Nitric Oxide Induces Hypoxia-inducible Factor 1 Activation That Is Dependent on MAPK and Phosphatidylinositol 3-Kinase Signaling*

Kenji Kasuno‡§, Satoshi Takabuchi§, Kazuhiko Fukuda‡, Shinae Kizaka-Kondoh**, Junji Yodoi‡§, Takehiko Adachi‡, Gregg L. Semenza‡‡, and Kiichi Hirota§§

From the ‡Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, IDECA, Osaka, Japan 563-0053, the §Department of Anesthesia, Tazuke Kofukai Medical Research Institute, Kitano Hospital, 2-4-20, Ōgimachi, Kita-ku, Osaka 530-8480, the ¶Institute for Virus Research and ‡Department of Anesthesia, Kyoto University Hospital, Kyoto 606-8507, Japan, the **Department of Molecular Oncology, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8507, Japan, and the §§McKusick-Nathans Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287

Hypoxia-inducible factor-1 (HIF-1) is a master regulator of cellular adaptive responses to hypoxia. Levels of the HIF-1α subunit increase under hypoxic conditions. Exposure of cells to certain nitric oxide (NO) donors also induces HIF-1α expression under nonhypoxic conditions. We demonstrate that exposure of cells to the NO donor NOC18 or S-nitrosoglutathione induces HIF-1α expression and transcriptional activity. In contrast, hypoxia, NOC18 did not inhibit HIF-1α hydroxylation, ubiquitination, and degradation, indicating an effect on HIF-1α protein synthesis that was confirmed by pulse labeling studies. NOC18 stimulation of HIF-1α protein and HIF-1-dependent gene expression was blocked by treating cells with an inhibitor of the phosphatidylinositol 3-kinase or MAPK-signaling pathway. These inhibitors also blocked NOC18-induced phosphorylation of the translational regulatory proteins 4E-BP1, p70 S6 kinase, and eIF-4E, thus providing a mechanism for the modulation of HIF-1α protein synthesis. In addition, expression of a dominant-negative form of Ras significantly suppressed HIF-1α activation by NOC18. We conclude that the NO donor NOC18 induces HIF-1α synthesis under conditions of NO formation during normoxia and that hydroxylation of HIF-1α is not regulated by NOC18.

Hypoxia induces a series of adaptive physiological responses (1). At the cellular level, the adaptation involves a switch of energy metabolism from oxidative phosphorylation to anaerobic glycolysis, increased glucose uptake, and the expression of stress proteins related to cell survival or death (2). At the molecular level, the adaptation involves changes in mRNA transcription and mRNA stability (2, 3). One of the most important transcription factors that activates the expression of oxygen-regulated genes including vascular endothelial growth factor (VEGF)1 and inducible nitric-oxide synthase is hypoxia-inducible factor 1 (HIF-1) (4–6). VEGF is a potent angiogenic and vascular permeability factor that plays critical roles in both physiological and pathological angiogenesis (7). Recently, the expression of VEGF in response to heregulin-induced activation of the HER2/neu receptor tyrosine kinase in breast cancer cells (8), IGF-1 stimulation of colon cancer cells (9), and insulin treatment of retinal pigment epithelial cells (10) was shown to be mediated by HIF-1 via the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. Thus, HIF-1 regulates both hypoxia- and growth factor-induced VEGF expression.

HIF-1 is a heterodimer composed of a constitutively expressed β subunit (HIF-1β) and an inducibly expressed α subunit (HIF-1α) (4). The regulation of HIF-1 activity occurs at multiple levels in vivo (11). Among these, the mechanisms regulating HIF-1α protein expression and transcriptional activity have been most extensively analyzed. The von Hippel-Lindau tumor suppressor protein (VHL) has been identified as the HIF-1α-binding component of a ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation in nonhypoxic cells (12–15). Under hypoxic conditions, the hydroxylation of specific proline and asparagine residues in HIF-1α is inhibited due to substrate (O2) limitation, resulting in HIF-1α protein stabilization and transcriptional activation (14, 16, 17). The iron chelator deferoxamine (DFX) inhibits the prolyl and asparaginyl hydroxylases, which contain Fe2+ at their catalytic sites, causing HIF-1α stabilization and transactivation under normoxic conditions (12, 13).

Signaling via the HER2/neu and IGF-1 receptor tyrosine kinases induces HIF-1 expression by an independent mechanism. HER2/neu activation increases the rate of HIF-1α protein synthesis via PI3K and the downstream serine-threonine kinases AKT (protein kinase B) and FKBP/rapamycin-associated protein (FRAP; also known as mammalian target of rapamycin (mTOR)) (8). IGF-1-induced HIF-1α synthesis is dependent upon both the PI3K and MAPK pathways (10). FRAP/mTOR phosphorylates and activates the translational eIF-4E-binding protein 1; FRAP, FKBP/rapamycin-associated protein; mTOR, mammalian target of rapamycin; VHL, von Hippel-Lindau; HRE, hypoxia-responsive element; DFX, deferoxamine; TAD, transactivation domain; HA, hemagglutinin; GST, glutathione S-transferase; GSNO, S-nitrosoglutathione; SNP, sodium nitroprusside; CHX, cycloheximide; RT, reverse transcriptase; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; NLS, nuclear localization signal; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; CREB, cAMP-response element-binding protein; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein.

