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iNOS Expression Inhibits Hypoxia-Inducible Factor-1 Activity

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Hypoxia-inducible factor-1 (HIF-1) activates genes important in vascular function such as vascular endothelial growth factor (VEGF), erythropoietin (EPO), and inducible nitric oxide synthase (iNOS). iNOS catalyzes the synthesis of nitric oxide (NO), a free radical gas that mediates a number of cellular processes, including regulation of gene expression, vasodilatation, and neurotransmission. Here we demonstrate that iNOS expression inhibits HIF-1 activity under hypoxia in C6 glioma cells transfected with an iNOS gene and a VEGF promoter-driven luciferase gene. HIF-1 induction of VEGF-luciferase activity in C6 cell is also inhibited by sodium nitroprusside (SNP). Furthermore, pretreatment of C6 cells with *N*-acetyl-L-cysteine (NAC), an antioxidant, nullified the inhibitory effect of iNOS on HIF-1 binding. These results demonstrate that NO generated by iNOS expression inhibits HIF-1 activity in hypoxic C6 cells and suggest a negative feedback loop in the HIF-1 → iNOS cascade. © 2000 Academic Press

Key Words: **HIF-1; hypoxia; glioma; iNOS; luciferase; NAC; NO; SNP.**

HIF-1, a heterodimeric protein complex consisting of alpha (HIF-1α) and aryl hydrocarbon receptor nuclear translocator (ARNT, or HIF-1β) subunits, is a key regulator of mammalian oxygen homeostasis. The expression of this basic helix-loop-helix transcription factor is increased in most cells in response to low oxygen tension (1, 2). Under hypoxic conditions, HIF-1 mediates the activation of several genes including EPO, VEGF, and iNOS (3). HIF-1α expression is tightly regulated by the cellular oxygen tension (4, 5), whereas the expression of the ARNT subunit is oxygen-independent. Hypoxic conditions prevent ubiquitination and subsequent proteasomal degradation of HIF-1α in cells.

HIF-1 appears to play a key role in the pathophysiology of diseases including cancer. Because hypoxia promotes tumor growth, invasion, and metastasis, the oxygen content in a tumor is a clinically important indicator of its malignancy (6). A growing body of evidence has shown a pivotal role for HIF-1 in adaptive responses of tumors to hypoxia (3, 7). Understanding the regulatory mechanisms of HIF-1 may broaden our insight into the development of better therapeutic strategies for cancers.

HIF-1 is essential for iNOS induction under hypoxic conditions (8, 9). Large quantities of NO may be derived from the expression of iNOS following cytokine exposure (10). NO exhibits tumoricidal activity both *in vitro* (11) and *in vivo* (12, 13). However, NO may also alter vascular reactivity or promote neovascularization in favor of tumor growth (14). Interaction of NO and HIF-1 has not been fully delineated. Specifically, no negative feedback loop on the HIF-1 → iNOS cascade has been clearly demonstrated previously. NO donors can inhibit (15, 16) or activate (17) HIF-1 activity. The conflicting roles of NO donors on HIF-1 activity may be related to their compounding pharmacological actions other than being a NO source. iNOS is expressed in glioma.

Lipopolysaccharide (LPS), a bacterial endotoxin, in combination with interferon-γ (IFN-γ) or tumor necrosis factor-α induces iNOS expression in rat C6 glioma cells at the mRNA and protein levels (18). Using C6 glioma cells as a model, we apply a direct genetic approach to demonstrate that induction of iNOS expression is accompanied by an inhibition of HIF-1 DNA binding activity, suggesting the existence of a negative feedback loop in the HIF → iNOS cascade.

Abbreviations used: ARNT, aryl hydrocarbon receptor nuclear translocator; EMSA, electrophoretic mobility shift assay; EPO, erythropoietin; GSH, reduced form of glutathione; GSNO, *S*-nitrosoglutathione; GSSG, oxidized form of glutathione; HIF-1, hypoxia inducible factor; IFN-γ, interferon-γ; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor.

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MATERIALS AND METHODS

Materials and experimental protocols. Rat C6 glioma cells were purchased from American Type Culture Collection (Rockland, MD) and cultured according to ATCC instructions. SNP and NAC were purchased from Sigma (St. Louis, MO). After C6 cells were cotransfection with VEGF-Luc and pCMV-RL (see below), SNP was added to the medium for 4 h before the cells were incubated at 37°C under humidified normoxia (95% air and 5% CO_2) or hypoxia (1% O_2, 94% N_2, and 5% CO_2) for an additional 16 h. In the NAC experiments, transfected cells were treated with NAC for 2 h followed by hypoxia for 16 h before the luciferase assay.

