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Normoxic Stabilization of Hypoxia-Inducible Factor-1 Expression and Activity: Redox-Dependent Effect of Nitrogen Oxides

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is an essential transcription factor involved in the oxygen-dependent regulation of gene expression. Thiol groups in HIF-1 or in proteins that modify HIF-1 are conventional targets for regulation by nitric oxide (NO). Moreover, NO delivery to tissue by hemoglobin appears to be oxygen dependent. Therefore, the role NO plays in regulating HIF-1 activity and expression was examined. The 1-substituted diazen-1-ium-1,2-diolate NOC-18 induced HIF-1 DNA-binding activity in normoxic bovine pulmonary artery endothelial cells and rat aortic smooth muscle cells in a time- and dose-dependent manner. Induction of HIF-1-binding activity was consistent with an increased expression of HIF-1 subunit proteins HIF-1α and HIF-1β. The effect of NOC-18 on HIF-1 activity was blocked by cycloheximide, consistent with a post-transcriptional effect. NOC-18 induction of HIF-1 DNA-binding activity was not blocked with oxyhemoglobin, nor was it related to the rate of NO evolution, arguing against NO-mediation of the effect. Additionally, the effect of NOC-18 could not be mimicked by Angeli's salt, arguing against nitroxyl mediation. However, the NOC-18 effect could be reproduced by *S*-nitrosoglutathione (GSNO), an endogenous nitrosonium donor formed in the presence of deoxyhemoglobin. Furthermore, the GSNO effect could be reversed by dithiothreitol as well as acivicin, an inhibitor of GSNO bioactivation. Taken together, these results suggest that an *S*-nitrosylation reaction stabilizes HIF-1 protein expression and activity. We speculate that one signaling mechanism by which deoxyhemoglobin may activate HIF-1 involves NO.

Hypoxia-inducible factor-1 (HIF-1) is a pivotal regulator of many hypoxia-regulated genes. This transcription factor is a heterodimeric DNA-binding protein consisting of 120-kDa HIF-1α and 91- to 94-kDa HIF-1β (Wang and Semenza, 1995). Both subunits are members of the basic helix-loop-helix/periodicity/aryl hydrocarbon receptor nuclear translocator(ARNT)/simple-minded family of transcription factors (Wang et al., 1995). The α-subunit is a unique member of this family and is stabilized in profound hypoxia (Huang et al., 1996). The β-subunit is identical to the aryl hydrocarbon nuclear translocator protein and is constitutively expressed (Wang et al., 1995). Whereas HIF-1α has been shown to be primarily stabilized by hypoxia, stabilization of ARNT by hypoxia is less clear with both induction (Gleadle et al., 1995; Wang et al., 1995) and no response (Huang et al., 1996) reported.

The formation of HIF-1 is primarily dependent on the stability of the HIF-1α subunit (Huang et al., 1996). However, the mechanism by which oxygen is sensed and HIF-1α is stabilized under physiological conditions is not clear. One current hypothesis suggests a model in which redox reactions play a role (Huang et al., 1996). Alterations in the redox state of the cell have been shown to impair the hypoxic signaling mechanism and expression of the HIF-1α protein (Salceda and Caro, 1997). HIF-1 DNA-binding activity and expression is minimal with exposure to oxygen tensions greater than 30 mm Hg (Guillemin and Krasnow, 1997) because of the degradation of HIF-1α protein (Salceda and Caro, 1997). Oxygen-dependent degradation (ODD) appears to require a region of HIF-1α that spans amino acids 401 to 603, as well as control degradation by the ubiquitin proteasome pathway (Huang et al., 1998). Thiol groups in HIF-1 or the proteins that are involved in the regulation of HIF-1 are potential targets for nitric oxide (NO). Currently, NO is known to modulate HIF-1 expression in hypoxia (Liu et al., 1998; Sogawa et al., 1998; Huang et al., 1999). In addition, NO has

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ABBREVIATIONS: HIF-1, hypoxia-inducible factor-1; NOC-18, (*Z*)-1–1[2-aminoethyl)amino]diazen-1-ium-1,2-diolate; ARNT, aryl hydrocarbon nuclear translocator; ODD, oxygen-dependent degradation; NO, nitric oxide; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; NO^-, nitroxyl; NO^+, nitrosonium; GSNO, *S*-nitrosoglutathione; pVHL, protein von Hipple Lindau; BLOTTO, bovine lacto transfer optimizer; TBS, Tris-buffered saline.

been shown to stimulate vascular endothelial growth factor expression by stabilizing HIF-1α expression (Kimura et al., 2000). However, the mechanism by which NO regulates HIF-1 expression in normoxia has not been determined. In this report, we describe the ability of NO, through a novel cGMP-independent mechanism (*S*-nitrosylation), to induce HIF-1 DNA-binding activity and protein expression under conditions of normal atmospheric oxygen tension. This is particularly interesting given the recent observation that NO delivery to tissues is enhanced and may be finely regulated by hemoglobin deoxygenation (Jia et al., 1996) under conditions less extreme than those currently used to induce HIF-1 activity in vitro.