This paper is available online at http://www.jbc.org

2550
Nitric Oxide Up-regulates HIF-1α Synthesis

NO has also been implicated in pathological conditions such as destruction of tumor cells by macrophages, rheumatoid arthritis, and focal brain ischemia. There are several reports demonstrating that exposure of cells to certain NO donors or gaseous NO modulates HIF-1 activity (19–23). S-nitrosoglutathione (GSNO) or NOC18 induces HIF-1 activity under nonhypoxic conditions (22). In contrast, sodium nitroprusside (SNP) inhibits hypoxia-induced HIF-1 activation (19–21). However, the molecular mechanisms that regulate HIF-1α expression and transactivation in response to NO donors are poorly defined. In this study, we found that NOC18 induces HIF-1 activity by increasing HIF-1α protein synthesis via PI3K- and MAPK-dependent pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**Hep3B cells, HEK293 cells, and HCT116 cells were maintained in minimal essential medium with Earle’s salts, Dulbecco’s modified Eagle’s medium, and McCoy’s 5A medium, respectively, supplemented with 10% fetal bovine serum and 100 units/ml penicillin, and 100 μg/ml streptomycin. Human umbilical vein endothelial cells were obtained from Kurabo (Osaka, Japan) and cultured with HuMedia-EG2 (Kurabo). DFX, the thiol-dependent NO releaser GSNO, and N-acetyl cysteine were obtained from Sigma. The spontaneous NO releasers NOB4 (half-life, 60 min), NOB5 (half-life, 20 h), NOC12 (half-life, 100 min), and NOC18 (half-life, 21 h) were obtained from Dainibo (Kumamoto, Japan). Cycloheximide (CHX), SNP, wortmannin, genistein, LY294002, PD98059, and rapamycin were obtained from Calbiochem.

**Plasmid Constructs—**Expression vectors pGAL4/HIF-1α (531–826), pGAL4/HIF-1α (531–726), pGAL4/HIF-1α (726–826) were described previously (24). Plasmid p2.1 contains a 68-bp hypoxia response element (HRE) from the E010 gene inserted upstream of an SV40 promoter in the luciferase reporter plasmid pGL2-Pr (27). The expression plasmid pVEGF-KpnI contains nucleotides 2274 to +379 of the human VEGF gene inserted into the luciferase reporter pGL2-Pr (28). The reporter GAL4E1bLuc contains five copies of a GAL4 binding site upstream of a TATA sequence and firefly luciferase coding sequences (24). A FLAG-tagged dominant negative form of HIF-1α pCMV-3XFLAG-HIF-1αΔNΔBαAB was generated based on pCp4-HIF-1αΔNΔBαAB (27). The expression plasmid pCH-NLS-HIF1α (548–603)–LaCZ was described elsewhere (25). Plasmids encoding p53, a dominant-negative form of the PI3K p85 regulatory subunit, and a kinase-dead form of Akt were gifts from Dr. Wataru Ogawa (Kobe University, Kobe, Japan) (29, 30). Plasmid encoding a dominant negative form of Ras (31) was a generous gift from Dr. Kaikobad Irahi (The Johns Hopkins University, Baltimore, MD).

**Hypoxic Treatment—**Tissue culture dishes were transferred to a modular incubator chamber (Billups-Rothenberg, Del Mar, CA), which was flushed with 1% O2, 5% CO2, 94% N2, and placed at 37 °C.

**Immunoblot Assays—**Whole cell lysates were prepared by incubating cells for 3 min in cold radioimmunoprecipitation assay buffer containing 2 mM dithiothreitol, 1 mM NaVO3, and Complete protease inhibitor.
Nitric Oxide Up-regulates HIF-1α Synthesis

Fig. 3. Effect of NO donors on HRE-dependent gene expression. HEK293 cells were transfected with pTK-RL encoding Renilla luciferase and one of the following plasmids encoding firefly luciferase: HRE reporter p2.1 (A–C), mutant HRE reporter p2.4 (B), or VEGF promoter reporter pVEGF-KpnI-Luc (D). Cells were exposed to 20% or 1% O2 with or without NO donors for 16 h and then harvested for luciferase assays. C, cells were co-transfected with p2.1, pTK-RL, and the indicated amount of expression vectors encoding either no protein (EV) or a dominant negative form of HIF-1α (DN). The total amount of expression vectors was adjusted to 500 ng with empty vector. The ratio of firefly to Renilla luciferase activity (RLA) was determined and normalized to the value obtained from nonhypoxic cells transfected with empty vector to obtain the relative luciferase activity. Results shown represent mean ± S.D. of three independent transfections.

The protocol of RT-PCR is described elsewhere (33). Samples were centrifuged at 10,000 × g to pellet cell debris. For HIF-1α and HIF-1β, 100-μg aliquots were fractionated by 7.5% SDS-PAGE and subjected to an immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA) or HIF-1β (H1624; Novus Biologicals, Littleton, CO) at 1:1000 dilution. Signal was developed using ECL reagent (Amersham Biosciences). For phosphorylated protein, HEK293 cells or HCT116 cells were serum-starved (0.1% fetal bovine serum for 24 h) and treated with NOC18, and 50-μg aliquots of cDNAs were amplified with TaqGold polymerase in a thermocycler with the following primer pairs: HIF1A, GAAAGCGCTCTGCACCCGAGAC, and GCCACCATGGGGTTGTAATG; GLUT1, GGGCATGTGCTTCCAGTATGT and ACGAGGAGCACCGTCCATGAACTTTCTGCTGTCTT and ATCGCATCAGGGGCACACAG; CAAGTCCTCAAA and CTATATGGTGATGATGTGGCACTA; VEGF, EV–HIF-1α, and one of the following plasmids encoding firefly luciferase: HRE reporter p2.1 (A–C), mutant HRE reporter p2.4 (B), or VEGF promoter reporter pVEGF-KpnI-Luc (D). Cells were exposed to 20% or 1% O2 with or without NO donors for 16 h and then harvested for luciferase assays. C, cells were co-transfected with p2.1, pTK-RL, and the indicated amount of expression vectors encoding either no protein (EV) or a dominant negative form of HIF-1α (DN). The total amount of expression vectors was adjusted to 500 ng with empty vector. The ratio of firefly to Renilla luciferase activity (RLA) was determined and normalized to the value obtained from nonhypoxic cells transfected with empty vector to obtain the relative luciferase activity. Results shown represent mean ± S.D. of three independent transfections.