Transient transfection. The NS05 plasmid, a mouse iNOS gene in the pcDNA1.1 expression vector (Oxford Biomedical Research, Oxford, MI) was introduced into C6 cells by transient transfection. The pcDNA1.1 parent vector was used as control. For transient transfection, 700 μl of growth medium containing 2 μg plasmid DNA and 4 μl SuperFect (QIAGEN Inc., Valencia, CA; DNA to SuperFect ratio, 1:2) was applied to C6 cells for 3 h at 37°C in 5% CO_2 according to the manufacturer's protocol. C6 cells were used for experiments 24 h after iNOS gene transfection. Using pTracer-SV40, with green fluorescent protein under fluorescence microscopy as an indicator, C6 cells had 30–40% transfection efficiency (data not shown). Nitrite accumulation in the culture medium was measured to monitor NS05 transfection and resultant iNOS overexpression (see nitrite assay below).

Luciferase reporter assay. A CMV promoter-driven *Renilla* luciferase DNA plasmid (pCMV-RL, 0.5 μg) was cotransfected with an equal amount of VEGF-Luc (a generous gift from Dr. Annette Damert, Max Planck Institute) to normalize variations in transfection efficiency. Briefly, 36–40 h posttransfection, the cells were washed with phosphate buffered saline (pH 7.4) and lysed with 0.5 ml of the passive lysis buffer provided by Promega. After centrifugation at 20,000*g* for 30 s, the supernatant was stored at −70°C until analysis. Twenty microliters of each sample was mixed with 100 μl of the assay buffer. The VEGF-luciferase activity was measured as light output (15 s) in a TD 20/20 luminometer (Turner Design, Sunnyvale, CA). Subsequently, the *Renilla* luciferase activity, as a standard, was determined after adding 100 μl of the Stop and Glo reagent and the light output (15 s) was measured separately.

Western blotting. Western blots for the HIF-1α protein were performed as described previously (19) using a primary polyclonal rabbit anti-HIF-1α antibody (1:600, Novus Biologicals, Littleton, CO) followed by a secondary alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (1:6000, Amersham Life Science Inc., Arlington Heights, IL).

Nitrite assay. The amount of NO formed was estimated by measuring nitrite levels in the culture medium. One hundred μl of cell-free supernatant was mixed with 100 μl Griess reagent, consisting of equal volumes of 1.32% sulfanilamide in 60% acetic acid and 0.1% *N*-1-naphthylethylenediamine–HCl (20). The samples were incubated for 10 min at room temperature and then the optical density was read at 540 nm wavelength. The standard curve was constructed using known concentrations of sodium nitrite.

Electrophoretic mobility shift assay (EMSA). Nuclear protein extracts from C6 cells incubated under normoxia or hypoxia were prepared as previously described (2, 21). An [γ-^{32}P]ATP-labeled oligonucleotide probe (5′-agcttGCCCTACGTGCTGTCTCAg-3′ and 5′-aattcTGAGACAGCACGTAGGGCa-3′) corresponding to the hypoxia-response element in the EPO gene was used.

Statistical analysis. Results are expressed as means ± SEM from triplicate samples. Each experimental paradigm was repeated at least on 3 separate occasions to confirm reproducibility of the findings. Statistical analysis was performed using Student's unpaired *t* test between two experimental groups. A *P* value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

HIF-1 Activity in Hypoxic C6 Cells

C6 and Hep3B cell lines were treated with $CoCl_2$ (0.1 mM for 12 h) to determine whether HIF-1 expression could be induced in these cells; $CoCl_2$ has been used previously to induce HIF-1 in Hep3B cells (19, 22). Western blotting demonstrated a significant increase in signal intensity of a 120-kDa band suggestive of HIF-1α accumulation in $CoCl_2$-treated C6 cells (Fig. 1A). Similar results were noted in $CoCl_2$-treated Hep3B cells (data not shown). EMSA further demonstrated that in $CoCl_2$-treated C6 cells an increase in DNA binding activity (data not shown) in accordance with HIF-1 expression.