Experimental Procedures

Materials. NOC-18 and GSNO were obtained from Alexis Corporation (San Diego, CA), Angeli's salt was from Cayman Chemical (Ann Arbor, MI), clasto-lactacystin β-lactone was from Calbiochem-Novabiochem Corp. (San Diego, CA), and MG132 was from Peptides International Inc. (Louisville, KY). All other materials were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Bovine pulmonary artery endothelial cells were grown in M199 medium supplemented with 10% fetal calf serum and 2.4 μg/ml thymidine and were characterized as described previously (Johns et al., 1990). Rat aortic smooth muscle cells were grown in M199 supplemented with 10% fetal bovine serum. Cells were maintained in a humidified 37°C, 5% CO_2, incubator and used between passages 7 and 13. For studies involving hypoxic conditions, cells were placed in a modular incubator and purged with 95% N_2, 5%CO_2 for 20 min as described previously (Palmer et al., 1998). Partial pressure of O_2 of the medium of the cells exposed to hypoxia was 15 mm Hg. In the studies using actinomycin D and cycloheximide, cells were treated with 15 μg/ml actinomycin D for 2 h or 60 μg/ml cycloheximide for 15 min before the addition of 500 μM NOC-18 for 4 h. For the studies using the proteasome inhibitors, 20 μM clasto-lactacystin β-lactone or 50 μM MG-132, was used. In these experiments, dimethyl sulfoxide was used as a vehicle control.

Nuclear Extract and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared from bovine pulmonary artery endothelial cells or rat aortic smooth muscle cells exposed to normoxia or hypoxia for 4 h as described previously (Palmer et al., 1998). EMSAs were performed as described previously (Palmer et al., 1998).

Isolation of RNA and Northern Analysis. Total RNA was purified from treated and untreated cells using Trireagent (Molecular Research Center, Inc., Cincinnati, OH) as described by the manufacturer. Aliquots of RNA (10 μg) were fractionated by formaldehyde gel electrophoresis and transferred to positively charged nylon membrane (Roche Molecular Biochemicals, Summerville, NJ). cDNA probes for heme oxygenase I and GAPDH (kind gifts from Victor Laubach, Department of Surgery, University of Virginia) were labeled with [γ-^{32}P]dCTP using Ready to Go labeling beads (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridizations were performed using Express Hyb (Clontech, Palo Alto, CA) as described by the manufacturer.

Western Blot Analysis. Nuclear protein (100 μg) was separated on a 6% (w/v) SDS polyacrylamide gel. The fractionated protein was transferred to nitrocellulose using an electrophoretic transfer cell. The blots were blocked in BLOTTO A (1× TBS, 5% milk, 0.05% Tween-20) for 1 h. TBS is 10 mM Tris-HCl, pH 8.0, 150 mM NaCl. The blots were probed with the anti-HIF-1α and -HIF-1β antibodies (Novus Biologicals, Littleton, CO) for 1 h at room temperature in BLOTTO A. Blots were washed several times with TBS plus 0.05% Tween 20, incubated for 30 min with a secondary antibody coupled to horseradish peroxidase, and washed with TBS plus 0.05% Tween 20 and once with TBS. Protein bands were visualized by chemiluminescence by ECL (Amersham Pharmacia Biotech). For experiments examining heme oxygenase 1 expression, 100 μg of total cellular protein was separated on a 12% (w/v) SDS polyacrylamide gel. Proteins were transferred and then probed with anti-heme oxygenase 1 antibody (Affinity Bioreagents Inc., Golden CO) as described above.

Detection of NO. Medium from NOC-18-exposed endothelial cells was assayed serially for NO by anaerobic chemiluminescence assay (NOA-28; Sievers, Boulder, CO) following injection into a helium-purged vessel containing PBS, pH 7.4. Standard curves were constructed from dilution of NO-saturated Argon-deaerated water as described previously (Brien et al., 1996).

Preparation of Oxyhemoglobin. Oxyhemoglobin was prepared from hemoglobin as described previously (Murphy and Noack, 1994). Briefly, 25 mg of hemoglobin was dissolved in 1 ml of 50 mM HEPES, pH 7.5, in a 25-ml flat-bottomed flask. To this solution was added 1 to 2 mg of sodium hydrosulfite powder. Oxygen was then blown into the flask. Oxyhemoglobin was purified by passing the solution over a Sephadex G-25 column. The concentration of oxyhemoglobin was measured at an absorbance of 415 nm. In the experiments described, final concentrations of 1 and 5 μM oxyhemoglobin were used with redosing after 2 h.