The protocol of RT-PCR is described elsewhere (33). Samples were centrifuged at 10,000 × g to pellet cell debris. For HIF-1α and HIF-1β, 100-μg aliquots were fractionated by 7.5% SDS-PAGE and subjected to an immunoblot assay using mouse monoclonal antibody against HIF-1α (BD Biosciences, San Jose, CA) or HIF-1β (H1624; Novus Biologicals, Littleton, CO) at 1:1000 dilution. Signal was developed using ECL reagent (Amersham Biosciences). For phosphorylated protein, HEK293 cells or HCT116 cells were serum-starved (0.1% fetal bovine serum for 24 h) and treated with NOC18, and 50-μg aliquots of cDNAs were amplified with TaqGold polymerase in a thermocycler with the following primer pairs: HIF1A, GAAAGCGCTCTGCACCCGAGAC, and GCCACCATGGGGTTGTAATG; GLUT1, GGGCATGTGCTTCCAGTATGT and ACGAGGAGCACCGTCCATGAACTTTCTGCTGTCTT and ATCGCATCAGGGGCACACAG; CAAGTCCTCAAA and CTATATGGTGATGATGTGGCACTA; VEGF, EV–HIF-1α, and one of the following plasmids encoding firefly luciferase: HRE reporter p2.1 (A–C), mutant HRE reporter p2.4 (B), or VEGF promoter reporter pVEGF-KpnI-Luc (D). Cells were exposed to 20% or 1% O2 with or without NO donors for 16 h and then harvested for luciferase assays. C, cells were co-transfected with p2.1, pTK-RL, and the indicated amount of expression vectors encoding either no protein (EV) or a dominant negative form of HIF-1α (DN). The total amount of expression vectors was adjusted to 500 ng with empty vector. The ratio of firefly to Renilla luciferase activity (RLA) was determined and normalized to the value obtained from nonhypoxic cells transfected with empty vector to obtain the relative luciferase activity. Results shown represent mean ± S.D. of three independent transfections.

A

B

C

D

Fig. 3. Effect of NO donors on HRE-dependent gene expression. HEK293 cells were transfected with pTK-RL encoding Renilla luciferase and one of the following plasmids encoding firefly luciferase: HRE reporter p2.1 (A–C), mutant HRE reporter p2.4 (B), or VEGF promoter reporter pVEGF-KpnI-Luc (D). Cells were exposed to 20% or 1% O2 with or without NO donors for 16 h and then harvested for luciferase assays. C, cells were co-transfected with p2.1, pTK-RL, and the indicated amount of expression vectors encoding either no protein (EV) or a dominant negative form of HIF-1α (DN). The total amount of expression vectors was adjusted to 500 ng with empty vector. The ratio of firefly to Renilla luciferase activity (RLA) was determined and normalized to the value obtained from nonhypoxic cells transfected with empty vector to obtain the relative luciferase activity. Results shown represent mean ± S.D. of three independent transfections.
were stained with X-gal to detect nuclear expression of using anti-HIF-1

HEK293 cells were exposed to 500

analysis (16).

of Laemmli sample buffer and analyzed by SDS-PAGE and immunoblot

In Vitro HIF-1

were transfected to polyvinylidene difluoride membrane and visualized using streptavidin-labeled horseradish peroxidase and ECL reagent (Amersham Biosciences).

In Vitro Ubiquitination Assay—HEK293 cells were washed twice with cold hypotonic extraction buffer (20 mM Tris (pH 7.5), 5 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol) and lysed in a Dounce homogenizer. The cell extract was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant was stored at 70 °C. Ubiquitination assays were performed as described previously (33). 2 μl of HA-HIF-1α that was in vitro translated (TNT Quick Coupled Transcription/Translation System; Promega) in the presence of [35S]methionine was incubated at 30 °C in a volume of 40 μl containing 21 μl (50 μg) of cell extract, 4 μl of 10× ATP-regenerating system (20 mM Tris (pH 7.5), 10 mM MgCl2, 10 mM magnesium acetate, 300 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase), 4 μl of 5 mg/ml ubiquitin (Sigma), 1 μl of 150 μM ubiquitin aldehyde (Sigma), and HA-HIF-1α was recovered using anti-HA-agarose beads, which were then mixed with SDS sample buffer and boiled for 5 min. The eluates were analyzed by SDS-PAGE and autoradiography.

Reporter Gene Assays—Reporter assays were performed in Hep3B cells and HEK293 cells (32, 34). 5 × 104 cells were plated per well on the day before transfection. In each transfection, the indicated dose of test plasmids, 200 ng of reporter gene plasmid, and 50 ng of the control plasmid ptk-RL (Promega), containing a thymidine kinase promoter upstream of Renilla reniformis (sea pansy) luciferase coding sequences, were premixed with Fugene 6 transfection reagent (Roche Applied Science). In each assay, the total amount of DNA was held constant by the addition of empty vector. After treatment, the cells were harvested, and the luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly to sea pansy luciferase activity was determined. For each experiment, at least two independent transfections were performed in triplicate.

X-gal Staining—HEK293 cells were washed twice with PBS, fixed with 1% formaldehyde, 0.2% glutaraldehyde solution, washed twice with PBS, and then treated with an X-gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM MgCl2, and 0.04% X-gal) at 37 °C (28).

RESULTS

NO Donors Activate HIF-1 under Nonhypoxic Conditions—To study the effect of NO on HIF-1 activation, we tested several NO donors. NORs and NOCs spontaneously release NO with different kinetics (see “Experimental Procedures”), whereas GSNO and SNP require cellular thiol for NO release. HEK293 cells were exposed to the compounds for 1–4 h at 20% O2, harvested, and subjected to immunoblot analysis using anti-HIF-1α or anti-HIF-1β antibody (Fig. 1A). Neither NOR4 nor NOR5 induced HIF-1α protein accumulation (lanes 2–5). In contrast, exposure of cells to NOC18 or GSNO efficiently induced HIF-1α protein accumulation comparably with 100 μM DFX (lanes 6–8, 12, and 13). SNP did not induce HIF-1α accumulation (lanes 10 and 11). Expression of HIF-1β was not affected by NO donors or DFX. NOCs induced accumulation of HIF-1α with quite different kinetics as compared with GSNO. NOC18-induced accumulation was detected as early as 30 min and lasted no less than 8 h. The effect of GSNO peaked at 1 h (lane 8) and was lost by 4 h after the addition (lane 9).

