBS and the HAS of several hypoxia-inducible genes are shown. Note that these two motifs are usually spaced by 8 nt, and all HASs, except for VEGF, contain CACGT/C or CACAG/T. The closed and open arrow indicates the HBS and the HAS, respectively. The arrowhead arbitrarily indicates the orientation of the half-site. Underlined nucleotides match the corresponding nucleotides in the remaining half-site as an inverted repeat.

Effect of NO on VEGF expression and angiogenesis

The role of NO in angiogenesis is controversial. Nitric oxide donors inhibit angiogenesis in the chick chorioallantoic membrane, tube formation in the matrigel tube formation assay (79), and the growth and metastatic properties of the Lewis lung tumor in mice (80). Nitric oxide donors inhibited VEGF expression in the arterial wall in response to balloon angioplasty (12), and in rat lungs during acute and chronic hypoxia (14). In contrast, there are some observations that NO enhances the expression of angiogenic activity. Nitric oxide synthase activity correlates positively with tumor growth and vascular density (36-38). Human colon tumor cell lines transfected with a NOS-encoding gene grew faster and were more vascularized than the parent cell lines *in vivo* (81). Nitric oxide produced in vascular endothelium has also been suggested as a downstream mediator for VEGF receptors in angiogenesis (82). Exogenous NO and endogenous NO, elicited by substance P, enhanced angiogenesis *in vivo*. Nitric oxide also enhanced the proliferation and migration of endothelial cells *in vitro* (83). Moreover, promoting endothelial NOS activity accelerated *in vivo* angiogenesis (84).

There are some recent reports showing an inhibitory effect of NO on VEGF expression (33-35). Sogawa *et al.* (33) and Huang *et al.* (35) demonstrated that SNP suppresses hypoxia-induced VEGF gene activation and HIF-1 binding activity. As shown in this work, SNP inhibits the hypoxic induction of the VEGF gene in a dose-dependent manner in glioblastoma and hepatoma cell lines, in contrast to the effects of SNAP and NOC5. This contradiction is clearly due to the specific nature of SNP. SNAP and NOC5 are chemically distinct compounds that generate NO radicals spontaneously. In contrast, after donating NO, SNP disintegrates into ferrocyanide, ferricyanide, iron ions, and cyanide, each of which has a variety of biological effects (85). There is no definite explanation for the cause of the inhibitory effect of SNP, as ferrocyanide and ferricyanide at concentrations up to 100 μM made no change on VEGF promoter activity in A-172 cells (our unpublished data). Therefore, SNP is far from an ideal NO donor.

Sogawa *et al.* (33) also used S-nitrosoglutathione (GSNO) and 3-morpholinosydnonimine (SIN-1) only under hypoxic conditions. We also examined the effects of these compounds, and found that they showed remarkable induction of the VEGF reporter gene under normoxic conditions in both A-172 and Hep3B cells (our unpublished data). These results suggest that SNP has a distinct effect on the promoter activity, when compared with other NO donors, and that its inhibitory effect may not simply be attributable to NO itself. In another recent report (34), the cell lines used were not tumor cells but were vascular endothelial and smooth muscle cells. SNAP downregulated VEGF expression by inhibiting PKC-induced AP-1 binding activity in smooth muscle cells (12). Nitric oxide inhibits proliferation and migration of endothelial cells (86,87). These contradictory data indicate that NO has both inhibitory and activating effects on angiogenesis, depending upon the cellular environment and the types of cells in which assays are performed. Nitric oxide chemistry is highly redox-sensitive. This may also explain the contradictory effects of NO on HIF-1 activation in different cell systems, and why MB and LY83583, but not ODQ, are inhibitory, as the former two compounds are known to generate superoxide anions (88,89).

Conclusions and implications

Our results indicates that NO and hypoxia commonly activates the VEGF gene transcription by enhancing HIF-1 activity, while in part NO mediates gene transcription by a mechanism distinct from hypoxia (Fig. 12). This is demonstrated by a difference in sensitivity to GC inhibitors and a different pattern of HIF-1 binding. Our findings imply a direct involvement of NO in the control of angiogenesis through its regulation of VEGF expression, where HIF-1α activity appears to be essential (90). Moreover, the identification of HIF-1 as an additional molecular target of NO opens a new way for the molecular characterization of the effects of this intercellular mediator on gene transcription. Furthermore, these findings also suggest a role of NO and its redox derivatives in tissue