Results

Induction of HIF-1 DNA-Binding Activity by the NO Donor NOC-18. HIF-1 DNA-binding activity was induced in extracts made from bovine pulmonary artery endothelial cells treated with 500 μM NOC-18 (Fig. 1A). The NOC-18-induced DNA-binding activity was due to HIF-1 because antibodies specific for HIF-1α and HIF-1β could supershift this complex (Fig. 2). In addition, this response was specific for HIF-1 because NOC-18 did not modulate SP-1 DNA-binding activity. To determine whether the induction of HIF-1 DNA-binding activity by NOC-18 was cell-type specific, similar experiments were performed in rat aortic smooth muscle cells. NOC-18 was found to induce HIF-1 DNA-binding activity in rat vascular smooth muscle cells (Fig. 1B). In both cell types, the NOC-18-induced complexes migrated to a position identical with the HIF-1 heterodimeric

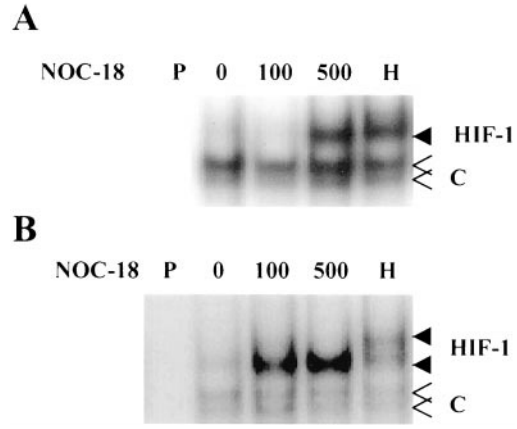

Fig. 1. NOC-18 induces HIF-1 DNA-binding activity in bovine pulmonary artery endothelial and rat aortic smooth muscle cells in a dose-dependent manner. Nuclear extracts were isolated from bovine pulmonary artery endothelial cells (A) or rat aortic smooth muscle cells (B) exposed to 0, 100, and 500 μM NOC-18 for 4 h. HIF-1 DNA-binding activity was determined by electrophoretic mobility shift analysis using 3 μg of nuclear protein and 1.5 fmol of a 30-bp oligonucleotide that contains the HIF-1 DNA-binding site. The HIF-1 DNA-binding complex is indicated. C, constitutive protein-DNA complexes; P, probe alone; H, hypoxic control.

complex (Fig. 1B, lower band) seen in hypoxia (Wang and Semenza, 1995). To determine whether the effects of NOC-18 affect the transcription of hypoxia regulated genes, we examined the ability of NOC-18 to induce the expression of the hypoxia-inducible gene, heme oxygenase I. NOC-18 (500 μM) dramatically induced mRNA and protein expression of heme oxygenase I (Fig. 3). This is consistent with previous reports showing that 1) hypoxic induction of heme oxygenase I is dependent on HIF-1 (Lee et al., 1997) and 2) NO donors can induce heme oxygenase I (Hara et al., 1999). Thus, the ability of NOC-18 to induce HIF-1 DNA-binding activity in normoxia is not limited to a particular cell type and is relevant to downstream gene expression.

The time course of NOC-18-induced HIF-1 DNA binding was examined (Fig. 4). HIF-1 DNA-binding activity was detected within 2 h. Maximum DNA-binding activity was detected between 3 and 4 h of exposure. No HIF-1 DNA-binding activity was detected after 24 h.

Stabilization of HIF-1α and Augmentation of HIF-1β Protein Expression by the NO Donor NOC-18. HIF-1α protein was stabilized by NOC-18 in a dose- and time-dependent manner (Fig. 5, A and B, respectively). HIF-1β protein expression was detected in the absence of NOC-18, consistent with previous reports (Huang et al., 1996, 1998). In addition, HIF-1β protein expression was augmented by NOC-18 in a dose- and time-dependent manner (Fig. 5, C and D, respectively). Taken together, these data demonstrate that NOC-18 stabilizes HIF-1α and augments HIF-1β protein expression. The increased protein expression of HIF-1α and HIF-1β by NOC-18 is consistent with the increase in DNA-binding activity detected.

The Effects of NOC-18 on HIF-1α and HIF-1β Protein Expression Is Not Transcriptional. To determine whether the stabilization of HIF-1α and the augmentation of HIF-1β proteins are transcriptional, cells were treated with actinomycin D, an inhibitor of transcription, or cycloheximide, an inhibitor of translation (Fig. 6). Neither actinomycin D nor cycloheximide had any effect on the protein DNA complexes formed with nuclear extracts isolated from untreated cells. Actinomycin D had no effect on the induction of HIF-1 DNA-binding activity induced by NOC-18. On the other hand, cycloheximide completely eliminated the induction of HIF-1 DNA-binding activity induced by NOC-18. These results suggest that the effects of NOC-18 on HIF-1 DNA-binding activity and protein expression are post-transcriptional, consistent with the reports that HIF-1α mRNA is constitutively expressed (Huang et al., 1996; Wood, 1996; Kallio et al., 1997) and that HIF-1α protein levels are regulated by proteolysis (Salceda and Caro, 1997; Huang et al., 1998).