NOC18 induced HIF-1α accumulation in a dose-dependent manner up to 500 μM (Fig. 1B). Induction by GSNO was saturated at a concentration of 100 μM. The accumulation of HIF-1α induced by NOC12, which releases NO by the same mechanism.

Fig. 4. Effect of NOC18 and GSNO on HIF-1 protein stability and synthesis. HEK293 cells were exposed to 500 μM NOC18 (A), 250 μM GSNO (B), or 100 μM DFX (A and B) for 4 h, and then CHX was added to a final concentration of 100 μM. The cells were incubated for 0–60 min, and whole cell lysates were subject to immunoblot (IB) assay using anti-HIF-1α (top) and HIF-1β (bottom) antibodies. C, pulse labeling of HEK293 cells. Serum-starved cells were pretreated with no drug, NOC18, or DFX for 30 min in Met-free medium, [35S]Met-Cys was added, and the cells were incubated for 20 or 40 min prior to preparation of cell lysates and immunoprecipitation of HIF-1α. D, HEK293 cells were transfected with plasmid pCH-NLS-HIF-1α (574–603)-LacZ (b–d) or empty vector (a) and treated with NOC18 (c) or DFX (d). The cells were stained with X-gal to detect nuclear expression of β-galactosidase.

In Vitro HIF-1α-VHL Interaction Assay—Glutathione S-transferase (GST)-HIF-1α(429–608) fusion protein was expressed in E. coli as described (16, 33). Biotinylated methionine-labeled proteins were generated in reticulocyte lysates using the TNT T7 coupled transcription/translation system using Transcend biotinylated tRNA (Promega). 25-μl aliquots of HEK293 cell lysate were preincubated with NO donor or DFX for 30 min at 30 °C, 2.5 μg of GST-HIF-1α(429–608) was added, and the mixture was incubated for 30 min at 30 °C. A 5-μl aliquot of in vitro translated biotinylated VHL protein was mixed with 4 μg of GST fusion protein in a final volume of 200 μl of binding buffer (Dulbecco’s PBS (pH 7.4), 0.1% Tween 20) and incubated for 2 h at 4 °C with rotation followed by the addition of 10 μl of glutathione-Sepharose 4B beads (Amersham Biosciences) and incubation at 4 °C for 1 h. The beads were pelleted, washed 3 times in binding buffer, pelleted again, resuspended in Laemmli sample buffer, and analyzed by SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane and visualized using streptavidin-labeled horseradish peroxidase and ECL reagent (Amersham Biosciences).

In Vitro HIF-1α protein synthesis was affected by NO donors or DFX. NOCs induced accumulation of HIF-1α as early as 30 min whereas GSNO and SNP required cellular thiol for NO release. In contrast, exposure of cells to NOC18 or GSNO efficiently induced HIF-1α protein accumulation comparably with 100 μM DFX. This effect was not detected when cells were treated with an X-gal staining solution (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 1 mM MgCl2, and 0.04% X-gal) at 37 °C (28).

Results

NO Donors Activate HIF-1 under Nonhypoxic Conditions—To study the effect of NO on HIF-1 activation, we tested several NO donors. NORs and NOCs spontaneously release NO with different kinetics (see “Experimental Procedures”), whereas GSNO and SNP require cellular thiol for NO release. HEK293 cells were exposed to the compounds for 1–4 h at 20% O2, harvested, and subjected to immunoblot analysis using anti-HIF-1α or anti-HIF-1β antibody (Fig. 1A). Neither NOR4 nor NOR5 induced HIF-1α protein accumulation (lanes 2–5). In contrast, exposure of cells to NOC18 or GSNO efficiently induced HIF-1α protein accumulation comparably with 100 μM DFX (lanes 6–8, 12, and 13). SNP did not induce HIF-1α accumulation (lanes 10 and 11). Expression of HIF-1β was not affected by NO donors or DFX. NOCs induced accumulation of HIF-1α with quite different kinetics as compared with GSNO. NOC18-induced accumulation was detected as early as 30 min and lasted no less than 8 h. The effect of GSNO peaked at 1 h (lane 8) and was lost by 4 h after the addition (lane 9).

NOC18 induced HIF-1α accumulation in a dose-dependent manner up to 500 μM (Fig. 1B). Induction by GSNO was saturated at a concentration of 100 μM. The accumulation of HIF-1α induced by NOC12, which releases NO by the same mechanism.
Nitric Oxide Up-regulates HIF-1α Synthesis

**A**

**Time (min)**

| NOC18 (μM) | 0   | 150 |
|------------|-----|-----|
| (-)        | (-) | (-) |
| 250        | (-) | (-) |
| 250        | (-) | 100 |

**UBI-HIF-1α**

- HIF-1α

**B**

**lysate**

| NOC18 | GSNO | SNP | DFX |
|-------|------|-----|-----|
| -     | -    | -   | +   |
| -     | -    | +   | -   |
| -     | +    | -   | -   |

**C**

**IP:**

- (+) α-FLAG (NOC18, 250, 500, 166 μM)
- (-) α-FLAG
- (-) α-HA

**IB:**

- (+) α-FLAG
- (-) α-FLAG
- (-) α-HA

**D**

**IP:**

- (+) α-FLAG (NOC18, 250, 500, 166 μM)
- (-) α-FLAG
- (-) α-HA

**IB:**

- (+) α-FLAG
- (-) α-FLAG
- (-) α-HA

**Fig. 5.** Analysis of HIF-1α ubiquitination and interaction with VHL. A, in vitro ubiquitination assay. Lysates prepared from cells exposed to vehicle (-) or NOC18 (+) for 4 h were incubated with in vitro translated HIF-1α in the presence of ubiquitin and ATP for 0 or 150 min. Polyubiquitinated forms of HIF-1α (Ubi-HIF-1α) were identified as NOC18 but has a different NO-releasing time constant, was stronger than that induced by NOC18 at 30 min at a concentration of 250 μM (Fig. 1C, top panel). However, the induction of HIF-1α was dose-dependent such that the effect of 40 μM NOC12 was weaker than 500 μM NOC18 (Fig. 1C, bottom panel).