In C6 cells, HIF-1 binding to its cognate DNA sequence increased after 5 and 16 h of hypoxia (Fig. 1B). The observed HIF-1 binding was specific because it could be inhibited effectively with excess unlabeled probe (lane 5, Fig. 1B). HIF-1 binding and subsequent transactivation of the downstream VEGF gene have been reported previously (23). In addition to the Western blot and EMSA analyses, a VEGF-Luc reporter plasmid was introduced into C6 cells to demonstrate that hypoxia-induced HIF-1 is able to transactivate the VEGF promoter. In VEGF-Luc transfectants, luciferase activity was increased 2- to 3-fold under hypoxia compared to normoxia (Fig. 1C). Collectively, these results (Fig. 1) demonstrate that in C6 cells under hypoxia HIF-1α protein accumulated with a concomitant increase in HIF-1 binding activity and transactivation of the VEGF promoter.

Increase in Cellular NO Contents by Overexpression of iNOS Gene

Substantially higher concentrations of cellular NO, as reflected by increased nitrite levels in the culture medium, were observed in C6 cells transfected with a murine iNOS expression plasmid, NS05, compared to those transfected with the pcDNA1.1 vector control (NS05: 7.30 ± 0.28 μM vs pcDNA1.1: 2.48 ± 0.14 μM, $P < 0.0001$). The iNOS expression levels in the NS05-transfected C6 cells were comparable to that induced in LPS and IFN-γ-treated C6 cells (data not shown). iNOS expression did not result from the transfection procedure because similar nitrite concentrations (2 to 3 μM) were detected in culture media from the pcDNA1.1-transfected and the nontransfected controls. Western blot analysis was used to further demonstrate iNOS expression in NS05-transfected C6 cells. A single immunoreactive 130-kDa band corresponding to iNOS protein was detected only in samples from NS05-transfectants but not pcDNA1.1-transfected controls (data not shown). Therefore, these results indicate that NS05-transfected C6 cells expressed functional iNOS protein.