NOC-18 Does Not Augment the Effects of Proteasome Inhibitors. Proteasome inhibitors were used to determine whether the action of NOC-18 was mediated through the ubiquitin-proteasome pathway. As expected, bovine pulmonary artery endothelial cells treated with the proteasome inhibitors clasto-lactacystin β-lactone or MG 132 showed an increase in HIF-1 DNA-binding activity (Fig. 7) and HIF-1α protein expression (not shown). Likewise, NOC-18 alone induced HIF-1 DNA-binding activity. However, NOC-18 did not augment the induction seen with the proteasome inhibitors. Taken together, these results suggest that the actions of NOC-18 are not independent of the ubiquitin-proteasome pathway.

The Effects of NOC-18 May Be Mediated through Electrophilic Reactivity of the NO Species. NOC-18 decomposes slowly into two NO molecules and a free amine (Keefer et al., 1996). Initial experiments examined the possibility that the effect of NOC-18 on HIF-1 activity was caused by the release of the free amine. Bovine pulmonary artery endothelial cells were treated with NOC-18 or NOC-18 that had been allowed to decay for a period of 24 h (NOC + 24). Neither HIF-1 DNA-binding activity (Fig. 8A) nor HIF-1α or β protein expression was induced with NOC + 24. Thus, the data suggest that the effect of NOC-18 was medi-

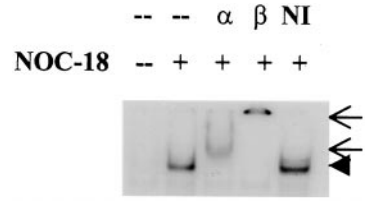

Fig. 2. NOC-18 induces HIF-1 expression in bovine pulmonary artery endothelial cells. EMSA was performed on nuclear extracts made from bovine pulmonary artery endothelial cells grown in the absence (--) or presence (+) of 500 μM NOC-18 for 4 h. Nuclear extracts were incubated with antibody directed against HIF-1α (α) or HIF-1β (β) or nonimmune serum (NI) 15 min before the addition of HIF-1 probe. The solid arrowhead indicates the location of HIF-1 DNA-binding activity. Arrows indicate the location of the supershifted protein-DNA complexes.

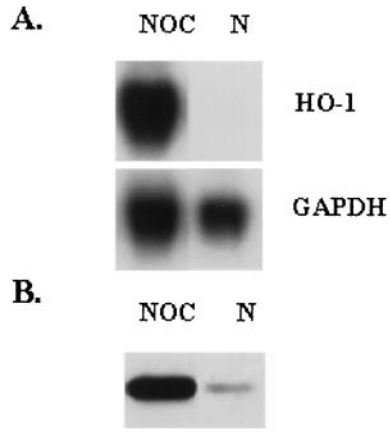

Fig. 3. NOC-18 induces the expression of heme oxygenase I RNA and protein. Northern (A) and Western (B) blot analyses were performed on RNA and protein isolated from rat aortic smooth muscle cells grown in the presence or absence of 500 μM NOC-18 for 4 h. N, normoxic. A, RNA (10 μg) isolated from untreated and NOC-18-treated (500 μM) cells was analyzed by Northern blot analysis. Northern blots were probed with both heme oxygenase I (HO-1) and GAPDH. B, nuclear protein (100 μg) was analyzed by Western blot analysis. The expression of HO-1 protein was detected by antibody directed against HO-1.

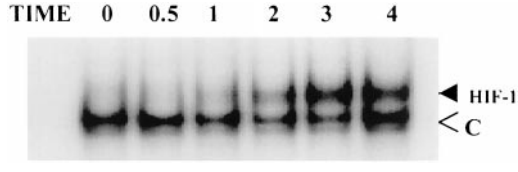

Fig. 4. NOC-18 induces HIF-1 DNA-binding activity in a time-dependent manner. Nuclear extracts were made from bovine pulmonary artery endothelial cells exposed to 500 μM NOC-18 for 0, 1, 2, 3, or 4 h. HIF-1 DNA-binding activity was determined by EMSA as described in Fig. 1. HIF-1 DNA-protein complexes are indicated. C, constitutive protein-DNA complexes.