We screened other cell lines for the effect of NO donors on HIF-1α protein levels. 500 μM NOC18 induced HIF-1α accumulation in Hep3B human hepatocellular carcinoma cells and HCT116 human colorectal carcinoma cells as strongly as 100 μM DFX (data not shown). NOC18 also induced HIF-1α in human umbilical vein endothelial cells (data not shown). These results indicate that the effect of NOC18 on HIF-1α expression is observed in multiple transformed and primary cell types.

We investigated by RT-PCR whether NO donors induced gene expression downstream of HIF-1. VEGF and GLUT1 mRNA expression was induced by NOC18 treatment under nonhypoxic conditions (Fig. 2). In contrast, HIF-1α mRNA expression was not affected by NOC18 treatment, indicating that the effect of NOC18 occurs at the level of HIF-1α protein expression. HEK293 cells were transfected with the reporter p2.1, containing a HIF-1α-dependent HRE, or p2.4, containing a mutated HRE. NOC18 induced HRE-dependent gene expression in a dose-dependent manner comparably with DFX or 1% O2 (Fig. 3A and B). The mutated reporter p2.4 was not activated by NOC18 (Fig. 3B), and expression of a dominant negative form of HIF-1α reduced p2.1 reporter gene expression (Fig. 3C), providing evidence that the gene activation was HRE- and HIF-1α-dependent. NOC18 also induced dose-dependent transcription of a reporter gene containing the VEGF promoter encompassing nucleotides −2274 to +379 relative to the transcription start site (Fig. 3D).

**NOC18 Does Not Prolong HIF-1α Protein Half-life**—To determine whether NOC18 treatment affected HIF-1α protein half-life, HEK293 cells were treated with NOC18 or DFX for 4 h to induce HIF-1α expression, and then CHX was added to block ongoing protein synthesis. In the presence of CHX, the half-life of HIF-1α was >30 min in DFX-treated cells but <15 min in NOC18-treated cells (Fig. 4A). Similarly, the half-life of GSNO-induced HIF-1α was >15 min in the presence of CHX (Fig. 4B). These results indicate that HIF-1α expression in NOC18-treated cells is dependent upon ongoing protein synthesis. Similar results were observed in Hep3B cells and human umbilical vein endothelial cells (data not shown).

To analyze the rate of HIF-1α synthesis, serum-starved HEK293 cells were pretreated with NOC18 or DFX for 30 min and then pulse-labeled with [35S]Met-Cys for 20 or 40 min, followed by immunoprecipitation of HIF-1α (Fig. 4C). In contrast to control serum-starved cells (Fig. 4C, lane 1), 35S-labeled HIF-1α was clearly increased in NOC18-treated cells (lanes 2 and 3), whereas the amount of labeled HIF-1α protein was not increased in cells treated with DFX (lanes 4). Thus, both the cycloheximide addition and metabolic labeling experiments provide evidence for increased synthesis of HIF-1α in response to NOC18 treatment.

by their reduced mobility after PAGE. B, GST-HIF-1α(429–608) fusion protein was incubated with in vitro translated VHL in the presence of PBS or lysates untreated or treated with the indicated reagents. Glutathione-Sepharose beads were used to capture GST-HIF-1α, and the presence of associated VHL in the samples was determined by PAGE. One-fifth of the input VHL protein was also analyzed. C and D, FLAG-tagged HIF-1α and HA-tagged VHL were expressed in HEK293 cells. Cells were treated or untreated with 250–500 μM NOC18 or 100 μM DFX for 2 h and harvested. Lysates were incubated with anti-FLAG affinity beads, and captured protein was eluted and analyzed by SDS-PAGE. Epitopes were detected with anti-FLAG (top) or anti-HA (bottom) antibody. IP, immunoprecipitation; IB, immunoblot.
Nitric Oxide Up-regulates HIF-1α Synthesis

Effects of NO Scavenger, Guanylate Cyclase Inhibitor, and Antioxidant on HIF-1α induction by NOC18. HEK293 cells were treated with 250 μM NOC18 or 100 μM DFX with or without carboxyl-PTIO (A), ODQ (B), or N-acetyl cysteine (NAC) (C) for 4 h and harvested. Then the lysates were subjected to immunoblot (IB) assay with anti-HIF-1α antibody.

We also assayed the stability of a fusion protein, consisting of a nuclear localization signal (NLS), β-galactosidase sequences (encoded by the lacZ gene), and HIF-1α residues 548–603. The NLS-LacZ-HIF1α(548–603) expression vector was transfected into HEK293 cells, and β-galactosidase activity was analyzed by X-gal staining after incubation of the cells in the presence of 500 μM NOC18 or 100 μM DFX. There was essentially no X-gal staining in cells that were transfected with empty vector, transfected with NLS-LacZ-HIF1α(548–603) without treatment, or transfected with NLS-LacZ-HIF1α(548–603) with NOC18 treatment (Fig. 4D). In contrast, significant X-gal staining was detected in NLS-LacZ-HIF1α(548–603)-transfected cells that were treated with DFX, which inhibits O2-dependent degradation mediated by the HIF-1α domain of the fusion protein.

NOC18 Does Not Affect the Interaction between HIF-1α and VHL in Vitro or in Vivo—Under hypoxic conditions, VHL-dependent ubiquitination of HIF-1α is inhibited (12–14). To determine whether NOC18 treatment affects ubiquitination, an in vitro assay was performed using lysates prepared from control and NOC18-treated cells. As shown in Fig. 5A, there was no significant difference detected between lysates from NOC18-treated or untreated cells with respect to their ability to ubiquitinate HIF-1α.