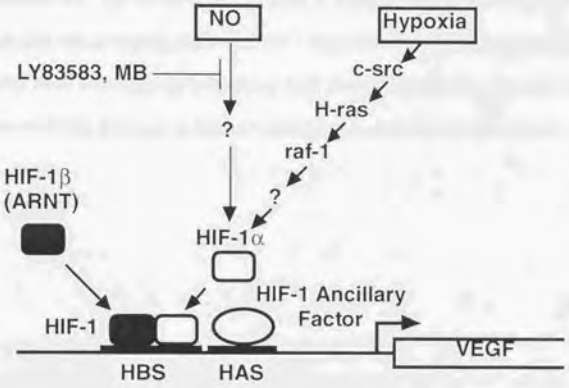

Figure 12. Hypoxic and NO pathways of VEGF gene induction. VEGF gene induction by NO and hypoxia is commonly mediated by the HIF-1 complex. A putative HIF-1 ancillary factor may bind to a novel cis-element HAS as a transactivator of the VEGF gene.

reactions to hypoxia. Indeed, given the importance of HIF-1 in the genomic responses of hypoxic cells, these results establish a direct link between NO and the adaptation of normal and neoplastic cells and tissues to low oxygen tension. This helps explain why NO donors can exert such diverse beneficial therapeutic actions, for example, in cardiovascular diseases (91), in ischemic brain injury (92) and following surgically related ischemic-reperfusion injuries(93). On the other hand, there is a strong positive correlation between NO production and tumor angiogenesis (36-39). These last observations suggest that there may be possible risks of long-term treatments with pharmacological agents that potentiate NO in patients suffering from, or at risk of cancer, where enhanced angiogenesis would be hazardous.

MATERIALS AND METHODS

Transient expression assays

The sequence of phVEGF1 (kindly provided by Dr. A. Minchenko (19)) contains the promoter and 5'-flanking sequence of human VEGF gene between positions -2,279 and +54, cloned into the pGL2-basic vector (Promega, Madison, WI). A series of deletion mutants was prepared by restriction endonuclease digestion and re-ligation. The sequence pT81luc0 (L. Cicatiello and A. Weisz: unpublished data), modified from pT81luc (kindly provided by Dr. S.K. Nordeen (94)) contains the herpes simplex virus thymidine kinase gene (HSV-TK) promoter, upstream of the luciferase coding sequence. The pHREL, pHRE, and related mutants of pHRE were prepared by amplifying a specific segment of the 5'-flanking region of the human VEGF gene with polymerase chain reaction (PCR) and cloning it in a single copy, upstream of the HSV-TK promoter of pT81luc0. The pHRA and pHRB sequences were prepared by ligating commercially synthesized oligonucleotides to pT81luc0. The pSV-nlslacZ sequence (SV40-driven promoter) was used as a control for monitoring transfection efficiency, and contains lacZ coding sequences. Constructs (5 μg of the reporter plasmid and 1 μg of pSV-nlslacZ) were transfected into human glioblastoma A-172 or hepatoma Hep3B cells (from the Japanese Collection of Research Bioresources, Tokyo, Japan) at 20-30% confluence in a 10-cm tissue culture plate, with 20 μl of lipofectin (GIBCO-BRL, Rockville, MD).

After incubation at 37°C for 15 h, the DNA-containing medium was replaced with normal culture medium. The cells were then incubated at 37°C before harvesting under normoxic conditions (21% O_2, 5% CO_2, balance N_2), or following exposure to hypoxic conditions (1% O_2, 5% CO_2, balance N_2), or to the NO donors, S-nitroso-N-acetyl-D, L-penicillamine (SNAP), 3-(hydroxy-1-(1-methylethyl)-2-nitrosohydrazino)-1-propanamine (NOC5) or sodium nitroprusside (SNP). SNAP was dissolved in dimethyl sulfoxide (DMSO), and NOC5 and SNP were dissolved in phosphate-buffered saline (PBS) immediately before use. An aliquot of SNAP was added 12 h before harvest. However,

because the half-life of NOC5 (25 min) is much shorter than that of SNAP (8 h), the first half dose of NOC5 was added at 12 h, and the remaining dose was added 6 h before cell harvest in A-172 cells. Harvested cells were dissolved in 200 µl of 0.25 M Tris-Cl, pH 7.5. Cell lysis was performed by four freeze-thaw cycles.