ated by the reactivity of the NO moiety. To determine whether the effects of NOC-18 were dependent on the release of NO from NOC-18, the concentration of NO in tissue culture medium was measured as a function of time by chemiluminescence. The concentration of NO released from NOC-18 in the culture medium was less than 2% of the concentration of NOC 18 after 30 min. This level of NO was maintained at 1, 2, 3, and 4 h ($n = 3$ for each time point examined). However, NOC-18 induced HIF-1 DNA-binding activity in a time-dependent manner with the maximum effect occurring between 3 and 4 h (Fig. 2). Therefore, the time course of NOC-18 bioactivity was not associated with its time course for release of NO. To further examine the role of NO in mediating the effect of NOC-18, the ability of oxyhemoglobin to block the action of NO was studied. Bovine pulmonary artery endothelial cells were treated with NOC-18 in the absence and presence of an excess of oxyhemoglobin. Oxyhemoglobin (5 μM) did not affect the ability of NOC-18 to induce HIF-1 DNA-binding activity (not shown), suggesting that a direct interaction with NO was not responsible for the effect of NOC-18. Finally, the downstream effects of NO mediated through cGMP were studied using 8-Br-cGMP (Fig. 8B). Treatment with 8-Br-cGMP could not mimic the effects of NOC-18. Taken together, the data suggest that the effects of NOC-18 are not mediated through release of the NO radical or through the downstream effector cGMP.

The biological action of "NO donors" can be mediated through transnitrosation reactions involving their nucleophilic (NO^-, nitroxyl equivalents) or electrophilic (NO^+, nitrosonium equivalents) activity (Hogg et al., 1997). To further examine the mechanism by which NOC-18 induced HIF-1 DNA-binding activity, bovine pulmonary artery endothelial cells were treated with the NO^- donor, Angeli's salt (Fig. 8C). There was no induction of HIF-1 DNA-binding activity after treatment with Angeli salt, suggesting that the effect of NOC-18 was not due to NO^-. To determine whether the effect of NOC-18 could be mediated through an electrophilic nitrosylation reaction involving NO^+ equivalents, the endogenous *S*-nitrosylating agent, *S*-nitrosoglutathione (GSNO) was examined. Unlike Angeli's salt, GSNO was found to reproduce the effect of NOC-18 (Fig. 8D). In addition, it appeared to be more potent than NOC-18. Moreover, this effect could be completely reversed by acivicin, an inhibitor of GSNO bioactivation (Fig. 9) (Hogg et al., 1997).

The Effects of NOC-18 Can Be Altered by Subsequent Treatment with Dithiothreitol. To determine whether the induction of HIF-1 DNA-binding activity by NOC-18 is dependent on the redox state of the cell, bovine pulmonary artery endothelial cells were treated with 200 μM dithiothreitol (DTT) 30 min before the addition of NOC-18 (Fig. 10A). DTT had no effect on HIF-1 DNA-binding activity seen in nuclear extracts isolated from untreated cells. Similarly, treatment with DTT before the addition of NOC-18 did not significantly alter the ability of NOC-18 to induce HIF-1 DNA-binding activity. Furthermore, the changes in DNA-binding activity seen in these treated cells were found to be consistent with the changes in the level of HIF-1α protein. However, if cells were treated with NOC-18 with the addition of DTT during the last 30 min of incubation, HIF-1 DNA-binding activity was eliminated by DTT (Fig. 10B). Again, the level of HIF-1α protein was found to be consistent with the changes in HIF-1 DNA-binding activity. These results suggest that DTT reverses the HIF-1-binding effect of NOC-18 and that this effect is not mimicked by pretreatment with DTT. The alternative explanation that DTT could interact with a nitrosating agent generated by oxidation of NOC-18, such as N_2O_3, is not supported by the following observations: 1) DTT completely reversed the effect of NOC-18 despite coincubation for only 13% of the NOC-18 incubation; 2) NOC-18 bioactivity is unrelated to its rate of NO generation. Taken together, these observations strongly suggest that DTT may re-reduce a sulfhydryl group nitrosylated and/or oxidized by NOC-18 (Gaston, 1999). Moreover, because this reaction is reversed by DTT, the most likely target of NO^+ reactivity involves *S*-nitrosylation or oxidation of protein thiols.

Discussion

The action of NO in biological systems can be mediated directly by NO or can be mediated through redox modulation of NO to the reactivity of NO^- or NO^+ equivalents (Stamler et al., 1992). The effects of NOC-18 did not seem to be related to free radical reaction of NO evolved from NOC-18 because 1) oxyhemoglobin was unable to block the effect, 2) the induction of HIF-1 activity did not coincide with the evolution of NO, and 3) the effects of the reaction were reversed by DTT. The effects of NOC-18 were not caused by NO-mediated downstream signaling events through cGMP. Additionally, the NO^- donor, Angeli's salt, had no effect on HIF-1 activity. On the other hand, the NOC-18 effect was mimicked by GSNO, an endogenous NO^+ donor, and reversed by DTT. Moreover, acivicin, which may inhibit GSNO bioactivation by inhibiting its γ-glutamyl transpeptidase-mediated cleavage to form cell-membrane-permeable *S*-nitrosocysteinyl glycine (Hogg et al., 1997) effectively blocked the action of GSNO. Taken together, these data strongly suggest that the action of NOC-18 on HIF-1 activation may be mediated through a transnitrosation reaction involving a NO^+ equivalent.