Incubation of a GST-HIF1α(429–608) fusion protein with lysate from untreated cells resulted in prolyl hydroxylation of HIF-1α and interaction with VHL (Fig. 5B, lane 2). Lysate from cells treated with DFX did not promote interaction of GST-HIF1α(429–608) with VHL (lane 6). In contrast, lysate from cells treated with NOC18 promoted the interaction (lane 3), similar to control lysates, again providing evidence that NOC18 treatment does not induce HIF-1α expression by inhibiting VHL-mediated ubiquitination. Similar results were obtained from experiments using rabbit reticulocyte lysates (which also have HIF-1α prolyl hydroxylase activity) instead of HEK293 cell lysates (data not shown). Remarkably, lysate from GSNO-treated cells partially inhibited the interaction of GST-HIF1α(429–608) with VHL (lane 4), whereas lysate from SNP-treated cells dramatically increased the interaction (lane 5).

HEK293 cells were co-transfected with expression vectors encoding HA-tagged VHL and FLAG-tagged HIF-1α. Aliquots of whole cell lysates were analyzed for expression of the proteins directly or following immunoprecipitation of HA-VHL or FLAG-HIF1α. HIF-1α was present in anti-HA immunoprecipitates from cells co-expressing HA-VHL and FLAG-HIF1α (Fig. 5C, lane 3). Exposure of cells to NOC18 did not alter the interaction of HA-VHL and FLAG-HIF1α (Fig. 5C, lanes 4 and 5), consistent with the inability of NOC18 to inhibit VHL and HIF-1α interaction in vitro. In contrast, DFX treatment inhibited the interaction (lane 6). FIH-1 is the asparagine hydroxylase that negatively regulates HIF-1α transactivation domain function under nonhypoxic conditions (16, 17). The interaction between HA-FIH-1 and FLAG-HIF1α was not affected by either NOC18 or DFX (Fig. 5D). Taken together, results presented in Fig. 5 indicate that the molecular mechanism of NOC18 action is distinct from the inhibition of hydroxylase activity that occurs in cells exposed to hypoxia or DFX.

Impact of NO Scavenger, Guanylate Cyclase Inhibitor, and Antioxidant on NOC18-induced HIF-1α Accumulation—To examine signal transduction pathways mediating effects of NO donors on HIF-1α protein induction, the NO scavenger carboxyl-PTIO was utilized (35). Carboxyl-PTIO significantly suppressed HIF-1α accumulation induced by NOC18 but not by DFX (Fig. 6A). Carboxyl-PTIO by itself did not have any effects. Next we examined the impact of guanylyl cyclase activity on HIF-1α accumulation. NO stimulates the activity of guanylyl cyclase, which catalyzes the production of cGMP, an important second messenger for signal transduction. In HEK293 cells, the specific guanylyl cyclase inhibitor ODQ did not affect NOC18-induced HIF-1α accumulation (Fig. 6B), providing evidence that the guanylyl cyclase-cGMP pathway does not contribute to
Effect of kinase inhibitors on the induction of HIF-1α protein and transactivation. A and B, HEK293 cells were exposed to vehicle, 500 μM NOC18, or DFX in the presence (+) of 100 μM genistein.

HIF-1α accumulation induced by NOC18.

NO is a radical, and equimolar amounts of O₂ and NO form peroxynitrite (ONOO⁻), which decomposes at physiological pH to generate oxidant with similar reactivity to the hydroxyl radical. To examine whether the intracellular redox state modulates NOC18-induced HIF-1α accumulation, HEK293 cells were treated with NOC18 in the presence of 50 mM N-acetyl cysteine (NAC) (Fig. 6C). N-acetyl cysteine treatment did not affect HIF-1α levels, suggesting that thiol-mediated redox status does not play a critical role in NOC18-induced HIF-1α expression. Transient overexpression of the intracellular redox regulator thioredoxin also did not affect induction of HIF-1α expression by NOC18 (data not shown).

**HIF-1α-mediated Transactivation in Response to NOC18 Treatment**—We next investigated the impact of NOC18 on HIF-1α transcriptional activity. There are two independent transactivation domains (TADs) present in HIF-1α, which are designated as the amino-terminal (amino acids 531–575) and carboxyl-terminal (amino acids 786–826) TADs (TAD-N and TAD-C, respectively) (24). Steady-state levels of proteins consisting of the GAL4 DNA-binding domain fused to HIF-1α TADs (GAL4-HIF-1α(531–575), GAL4-HIF-1α(531–826), and GAL4-HIF-1α(786–826)) are similar under hypoxic and non-hypoxic conditions (24). These GAL4-HIF-1α fusion constructs can thus be used to examine the transcriptional activity of HIF-1α independent of its protein expression (24, 32). Transactivation mediated by GAL4-HIF-1α(531–826), which contains both TAD-N and TAD-C, or GAL4-HIF-1α(531–575), which contains only TAD-N, is increased in cells exposed to hypoxia or DFX (24). In contrast, GAL4-HIF-1α(786–826), which contains only TAD-C, is constitutively active in untreated cells. NOC18 treatment increased transactivation mediated by GAL4-HIF-1α(531–826) or GAL4-HIF-1α(531–575) in a dose-dependent manner, whereas transactivation mediated by GAL4-HIF-1α(786–826) was not increased by exposure of cells to NOC18 or DFX (Fig. 7). These results demonstrate that NOC18 not only promotes accumulation of HIF-1α but also enhances HIF-1α transcriptional activity.