Luciferase activity was determined by mixing 100 µl of cell extract with 225 µl of luciferin reagent containing 115 µM D-luciferin (Sigma, St. Louis, MO), 11.5 mM glycylglycine, 6.9 mM KPO_4, 6.9 mM $MgSO_4$, 1.9 mM EGTA, 0.9 mM ATP and 0.5 mM dithiotreitol (DTT). Luminescence was measured for 20 s in a luminometer (Luminescencer-JNR; ATTO, Tokyo, Japan), and results were expressed as relative light units. We measured β-galactosidase activity by using 50 µl of cell extract and 690 µl of mixture of 23.6 mM Tris-Cl, pH 7.5, 1.4 mM $MgCl_2$, 54 mM $NaPO_4$, pH 7.5, 1.4% β-mercaptoethanol and 1.1 mg/ml *o*-nitrophenyl-β-D-galactopyranoside (Sigma), incubated at 37°C for 0.5-1 h. The A_{420} was determined after stopping the reaction by addition of 1 M Na_2CO_3. The relative luc activity (mean ± standard error of the mean) was defined as luciferase activity standardized by β-galactosidase activity. Fold induction was defined as the ratio of the relative luc activity of stimulated cells to that of unstimulated controls.

Griess reaction was performed by using NO_2/NO_3 Assay Kit-C (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

Preparation of nuclear and whole-cell extracts

Cells at 60-70% confluence were incubated under normoxic (21% O_2) or hypoxic (1% O_2) conditions (8 h for A-172 and 12 h for Hep3B cells), or with DMSO (0.1%), or with SNAP (0.5 mM in 0.1% DMSO) under normoxic conditions (3 h for A-172 and 8 h for Hep3B cells) at 37°C before harvest. The cells were scraped free and centrifuged at 270G

for 10 min at 4°C. Nuclear extracts were prepared with buffers A and C as described previously (95), except that dialysis procedures were omitted. The pellet was re-suspended in buffer A, and incubated on ice for 10 min before being homogenized by pipetting 5-8 times with a syringe. The nuclei were pelleted by centrifugation at 12,000G for 2 min at 4°C. They were then re-suspended in ice-cold buffer C, and mixed by rotation for 30 min at 4°C. After centrifugation at 16,000G for 10 min at 4°C, the supernatant was stored at -70°C pending EMSA and Western blot analysis. Whole-cell extracts were prepared as described previously (52). In brief, the cells were harvested in TEN buffer (40 mM Tris-Cl, pH 7.9/10 mM EDTA, pH 8.0/150 mM NaCl). The cell pellet was resuspended in whole-cell extract buffer (10 mM Hepes, pH 7.9/400 mM NaCl/0.1 mM EDTA/5% (vol/vol) glycerol/1 mM DTT/1 mM phenylmethylsulfonyl fluoride). It was then centrifuged at 16,000G for 30 min at 4°C. The supernatant was stored at -70°C until required. Protein concentration was determined by the Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA).

EMSA

Nuclear extracts (5 μg) from the control or stimulated cells were incubated with 3 x 10^4 cpm of a ^{32}P-labeled double-stranded oligonucleotide probe and 0.1 μg of denatured calf thymus DNA, in modified buffer Z+ (58.5 mM KCl), for 30 min at room temperature, as described previously (23). Electrophoresis was performed on 5 % non-denaturing polyacrylamide gels at 25 mA in 1x TAE at 4°C. Autoradiography of gels was performed with a Bioimage Analyzer BAS 2000 (Fuji Photo Film Co., Tokyo, Japan). Competition experiments were performed with 10-fold to 250-fold molar excess of unlabeled oligonucleotides, relative to the labeled probe. For supershift assays, 1 μl each of antiserum specific for HIF-1α (kindly provided by Dr. D.M. Livingston(54)), or HIF-1β (ARNT) (Affinity Bioreagents Inc., Golden, CO), or c-Myc (Calbiochem, La Jolla, CA) were added to the binding reaction mixture without the labeled probe. These mixtures were

incubated for 30 min at 4°C. The labeled probe was then added and incubation continued for 30 min at room temperature.