The mechanism for the normoxic effect of NOC-18 on HIF-1 expression appears to involve HIF-1α, because HIF-1

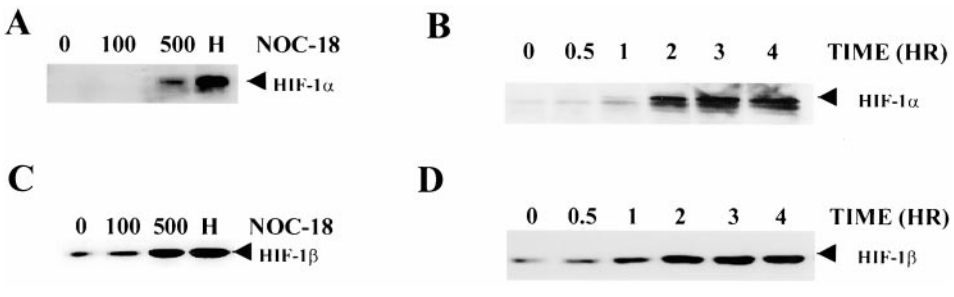

Fig. 5. NOC-18 stabilizes HIF-1α and augments HIF-1β protein expression. Western blot analysis was performed on nuclear extracts (100 μg) isolated from bovine pulmonary artery endothelial cells treated as described in Figs. 1 and 2. A and C show the increased expression of the α- and β-subunits of HIF-1, respectively, as a function of the dose of NOC-18. B and D show the change in the abundance of α- and β-subunits of HIF-1, respectively, as a function of time (hours). H, hypoxia.

activity is primarily determined by the stability of this subunit (Huang et al., 1996). One potential mechanism is that NOC-18 could stabilize HIF-1α by inhibiting its ubiquitin-proteasome-mediated degradation. In this regard, the effect of NOC-18 on HIF-1-binding action was not transcriptional.

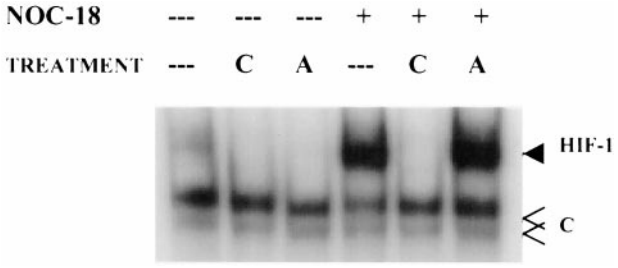

Fig. 6. The effects of NOC-18 on HIF-1 DNA-binding activity are not transcriptional. Bovine pulmonary artery endothelial cells were treated with 15 μg/ml actinomycin D (A) for 2 h or 60 μg/ml cycloheximide (C) for 15 min before the addition of 500 μM NOC-18 for 4 h. Nuclear proteins were isolated, and HIF-1 DNA-binding activity was determined by EMSA as described in Fig. 1. C, constitutive protein-DNA complexes. ---, no addition or no treatment.

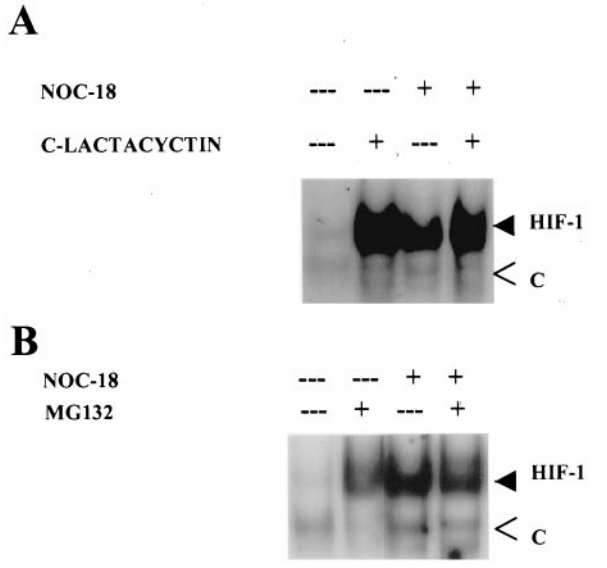

Fig. 7. NOC-18 does not augment the effect of proteasome inhibitors. Nuclear proteins were isolated from bovine pulmonary artery endothelial cells treated with 20 μM clasto-lactacystin β-lactone (A) or 50 μM MG132 (B) in the presence (+) or absence (---) of 500 μM NOC-18 for 4 h. HIF-1 DNA-binding activity was determined by EMSA as described in Fig. 1. C, control.