**Effect of Kinase Inhibitors on NOC18-induced HIF-1 Activation**—HEK293 cells were pretreated with LY294002, genistein, PD98059, or rapamycin, which are selective pharmacologic inhibitors of PI3K, tyrosine kinases, MEK, and FRAP/mTOR kinase activity, respectively. All of the agents inhibited the induction of HIF-1α protein expression in NOC18-treated cells (Fig. 8A). The combination of LY294002, PD98059, and rapamycin markedly inhibited NOC18-induced HIF-1α expression (Fig. 8B). In contrast to their effects on HIF-1α protein expression induced by NOC18 treatment, LY294002 or PD98059 had little inhibitory effect on the expression of HIF-1α in DFX-treated HEK293 cells (Fig. 8A, lanes 9–14).

LY294002 and rapamycin inhibited expression of the HIF-1α-dependent reporter gene p2.1 induced by NOC18 but not by DFX, whereas genistein and PD98059 inhibited both NOC18- and DFX-induced reporter gene expression (Fig. 8C, top). Interestingly, the stimulation of HIF-1α transactivation domain function by NOC18 was also blocked by kinase inhibitors, whereas only genistein blocked DFX-induced transactivation

**Fig. 8.** Effect of kinase inhibitors on the induction of HIF-1α protein and transactivation. A and B, HEK293 cells were exposed to vehicle, 500 μM NOC18, or DFX in the presence (+) of 100 μM genistein (GEN), 5 μM LY294002 (LY), 50 μM PD98059 (PD), 100 nM wortmannin (WT), or 200 nM rapamycin (Rap) and harvested after 4 h for analysis of HIF-1α protein. HRE-dependent gene expression (C (top) and D) or HIF-1α transactivation domain function (C, bottom) were analyzed using p2.1 or Gal4-HIF-1α(531–926) and Gal4E1bLuc, respectively. Cells were pretreated with LY294002, genistein, PD98059, or rapamycin and exposed to 250 μM NOC18 or 100 μM DFX. D, cells were transfected with an expression vector encoding a dominant negative form of p85 PI3K, Akt, or Ras. IB, immunoblot.
Nitric Oxide Up-regulates HIF-1α Synthesis

Fig. 9. MAPK and PI3K pathway signaling in NOC18-treated cells. HEK293 and HCT116 cells were exposed to 100 or 500 μM NOC18. Whole cell lysates were prepared after 15, 30, or 60 min and subjected to immunoblot (IB) assays using antibodies specific for phosphorylated (Thr-202/Tyr-204) or total p42 ERK2/p44 ERK1 MAPK (A) and phosphorylated (Ser-473) or total AKT (B).

(Fig. 8C, bottom). These results provide further evidence that NOC18 and DFX induce HIF-1α by distinct molecular mechanisms. Moreover, NOC18-induced HRE-dependent gene expression was suppressed by a dominant negative form of PI3K p85 subunit, AKT, or Ras, indicating critical roles of these signaling proteins in transducing the effects of NOC18 to HIF-1α (Fig. 8D).

NOC18-induced Activation of MAPK, PI3K, and Translational Regulators—HIF-1α activity induced by the stimulation of receptor tyrosine kinases or G protein-coupled receptors requires MAPK and PI3K signaling (10, 36). To determine whether the MAPK and PI3K pathways were activated in NOC18-treated cells, the phosphorylation of p42ERK2/p44ERK1 and AKT were analyzed in HEK293 cells and HCT116 cells. Increased phosphorylation of p42ERK2/p44ERK1 (Fig. 9A) and AKT (Fig. 9B) was induced by NOC18 treatment in both cell types.

The signal transduction pathway involving PI3K, AKT, and mTOR has been shown to regulate protein translation via phosphorylation of p70S6K, the S6 ribosomal protein, and eIF-4E. In both HCT116 cells (Fig. 10) and HEK293 cells (data not shown), the phosphorylation of p70S6K, S6, and eIF-4E was induced by NOC18 stimulation in a dose- and time-dependent manner. The mRNA cap-binding protein eIF-4E was also phosphorylated by NOC18 treatment of HCT116 cells (Fig. 10). This result is consistent with studies indicating that ERK activates the MAPK signal-integrating kinases, MNK1 and MNK2, which in turn phosphorylate eIF-4E (37, 38).

**DISCUSSION**

The studies reported above demonstrate that treatment of several different cell types with the NO donor NOC18 induces HIF-1α protein expression and HIF-1α transcriptional activation, resulting in VEGF and GLUT1 mRNA expression. NOC18 treatment did not increase the half-life of HIF-1α protein, did not inhibit the interaction between HIF-1α and VHL, and did not inhibit the ubiquitination of HIF-1α, indicating that the mechanism of NOC18 action does not involve inhibition of HIF-1α prolyl hydroxylation. Rather than increasing the stability of HIF-1α, the data suggest that NOC18 increases the rate of HIF-1α protein synthesis.

Whereas exposure of cells to hypoxia or DFX decreases HIF-1α protein degradation, exposure of cells to heparulin, IGF-1, insulin, or prostaglandin E2 increases HIF-1α protein synthesis (8–10, 36). In previous studies of MCF-7 and HCT116 cells, the effect on protein synthesis was documented by cycloheximide inhibition and by pulse-chase experiments (8). In the present study, we also confirmed that NOC18 treatment stimulated the synthesis of HIF-1α but had no effect on HIF-1α protein stability in HEK293 cells. Thus, as in the case of growth factor-treated cells, the increased expression of HIF-1α protein in NOC18-treated cells is due to increased synthesis.

As previously observed in growth factor-treated cells, the effect of NOC18 is dependent upon its activation of the PI3K and MAPK pathways. Dependence on MEK activity for phosphorylation of 4E-BP1 and p70S6K has been demonstrated in other cellular contexts. In the case of IGF-1-stimulated colon cancer cells, both MEK and PI3K are required for activation of p70S6K, with MEK inhibitors preventing the phosphorylation of Thr-241/Ser-242 in the Thr-389 by mTOR (39). ERK activity also stimulates the phosphorylation of eIF-4E, which is required for its mRNA cap binding activity (37). Thus, NOC18 signaling both derepresses (via phosphorylation of 4E-BP1) and activates (via phosphorylation of eIF-4E and p70S6K) protein synthesis. The effects of NO donors may not be specific for HIF-1α. The known targets for phosphorylation by mTOR are regulators of protein synthesis. The translation of several...
dozen different mRNAs are known to be regulated by this pathway, and sequences in the 5′-untranslated region of the respective mRNAs may determine the degree to which the translation of any particular mRNA can be modulated by mTOR signaling. HIF-1α protein expression is likely to be particularly sensitive to changes in the rate of synthesis because of its extremely short half-life under hypoxic conditions.