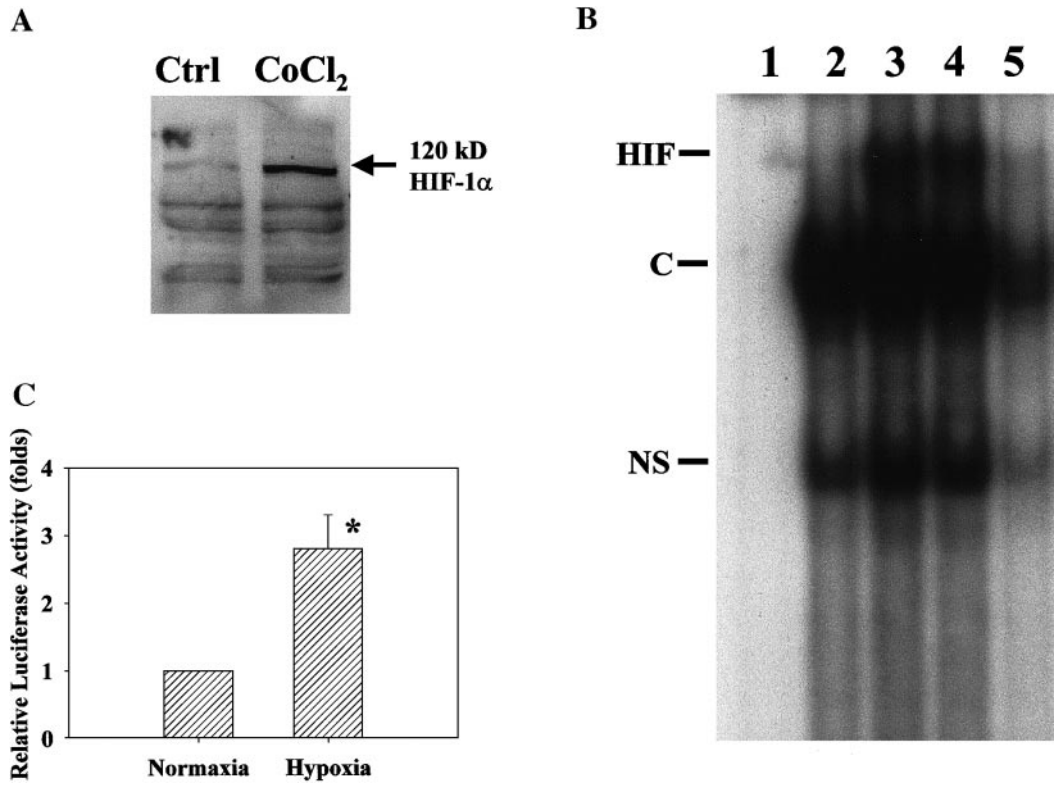

FIG. 1. Induction of HIF-1α activity under hypoxia or $CoCl_2$ treatment in C6 cells. (A) Western blot showing the induction of the 120-kDa HIF-1α protein in nuclear extracts from C6 cells treated with 0.1 mM $CoCl_2$ for 12 h (arrow; lanes 1, 2). (B) EMSA showing HIF-1 binding activity under hypoxia. Lane 1, probe alone; lane 2, normoxia for 5 h; lane 3, hypoxia for 5 h; lane 4, hypoxia for 16 h; lane 5, hypoxia for 5 h in the presence of excess unlabeled probe. C, constitutive binding; NS, nonspecific binding. (C) Luciferase reporter gene activity in C6 cells under hypoxia. Cells cotransfected with 0.5 μg each of VEGF-Luc and pCMV-RL plasmids were incubated under hypoxia or normoxia for an additional 16 h before luciferase assay. Data are representative of 3 separate experiments with similar results. $^*P < 0.05$.

Inhibition of HIF-1 Activity by iNOS under Hypoxia

To determine that NO could suppress HIF-1-mediated transactivation of VEGF promoter in C6 cells, a chemical NO donor SNP was used. SNP increased cellular nitrite content as well as inhibited the luciferase activity induced by HIF-1 under hypoxia in a dose-dependent manner (Fig. 2A). To examine the effect of iNOS on HIF-1 binding under hypoxia, C6 cells were transiently cotransfected with VEGF-Luc, pCMV-RL, and NS05. Because LPS and IFN-γ-treated C6 cells produced iNOS as well as other cellular factors that may alter HIF-1 activity, the direct transfection of the NS05 plasmid was considered to be more specific. HIF-1-mediated luciferase activity was reduced to less than half in NS05-transfected cells compared to the pcDNA1.1 transfectants grown under hypoxic conditions (Fig. 2B). Thus, results from molecular biological and chemical approaches demonstrate that NO inhibited HIF-1 activation in the C6 cells cultured under hypoxia.

NO donors have been shown to inhibit (15, 16) or increase (17) HIF-1α activation. Although such discrepancies may be due to the use of different experimental systems including different cell types and variable hypoxic/normoxic conditions, one major concern is that NO donors are chemically distinct in their mechanisms in increasing NO formation. Following the release of NO, SNP decomposes to ferrocyanide, ferricyanide, iron ions, and cyanide, each of which may have biological effects on HIF-1 binding activity (24). Other chemical NO donors can cause changes in cellular redox after NO release; for example, *S*-nitrosoglutathione (GSNO) decomposes to NO and the oxidized glutathione (GSSG) (25). A high concentration of GSSG alone is a prominent source of oxidative stress. Another commonly used NO donor, SIN-1, generates peroxynitrite (26), a strong oxidant that can damage cellular enzymes, membranes, and subcellular organelles through the nitration of tyrosine residues on proteins. Finally, any chemical NO donor applied at a sufficiently high concentration may prove cytotoxic.

In this study, a molecular biological approach was used to overexpress iNOS so that a more specific role of NO on HIF-1 transactivation of a downstream gene could be demonstrated. Direct iNOS expression *in situ* avoids the compounding effects of NO donors as well as the unpredictable actions of their side products on

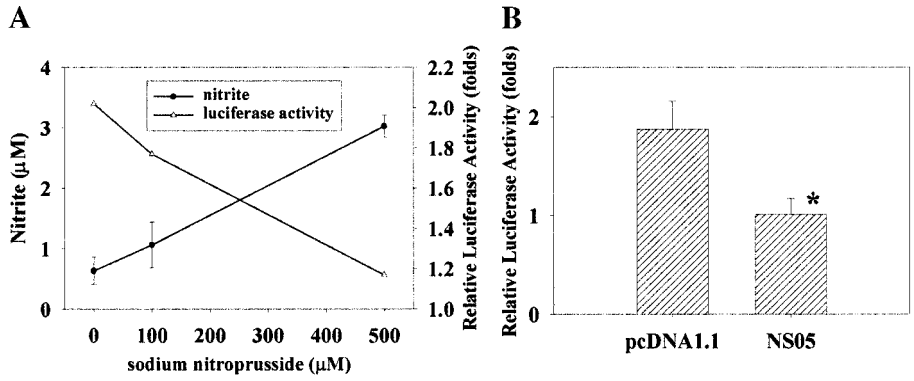

FIG. 2. Inhibition of HIF-1 activity under hypoxia by NO. (A) SNP effect on VEGF-luciferase activity activated by HIF-1. Cells were cotransfected with VEGF-Luc and pCMV-RL plasmids and treated with the indicated concentrations of SNP under hypoxia for 16 h. The nitrite concentration (●) as represented on the left ordinate in the culture medium was determined by the Griess reaction. The cells were lysed to measure the luciferase activity (△) as represented on the right ordinate. (B) Effect of iNOS overexpression on luciferase activity activated by HIF-1. Cells were cotransfected with VEGF-Luc, pCMV-RL, and the construct overexpressing iNOS (NS05) or the parent vector (pcDNA1.1). Following transfection, cells were incubated under hypoxia for additional 16 h before the luciferase assay. Data shown in (A) and (B) are representatives of 3 separate experiments with similar results. $*P < 0.05$.