Its inhibition by cycloheximide does not reflect the increase in translation of constitutively expressed HIF-1α mRNA (Huang et al., 1996; Wood et al., 1996; Kallio et al., 1997). Specific inhibitors of the ubiquitin-proteasome system, clasto-lactacystin β-lactone and MG132, have been shown to protect HIF-1α protein from degradation (Salceda and Caro, 1997). In these studies, NOC-18 did not augment the effects of these proteasome inhibitors, suggesting the action of NOC-18 may be mediated through an effect on the ubiquitin-proteasome pathway.

Two enzymes in the ubiquitin-proteasome pathway, E1 and E2, contain thiols in their active sites (Jahngen-Hodge et al., 1997). *S*-Nitrosylation of these critical thiol residues could modify the activity of these enzymes resulting in HIF-1α stabilization. Preliminary studies indicate that E1 can be nitrosylated in vitro (L. A. Palmer, K. Fang and B. Gaston, unpublished observations). However, it is not known whether *S*-nitrosylation of E1 alters the stability of HIF-1α in vivo.

Oxygen-dependent proteolysis of HIF-1 has also been associated with the tumor suppressor protein von Hipple Lindau (pVHL) (Maxwell et al., 1999; Richard et al., 1999). pVHL associates with Cullin-2 and elongins B and C to form a multiprotein complex homologous to the ubiquitin-ligase (E3)/proteasome-targeting complexes known as the anaphase promoting complex and the Skp1-Cullin-F box (Richard et al., 1999). The anaphase promoting complex and SCF complexes have not been shown to form thioester intermediates with ubiquitin (Huang et al., 1999). However, the association of HIF-1α with pVHL is dependent on the HIF-1α ODD domain (Maxwell et al., 1999). Interestingly, the ODD domain contains an unpaired cysteine at residue 520 (Huang et al., 1999). Thus, it is possible that *S*-nitrosylation of HIF-1α at this cysteine could interfere with the ability of the ODD domain to interact with pVHL and target HIF-1α degradation.

HIF-1 activity is primarily determined by the stability of the HIF-1α subunit. However, it appears that NOC-18 is less effective than hypoxia at stabilizing HIF-1α but equally effective at inducing HIF-1 DNA-binding activity. In this manuscript, we show that, in addition to the action of NOC-18 on HIF-1α protein expression, NOC-18 was able to augment the expression of HIF-1β. Although HIF-1β is a necessary component in the formation and the activity of HIF-1, it has been reported that HIF-1α acquires a new conformational state upon binding to HIF-1β in vitro (Kallio

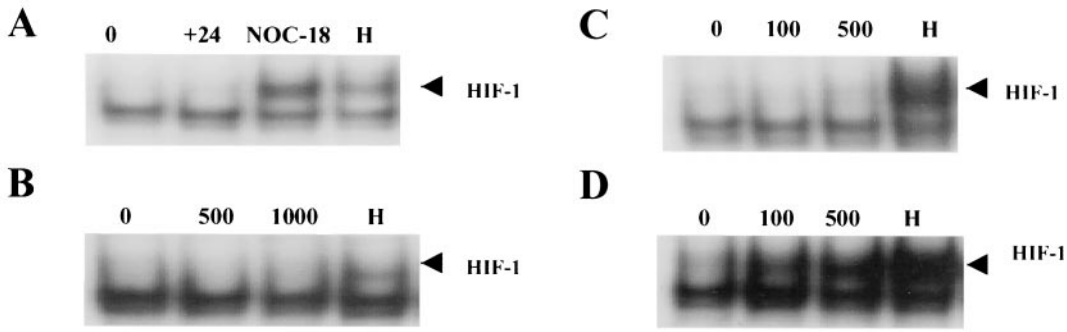

Fig. 8. The effect of NOC-18 is mediated by reactions of the nitrosonium ion. Nuclear proteins isolated from bovine pulmonary artery endothelial cells treated with 500 μM NOC-18 that was allowed to decay for 24 h (+24) (A), 8-Br cGMP (B), Angeli's salt (C), or GSNO (D) for a period of 4 h. HIF-1 DNA-binding activity was determined by EMSA as described in Fig. 1. C, constitutive protein-DNA complexes; H, hypoxia.

et al., 1997). The allosteric modification of HIF-1α results in a protein that is more resistant to proteolytic cleavage. Thus, it is possible that the increased expression of HIF-1β protein may also contribute to the mechanism by which NOC-18 induces HIF-1 expression in normoxia by stabilizing HIF-1α/ARNT heterodimer formation.