HIF-1 activity is regulated not only by HIF-1α protein expression but also by HIF-1α transcriptional activity. Our data analyzing transcription activity mediated by Gal4-HIF-1α-TAD fusion proteins demonstrate that NOC18 treatment also induces HIF-1α TAD activity under hypoxic conditions. A regulatory switch controlling TAD activity involves O2-dependent hydroxylation of Asn-803 by FIH-1. NOC18 treatment did not promote dissociation of FIH-1 and HIF-1α. TAD activity is also regulated by a MAPK-dependent mechanism (41). The MEK-1 inhibitor PD98059 blocked NOC18-induced HIF-1α activation and NOC18-induced MAPK activation, suggesting a link between NOC18, MEK, ERK, and HIF-1α. Published data suggest that the direct target of MEK/ERK may be the coactivators CREB-binding protein and p300, which interact with the TADs (42).

The action of NO in biological systems can be mediated directly by NO or by conversion of NO to NO− or NO2− equivalents (43). Because two enzymes in the ubiquitin-proteasome pathway, E1 and E2, contain thiols in their active sites, these thiols were a priori candidates as targets of NO donors. However, our experimental results do not support this mechanism of action for NOC18. Another potential target is HIF-1α itself, since there is a report that GSNO induces nitrosylation of HIF-1α (44). However, our results indicate that if NOC18 induces nitrosylation of HIF-1α, this modification does not lead to accumulation of the protein. NOC18 treatment had no effect on the interaction of HIF-1α and VHL, whereas GSNO partially inhibited the interaction, and SNP may stimulate the prolyl hydroxylation-ubiquitination system and promote increased HIF-1α degradation. Consistent with this hypothesis, SNP inhibited HIF-1α accumulation induced by DPX. Thus, different NO donors activate or inhibit HIF-1 through different molecular mechanisms.

Recent studies have demonstrated that NO donors stimulate cellular signaling cascades (45–47). Overexpression of a dominant negative form of Ras significantly inhibited NOC18-induced HRE-dependent gene expression, and the tyrosine kinase inhibitor genistein almost completely abolished NOC18-induced HRE-dependent gene expression, and the tyrosine kinase inhibitor genistein almost completely abolished NOC18-induced HRE-dependent gene expression, and the tyrosine kinase inhibitor genistein almost completely abolished NOC18-induced HRE-dependent gene expression. Thus, NOC18 treatment modulates protein kinase signaling pathways similar to the effects of growth factor treatment. Determination of the extent to which NO signaling to HIF-1 participates in physiological and pathophysiologic processes will require further investigation.

Acknowledgments—We are grateful to Drs. Wataru Ogawa and Kaikobad Irani for providing plasmid vectors and to Keiko Nishio for technical assistance.

REFERENCES
1. Hochachka, P. W., Buck, L. T., Dole, C. J., and Land, S. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9493–9498
2. Guillemin, K., and Krasnow, M. A. (1997) Cell 88, 9–12
3. Paukling, W. R., and Czyzyk-Krzeska, M. F. (2000) Adv. Exp. Med. Biol. 475, 111–121
4. Wang, G. L., Jiang, B. H., Rue, E. A., and Semenza, G. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5510–5514
5. Semenza, G. L. (2000) Gene 249, 1983–1991
6. Hirota, K. (2002) J. Anesthesiology 16, 150–159
7. Carmeliet, P., and Jain, R. K. (2000) Nature 407, 249–257
8. Laughner, E., Taghavi, P., Mahal, M. C., and Semenza, G. L. (2001) Mol. Cell. Biol. 21, 3995–4004
9. Treiss, C., Giorgetti-Peraldi, S., Murdana, J. S., and Van Obberghen, E. (2002) J. Biol. Chem. 277, 27975–27981
10. Fukuda, R., Hirota, K., Fan, F., Jung, Y. D., Ellis, L. M., and Semenza, G. L. (2002) J. Biol. Chem. 277, 38205–38211
11. Semenza, G. L. (1996) Curro J. Genet. Dev. 8, 588–594
12. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Dhanda, A., and Semenza, G. L. (2001) Cell 107, 43–54
13. Mahon, P. C., Hirota, K., and Semenza, G. L. (2001) Genes Dev. 15, 2675–2685
14. Landlo, D., Pest, D. J., Gordon, J. L., Weland, D. A., Whitelaw, M. L., and Bruick, R. K. (2002) Genes Dev. 16, 1466–1471
15. Ignarro, L. J., Cirino, G., Casini, A., and Napoli, C. (1999) J. Cardiovasc. Pharmacol. 34, 879–886
16. Liu, Y., Christon, H., Morita, T., Laughner, E., Semenza, G. L., and Kourembanas, S. (1998) J. Biol. Chem. 273, 15257–15262
17. Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A., and Bunn, H. F. (1999) J. Biol. Chem. 274, 9033–9044
18. Sogawa, K., Numayama-Tsurtai, K., Ema, M., Abe, M., Abe, H., and Fujiyuryama, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7368–7373
19. Palmer, L. A., Gaston, B., and Johns, R. A. (2000) Mol. Pharmacol. 58, 1197–1203
20. Kimura, H., Weisz, A., Kurashima, Y., Hashimoto, K., Ogura, T., D’Aquisto, F., Addeo, R., Maksymiuk, M., and Esami, H. (2000) Blood 95, 189–197
21. Huang, L. E., Willmore, W. G., Gu, J., Goldberg, M. A., and Bunn, H. F. (1999) J. Biol. Chem. 274, 9033–9044