HIF-1 binding activity. Thus, results based iNOS expression provides the direct evidence to support the argument that excessive formation of NO inhibits HIF-1-mediated transcription of a downstream gene.

The molecular mechanism of NO-mediated inhibition of HIF-1 binding remains unknown, but several scenarios are plausible. NO stimulates soluble guanylyl cyclase (27) to generate cyclic GMP (cGMP), which in turn activates cGMP-dependent protein kinases. NO may also bind to ferric heme oxygen and superoxide (28), possibly affecting the signal pathway for HIF-1 activation. NO reacting with superoxide anion resulting in peroxynitrite formation could in turn generate nitrosylated tyrosine residues in proteins and interrupt signal transduction pathways mediated by tyrosine phosphorylation. NO has also been shown to directly nitrosylate the sulfhydryl groups of cysteine residues on numerous proteins thereby inhibiting their enzymatic activity (29). Further experimental investigation is required to delineate which of these mechanisms are involved in NO inhibition of HIF-1 DNA binding.

NAC Effect on Induction of HIF-1α Activity

Because the cellular redox state dictates HIF-1α binding activity (30, 31) and iNOS expression with resultant NO production is expected to enhance oxidative stress, the effect of an antioxidant on iNOS inhibition of HIF-1α activation under hypoxia was explored. C6 cells were pretreated with NAC at different concentrations (1, 5, 10 mM) for 2 h followed by incubation under hypoxia for 16 h. NAC, at 5 and 10 mM, partially reversed the inhibition of luciferase activity induced by iNOS overexpression in C6 cells. At least two mechanisms may account for the NAC effect (Fig. 3). It has been reported in aortic endothelial cells that hypoxia decreases GSH/GSSG ratio while enhancing NO synthesis, leading to nitrosothiol formation (32). Hypoxic conditions coupled with an exogenous glutathione precursor (NAC) may increase total intracellular glutathione concentrations with a lowered GSH/GSSG ratio to favor the formation of GSNO, thus preventing the direct interaction of NO with HIF-1α. Alternatively, GSH/GSSG may stabilize the HIF-1 protein to enhance its DNA binding ability. NAC, a glutathione precursor and free radical scavenger, has been shown to stabilize the HIF-1α protein under hypoxia, thereby facilitating the HIF-1 DNA-binding activity by

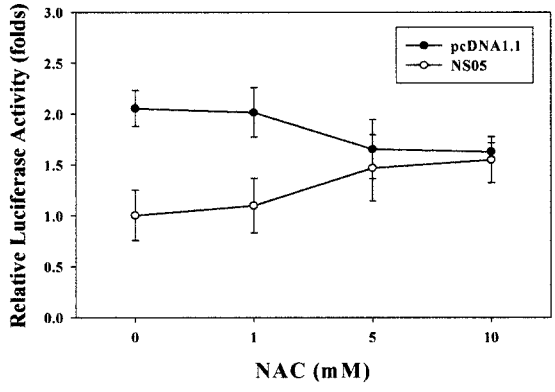

FIG. 3. NAC effect on the inhibition of HIF-1 activity by iNOS overexpression. C6 cells were transfected with NS05, VEGF-Luc, and pCMV-RL. Transfectants (○) or control cells (●) were then treated with NAC at indicated concentrations for 2 h followed by hypoxia for 16 h before the luciferase assay. Relative luciferase activity was assessed versus the activity obtained in C6 cells transfected with pcDNA1.1, VEGF-Luc, and pCMV-RL without NAC treatment. Results shown are representative of 3 separate experiments with similar findings. $*P < 0.05$ compared to NS05 transfectants without NAC treatment. Note that NAC at 5 or 10 mM significantly counteracts the iNOS-dependent inhibitory effect on HIF-1-mediated activation of VEGF-Luc reporter gene.

an increased GSH/GSSG ratio in the fetal alveolar epithelial cells (33). At present time, the exact molecular mechanism by which NAC reverses NO inhibition of HIF-1 DNA binding remains to be defined.

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