The mechanism by which NOC-18 activates HIF-1 activity in normoxia is likely to be different from the mechanism by which NO reduces HIF-1 activity in hypoxia (Liu et al., 1998; Sogawa et al., 1998; Huang et al., 1999) because the effects of NO are opposite under the two conditions. This article describes the ability of NO to mediate a cGMP-independent change in HIF-1α stability in normoxia through [NO^+] equivalents and *S*-nitrosylation. At this time, the site of action in normoxia is unknown and is currently under investigation in our laboratory. In contrast, the ability of NO to mediate changes in HIF-1 activity in hypoxia appears to be mediated through cGMP (Liu et al., 1998), suggesting that the effect in hypoxia involves activation of guanylate cyclase. Moreover, recent studies indicate NO targets the ODD domain as well as the C-terminal *trans*-activation domain of HIF-1α in hypoxia (Huang et al., 1999). However, the reduction in HIF-1 activity seen with NO donors in hypoxia is not affected by a mutation of the cysteine contained within the ODD domain, suggesting that the effect is not caused by *S*-nitrosylation of HIF-1α. In addition, the effect on the C-terminal *trans*-activation domain was found to be independent of HIF-1α stability. Although the reasons for these differences are unknown, one could hypothesize that the differences could be caused by 1) redox differences in NO and 2) the site of action of NO under a different oxygen concentration.

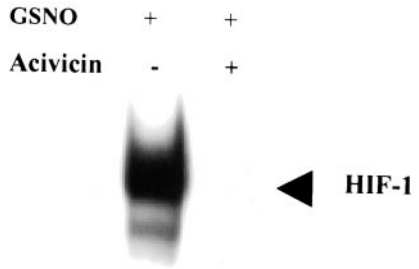

Fig. 9. Acivicin reverses the effect of GSNO. Nuclear extracts were made from bovine pulmonary artery endothelial cells treated with 100 μM GSNO in the presence (+) or absence (−) of 100 μM acivicin for 4 h. HIF-1 DNA-binding activity was determined by EMSA as described in Fig. 1.

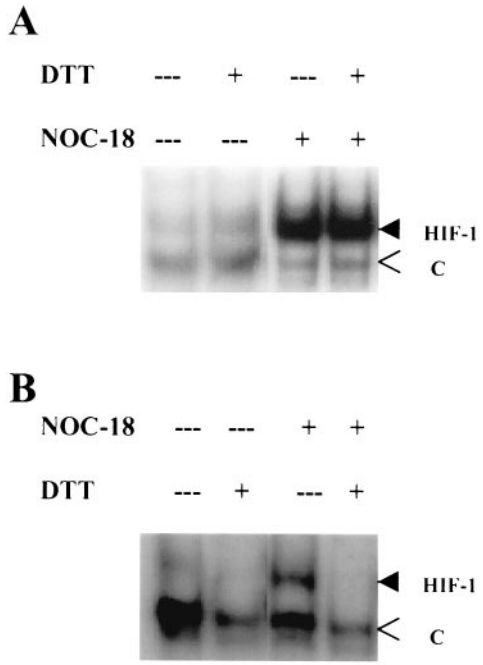

Fig. 10. DTT reverses HIF-1 DNA-binding activity induced by NOC-18. Nuclear extracts were made from bovine pulmonary artery endothelial cells exposed to 200 μM DTT 30 before the addition of 500 μM NOC-18 (A) or during the last 30 min of the 500 μM NOC-18 exposure (B). HIF-1 DNA-binding activity was determined EMSA as described in Fig. 1. ---, no addition; +, addition.

The observation that GSNO activated HIF-1 binding under normoxic conditions is particularly relevant biologically given recent observations that GSNO may be rapidly formed from *S*-nitrosohemoglobin and released from erythrocytes under conditions of physiological deoxygenation (Jia et al., 1996). Note that plasma *S*-nitrosothiol levels returning to the neonatal human pulmonary vasculature are relatively greater during hypoxemia than during normoxia (Gaston et al., 1998), consistent with evidence that glutathione exposed to deoxygenated blood forms 3- to 5-fold more GSNO than that exposed to oxygenated blood (B. Gaston, unpublished observations). Therefore, GSNO may serve as a signaling molecule in the vascular endothelium in vivo, where the oxygen concentrations are higher than those routinely used in vitro to demonstrate hypoxic HIF-1 activity (15 mm Hg). These observations suggest that endogenous NO donors arising from deoxyhemoglobin, such as GSNO, may have a role in regulating hypoxia-regulated genes under physiological conditions.

In summary, the NO donor, NOC-18, was found to increase HIF-1 activity in ambient oxygen tension. This effect is mediated, at least in part, by the stabilization of the HIF-1α subunit. It did not seem to be mediated by the classical pathway involving homolytic liberation of the NO radical with the activation of guanylate cyclase. Additionally, the mechanism did not seem to involve a reaction with NO^-, because the effect could not be reproduced with Angeli's salt. The most likely mechanism for increased HIF-1α protein stabilization and HIF-1 activity seems to involve an intracellular *S*-nitrosylation reaction because the effect was reversed by DTT and mimicked by the endogenous *S*-nitrosylating agent GSNO (Jia et al., 1996; Lee et al., 1997). We speculate that potential targets for *S*-nitrosylation-mediated normoxic stabilization of HIF-1α may involve the cysteine in the ODD domain of HIF-1α or the critical thiols of ubiquitin-activating enzymes.

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