Western blot analysis

For Western blots, anti-HIF-1α monoclonal antibody (Novus Biologicals Inc., Littleton, CO) was used according to the manufacturer's protocol. In brief, 30 μg of the nuclear or whole-cell extracts per lane were resolved using SDS/6% polyacrylamide gels. The proteins were then transferred onto nitrocellulose membranes by electrophoresis in the blotting buffer (5% (vol/vol) methanol/25 mM Tris/120 mM glycine). Membranes were blocked with 5% nonfat dried milk/2% bovine serum albumin/TBS-T (50 mM Tirs-Cl, pH 7.5/150 mM NaCl/0.1% Tween-20). Endogenous HIF-1α protein was probed with 1:1000 dilution of anti-HIF-1α monoclonal antibody. Horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as a secondary antibody at a dilution of 1 in 5000 in nonfat dried milk/TBS-T. The protein complexes were visualized by enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Comparison of two means was performed by the use of unpaired Student's *t* tests. Statistical significance was assumed at a value of $p<0.05$.

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ABBREVIATIONS

VEGF, vascular endothelial growth factor; NO, nitric oxide; HRE, hypoxia-response element; HIF-1, hypoxia-inducible factor 1; bHLH-PAS, basic helix-loop-helix-per-arnt-sim; ARNT, aryl hydrocarbon receptor nuclear translocator; HBS, HIF-1 binding site; GC/cGMP, guanylate cyclase/3', 5'-cyclic guanosine monophosphate; NOS, nitric oxide synthase; Epo, erythropoietin; SNAP, S-nitroso-N-acetyl-D, L-penicillamine; NOC5, 3-(2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino)-1-propanamine; MB, methylene blue; LY83583, 6-anilino-5,8-quinolinequinone; ODQ, 1H-[1,2,4]oxidiazolo[4,3-a]quinoxalin-1-one; SNP, sodium nitroprusside; HSV-TK, herpes simplex virus thymidine kinase; HAS, HIF-1 ancillary sequence; nt, nucleotide; HAF, HIF-1 ancillary factor; EMSA, electrophoretic mobility shift assay; LDHA, lactate dehydrogenase A; ALDA, aldolase A; ENO-1, enolase 1; GLUT-1, glucose transporter 1; PFKL, phosphofructokinase L; iNOS, inducible nitric oxide synthase; PGK-1, phosphoglycerate kinase 1; HO-1, heme oxygenase 1; Tf, transferrin; ET-1, endothelin 1; TH, tyrosine hydroxylase; GSNO, S-nitrosoglutathione; SIN-1, 3-morpholinosydnonimine; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; DTT, dithiotreitol; SEM, standard error of the mean

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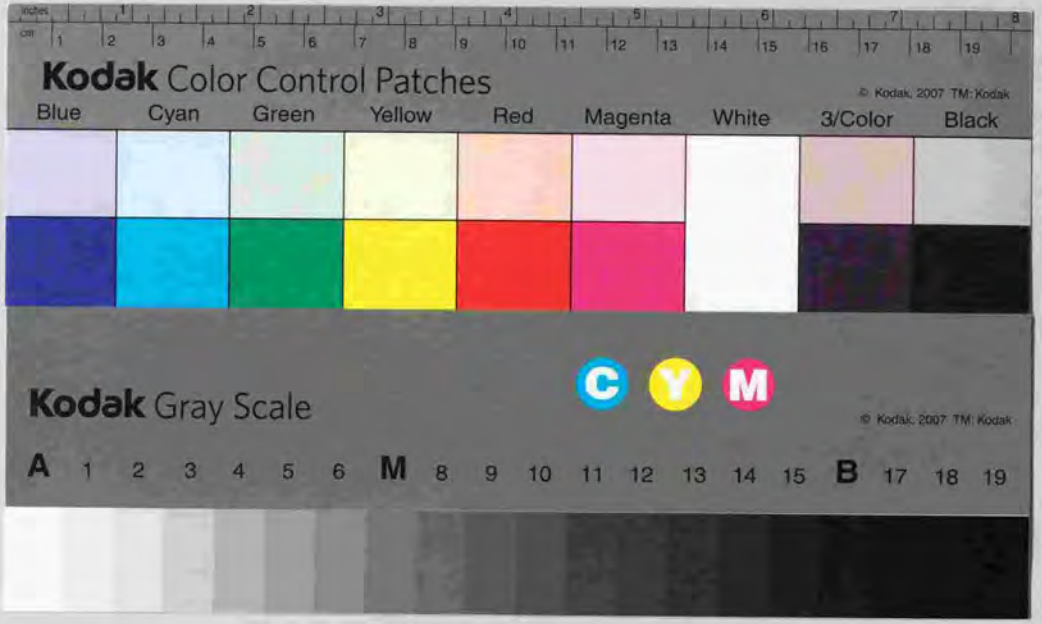
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